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Unleashing the future: Exploring the transformative prospects of artificial intelligence in veterinary science

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ABSTRACT

Artificial intelligence (AI) has emerged as a transformative paradigm, promising revolutionary advancements in animal healthcare. Leveraging AI's unparalleled capacity for rapid data analysis significantly enhances diagnostic precision and speed, thereby facilitating informed decision-making by veterinarians. Predictive medicine powered by AI not only anticipates disease outbreaks but also enables tracking zoonotic diseases and predicting individual health risks for animals. AI helps to generate personalized treatment plans by analyzing genetic, environmental, and historical data. Remote monitoring and telemedicine, empowered by AI, overcome geographical constraints and offer continuous care, enabling veterinarians to track vital signs and intervene promptly. However, as AI becomes integral to veterinary practice, ethical considerations surrounding data privacy, transparency, and responsible AI use are crucial. This review explores the scope of AI in enhancing research and drug development, highlighting its ability to improve the discovery process and contribute to novel therapeutic interventions. It emphasizes the necessity of maintaining a delicate balance between AI-driven automation and the expertise of veterinary professionals. As the veterinary community moves toward embracing

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the transformative potential of AI, this comprehensive examination provides valuable insights into the current scenario. It discusses the challenges, opportunities, implications, and ethical considerations that shape the future of AI in veterinary science.

1 Introduction

The field of veterinary science has traditionally relied on empirical observations, clinical expertise, and diagnostic tests to diagnose and treat animal diseases (Perera et al. 2022). While these methods have served veterinarians well for centuries, the rapid progression of technology over the years has opened up new possibilities for enhancing veterinary care (Ogilvie and Kastelic 2022). One such technological advancement is artificial intelligence (AI), which utilizes various computational techniques enabling machines to replicate human cognitive functions such as decision-making and problem-solving (Sarker 2022). AI has already improved various sectors, including healthcare, entertainment, finance, and transportation (Davenport and Kalakota 2019).

In veterinary science, AI holds immense scope to revolutionize treatment and diagnostics modalities and overall patient care (Appleby and Basran 2022). With the world becoming progressively interconnected and reliant on data-driven processes, integrating AI technologies promises to unlock new insights, enhance efficiency, and improve outcomes in veterinary practice (Appleby and Basran 2022; Akinsulie et al. 2024). The application of AI in veterinary medicine includes a wide range of areas, including genetic analysis, treatment planning, drug discovery, and personalized medicine (Akinsulie et al. 2024). Machine learning (ML) algorithms, deep learning (DL) networks, natural language processing (NLP), and computer vision techniques are among the many AI tools that are being leveraged to analyze vast amounts of veterinary data, extract meaningful patterns, and generate actionable insights (Sarker 2021a, 2021b; Sharma et al. 2021).

While the potential benefits of integrating AI into veterinary medicine are considerable, numerous issues must be addressed to harness its full potential effectively (Akinsulie et al. 2024; Bellamy 2023). Data availability and quality stand out as primary challenges. While vast amounts of data are generated in veterinary practice, including patient records, diagnostic test results, and imaging studies, much remains fragmented, unstructured, or stored in incompatible formats (Cravero et al. 2022; Paynter et al. 2021). Additionally, variations in data quality and completeness can hinder the development and performance of AI algorithms, as they rely heavily on high-quality, standardized data for training and validation (Aldoseri et al. 2023). The cost of implementing AI technologies poses another significant barrier (Javaid et al. 2023; Neethirajan 2023). Developing and deploying AI-powered solutions in veterinary practice requires substantial financial investment in hardware, software, training, and infrastructure, which may limit its access at the field level (Javaid et al. 2023).

Ethical considerations also loom large in integrating AI into veterinary medicine (Mennella et al. 2024). Data security, patient privacy, and informed consent concerns must be addressed to ensure that AI-driven systems adhere to ethical principles and respect the rights and welfare of animal patients and their owners (Gerke et al. 2020). Additionally, issues surrounding algorithmic bias, fairness, and accountability demand attention to prevent unintended consequences and ensure equitable access to veterinary care. Regulatory oversight is another critical aspect of AI integration in veterinary practice. Regulatory agencies must develop precise guidelines for validating and establishing AI technologies in veterinary medicine (Bellamy 2023; Mennella et al. 2024). These regulations should address data safety, privacy, and transparency to protect animal patients, their owners, and veterinary professionals (Mennella et al. 2024). Moreover, effective collaboration between veterinarians, data scientists, and technology developers is essential to overcome these challenges and tailor AI solutions to veterinary practice's unique needs and challenges (Bellamy 2023; Mennella et al. 2024). By fostering interdisciplinary partnerships and knowledge exchange, stakeholders can leverage their expertise to develop AI-driven tools and applications that address specific clinical needs and improve the overall quality of veterinary care (Yelne et al. 2023).

The rapid advancement of AI has permeated diverse sectors, and its transformative impact is increasingly evident in veterinary science (Appleby and Basran 2022). The integration of AI promises to revolutionize the field, offering innovative solutions, efficient diagnostics, and personalized treatments (Akinsulie et al. 2024; Appleby and Basran 2022). This paper explores the transformative prospects of AI in veterinary science, highlighting recent advancements and future directions in the field. By synthesizing the latest research findings and industry developments, this paper highlights the current scenario of AI-driven veterinary medicine and envisions the future trajectory of this rapidly evolving field.

2 Artificial intelligence, machine learning, and deep learning

AI represents a shift in computing where machines are endowed with capabilities traditionally associated with human intelligence (Xu et al. 2021). It involves various techniques and technologies to enable computers to perform tasks that usually require human intelligence (making decisions, identifying data patterns, and

experience-based learning (Najjar 2023; Xu et al. 2021). AI systems are classified into two main categories: Narrow AI, which performs specific tasks, and General AI, which exhibits human-like intelligence in different domains (Elahi et al. 2023). While General AI remains a theoretical concept, Narrow AI has widespread adoption in various applications, including virtual assistants, recommendation systems, and image recognition algorithms (Xu et al. 2021).

ML represents a subset of AI dedicated to crafting algorithms and statistical models. These models empower computers to obtain insights from data, making decisions autonomously without the need for programming (Sarker 2021b). ML algorithms learn from example data, known as training data, and iteratively improve performance through experience (Taye 2023). ML has found applications in diverse fields, including healthcare, finance, e-commerce, and autonomous vehicles (Jiang et al. 2020a).

DL concentrates on neural networks comprising numerous layers, known as deep neural networks (Najjar 2023). These DL algorithms can autonomously acquire hierarchical data representations from raw inputs, obviating the necessity for manual feature engineering (Taye 2023). CNNs, RNNs, and GANs are some of the most widely used architectures in DL (Alzubaidi et al. 2021). DL has attained notable success in various tasks, particularly in image recognition, NLP, speech recognition, and autonomous driving, surpassing human performance in certain domains (Alzubaidi et al. 2021; Sharma et al. 2021).

3 Diagnostics and disease prediction

AI-driven diagnostic tools represent a revolutionary advancement in veterinary science, leveraging the ability to process vast datasets encompassing diverse information such as medical records, diagnostic imaging, and genetic profiles (Akinsulie et al. 2024; Bellamy 2023). The integration of AI in diagnostics has noteworthy advantages beyond accelerating the diagnostic process (Bouhali et al. 2022; Hespel et al. 2022). AI algorithms excel in swiftly analyzing intricate datasets, providing veterinarians with near-instantaneous insights into the health status of animals. This is valuable in critical situations requiring quick decision-making (Akinsulie et al. 2024; Alowais et al. 2023). The precision achieved by AI-driven diagnostics is unparalleled. By scrutinizing numerous data points with high accuracy, these tools contribute to more nuanced and accurate diagnoses (Johnson et al. 2021). This heightened precision minimizes the margin of error in identifying health issues, enabling veterinarians to formulate precise treatment plans (Johnson et al. 2021; Najjar 2023). AI systems can seamlessly integrate various types of information, including medical histories, diagnostic images, and genetic data(Aldoseri et al. 2023). This holistic approach ensures that veterinarians understand an animal's health comprehensively, allowing for more informed decisions regarding treatment and care.

AI detects subtle anomalies in diagnostic images and genetic markers, enhancing early disease detection capacity (Bouhali et al. 2022; Dumortier et al. 2022; Nyquist et al. 2024). This early identification is pivotal in initiating timely interventions, potentially preventing the progression of diseases or enabling more effective management strategies. AI-driven diagnostics identify health issues and contribute to personalized treatment recommendations (Johnson et al. 2021; Leary and Basran 2022). By considering individual variations in genetic makeup and response to therapies, these tools assist veterinarians in tailoring treatment plans that are more likely to yield positive outcomes(Nosrati and Nosrati 2023). One of the remarkable features of AI is its ability to continuously learn and improve its diagnostic capabilities (Hespel et al. 2022). As these systems encounter more cases and receive feedback from veterinary professionals, they become even more adept at identifying patterns and making accurate predictions (Schofield et al. 2021; Yoon et al. 2018). AI-driven diagnostics have the potential to enhance accessibility to advanced veterinary care (Alowais et al. 2023). By streamlining diagnostic processes, these tools can reduce cost, making advanced diagnostics more affordable and widely available, ultimately benefiting a larger population of animals.

The utilization of AI in diagnostic imaging is poised for significant growth, driven by the digitization of medical data and advancements in AI technology (Cohen and Gordon 2022). Clinical diagnostic imaging encompasses various technologies, including radiography, ultrasound, MRI, CT, and nuclear medicine (Bouhali et al. 2022; Pereira et al. 2023). With the widespread adoption of digital imaging technologies, vast amounts of digital data are generated from these modalities and their corresponding reports (Alhasan and Hasaneen 2021). Radiography, which involves using X-rays to generate internal structure images of the body, has transitioned from traditional film-based imaging to digital radiography (Bansal 2006). Digital radiography systems produce high-resolution images that is stored, transmitted, and analyzed using AI algorithms (Alhasan and Hasaneen 2021; Bansal 2006). Similarly, ultrasound imaging, which utilizes sound waves to visualize internal organs and tissues, has evolved with the development of digital ultrasound machines (Carovac et al. 2011). These machines generate digital images that can be processed and interpreted using AI-based image analysis algorithms (Figure 1 and 2) (Alhasan and Hasaneen 2021). CT and MRI are advanced imaging modalities that provide cross-sectional images of the internal structures (Paudyal et al. 2023). Digital CT and MRI scans produce volumetric datasets that contain a wealth of information about anatomical and pathological features (Bouhali et al. 2022; Paudyal et al. 2023). AI algorithms can analyze these datasets to assist radiologists in detecting abnormalities, quantifying disease

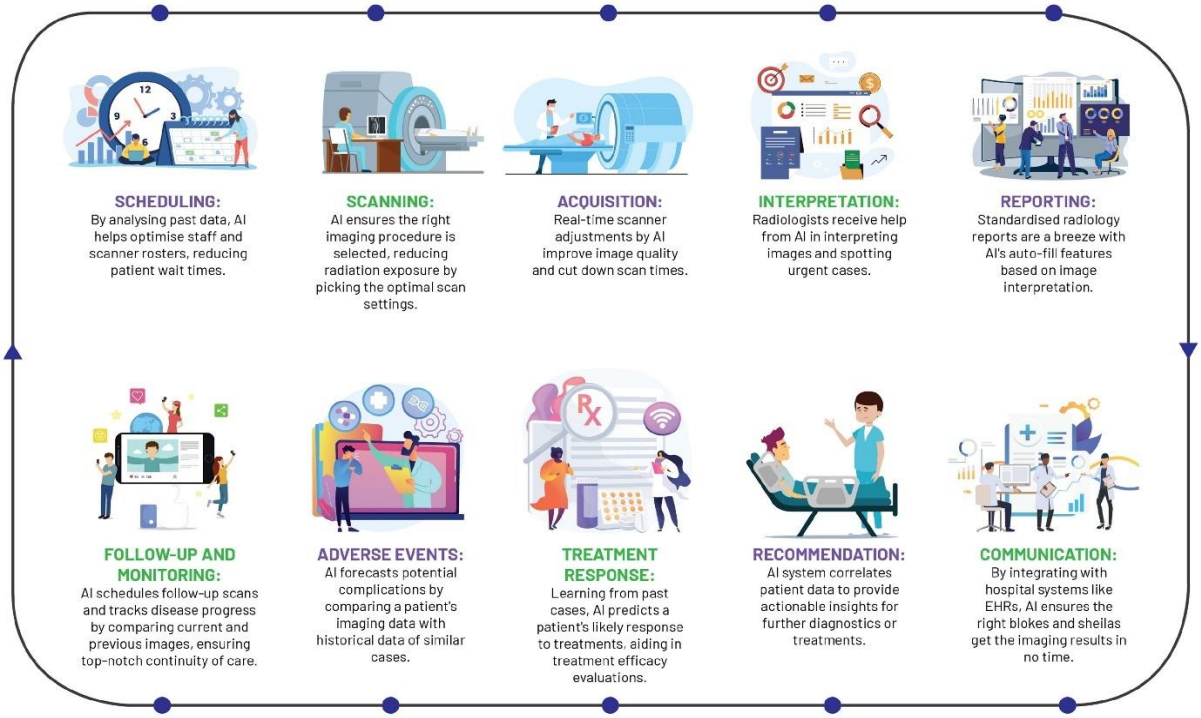

Figure 1 A schematic workflow diagram demonstrating the integration of AI into radiological practice. AI has catalyzed a transformative shift within radiology, reshaping conventional workflows and enhancing the role of radiologists. Reproduced from (Najjar, 2023) under CC BY license.

severity, and predicting treatment outcomes (Cohen and Gordon 2022). Nuclear medicine imaging techniques, such as SPECT and PET, involve using radioactive tracers to visualize metabolic processes and detect abnormalities at the molecular level. Digital nuclear medicine images can be processed and analyzed using AI algorithms to improve diagnostic accuracy and facilitate personalized treatment planning. The digitization of medical imaging data and reports has created opportunities for AI to enhance clinical diagnostic practices across various imaging modalities (Bouhali et al. 2022; Pereira et al. 2023). AI algorithms can automate image analysis tasks, identify specific patterns and issues in imaging data, and provide quantitative assessments of disease severity (Pereira et al. 2023).

The rapid progress in AI technology and computational capabilities has spurred the creation of a multitude of automated solutions for livestock monitoring (Jiang et al. 2020b; Siachos et al. 2024). Among these innovations are sophisticated systems employing AI algorithms explicitly designed to detect lameness in farm animals (Jiang et al. 2020b). To conduct comprehensive gait analysis, these cutting-edge systems utilize various tools and techniques, including accelerometers, radar sensors, body weight trackers, acoustic analysis, and advanced computer vision technology (Siachos et al. 2024). AI-driven diagnostic tools, powered by sophisticated algorithms, possess an unparalleled capacity to evaluate vast datasets, including diagnostic imaging records (Pereira et al. 2023). Their excellence in identifying intricate patterns and trends goes beyond mere diagnostic acceleration, extending to predictive capabilities (Dumortier et al. 2022; Yoon et al. 2018). Through analyzing historical data and ongoing health trends, AI algorithms can predict and forecast potential disease outbreaks in animal populations (Cravero et al. 2022). This foresight allows veterinary professionals to implement vaccination campaigns or quarantine protocols to contain and mitigate the impact of infectious diseases (de Melo et al. 2020; Nyquist et al. 2024).

Zoonotic diseases pose significant public health risks (Elsohaby and Villa 2023). AI predictive models can track the spread of such diseases, identifying potential hotspots and vulnerable populations (Ganasegeran and Abdulrahman 2019). This information is invaluable for implementing proactive measures to prevent cross-species transmission and protect animal and human health. AI-driven tools can assess animal health risks based on various factors, including genetic predispositions, environmental exposures, and lifestyle (Ezanno et al. 2021; Kamel Boulos et al. 2019). This personalized approach enables veterinarians to anticipate potential health issues in specific animals, facilitating early interventions and tailored preventive care plans (Ezanno et al. 2021). The predictive capabilities empower veterinary professionals to adopt a proactive approach to animal healthcare (Alowais et al. 2023; Johnson et al. 2021). By anticipating health risks and potential

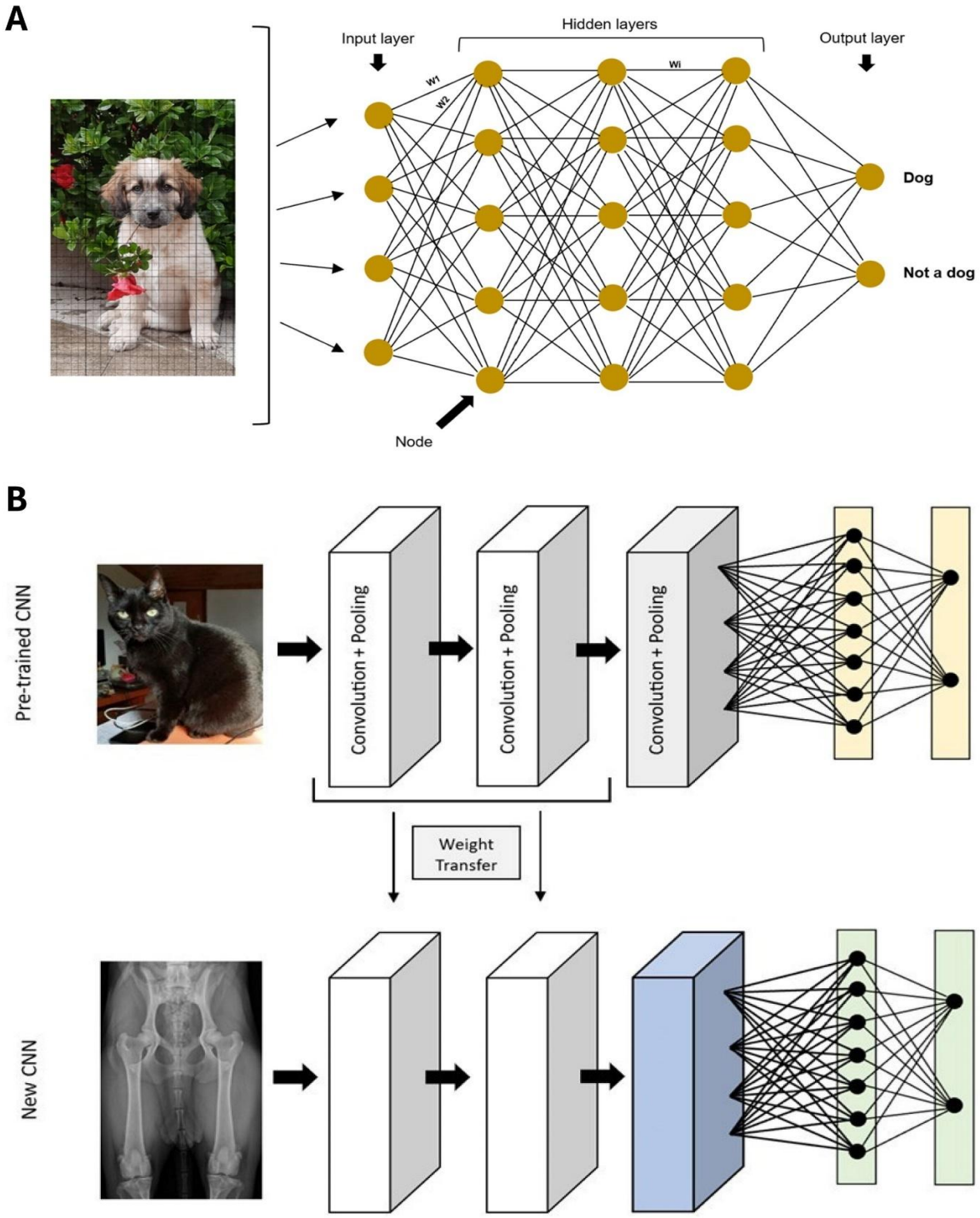

Figure 2 (A) Illustration depicting the artificial neural network architecture, with the pixels of a digital image of a dog serving as input. The network comprises four hidden layers and offers two potential outputs: "dog" or "not dog." Nodes are organized in layers and connected through connections, with weights denoted by the letter W (W1, W2, and Wi); (B) Depiction of the transfer learning process, where a portion of the weights from a CNN trained to analyze non-medical images is leveraged within a CNN tasked with classifying radiographs. Reproduced from (Pereira et al., 2023) under CC BY license.

complications, veterinarians can implement preventive strategies, such as targeted screenings, dietary adjustments, and lifestyle modifications, to maintain and enhance the overall well-being of animals (Appleby and Basran 2022; Guitian et al. 2023). The University of Calgary has developed a specialized data extraction software called the UCDEP (Anholt et al. 2014). This software has been designed to extract and store electronic health records (HER) from veterinary practices participating in the program. The primary goal of UCDEP is to make these medical records readily available for disease surveillance and to facilitate knowledge generation for evidence-based practice in veterinary medicine (Anholt et al. 2014). AI predictive models can continuously monitor and analyze such databases in real-time.

The effectiveness of surveillance systems for animal and zoonotic diseases hinges on completing a wide array of tasks, many of which can benefit from applying ML algorithms. Similar to other domains, the utilization of ML in surveillance has experienced significant growth in the last decade (Guitian et al. 2023). This expansion is mainly attributable to datasets' availability, advancements in data analysis techniques, and increased computational capabilities. ML algorithms are now being employed to tackle previously unfeasible tasks, including identifying underlying patterns within extensive datasets derived from ongoing streams of abattoir condemnation records (Guitian et al. 2023). Furthermore, DL techniques facilitate the identification of lesions in images captured during the slaughtering process, while the analysis of free text within EHR from veterinary practices enables sentinel surveillance (Guitian et al. 2023). Beyond these novel applications, ML augments tasks traditionally reliant on statistical data analysis. While statistical models have traditionally been used to deduce relationships between predictors and diseases for risk-based surveillance, there is a growing trend toward employing ML algorithms for disease prediction and forecasting (Guitian et al. 2023). This shift toward ML-based prediction and forecasting enhances the precision and efficiency of surveillance efforts and enables more targeted interventions.

A system was developed to discern the presence of respiratory, gastrointestinal, or urinary pathology within necropsy reports (Bollig et al. 2020). Various ML algorithms, including DL were assessed for their performance in this task. This approach represents a novel ML application for syndromic surveillance utilizing necropsy reports (Bollig et al. 2020). The developed model was then applied to a dataset comprising over 33,000 necropsy reports spanning 14 years. This analysis revealed temporal and spatial patterns of diseases, providing valuable insights into epidemiological trends (Bollig et al. 2020). Notably, the model identified a potential cluster of gastrointestinal diseases from a single submitting producer in 2016, highlighting its utility in detecting and tracking disease outbreaks within veterinary populations (Bollig et al. 2020).

Confirmatory diagnosis of Cushing's syndrome (CS) can be challenging in dogs, necessitating exploring novel diagnostic approaches (Carotenuto et al. 2019). Four ML algorithms were employed to predict the likelihood of a future diagnosis of CS (Schofield et al. 2021). Utilizing structured clinical data from the VetCompass program in the UK, dogs flagged as suspected cases of CS were analyzed and categorized based on their final diagnosis in records (Schofield et al. 2021). The models incorporated clinical and demographic features available at initial suspicion by attending veterinarians. Remarkably, the ML methods demonstrated the ability to accurately classify recorded diagnoses of CS, exhibiting robust predictive performance (Schofield et al. 2021).

Significant efforts have been dedicated to developing computer-based decision support tools to aid veterinary clinicians across various aspects of patient care (Hennessey et al. 2022). Such applications enhance the accuracy of medical diagnoses and ultimately improve patient outcomes (La Perle 2019). While ample supporting evidence exists for the former assertion, the latter remains a more challenging endpoint to assess comprehensively (Awaysheh et al. 2019; La Perle 2019). As these tools become increasingly integrated into veterinary pathology, evidence-based outcome assessments will be possible, shedding further light on their true efficacy in clinical practice (Awaysheh et al. 2019; Zuraw and Aeffner 2022). Following the training of ML models, they function collaboratively with pathologists to enhance diagnostic outcomes (Zuraw and Aeffner 2022). By leveraging the vast amounts of data available, these models provide insights and support to pathologists during the diagnostic process (La Perle 2019). ML algorithms refine their predictions through continuous interaction and feedback loops, leading to accurate and reliable results (Awaysheh et al. 2019; La Perle 2019). Additionally, these models can assist in identifying subtle patterns not be apparent to human observers, thereby augmenting the diagnostic capabilities of pathologists (La Perle 2019).

In assessing tumor grading schemes, the manual count of mitotic figures holds significant importance, serving as a critical parameter in evaluating tumor aggressiveness (Ibrahim et al. 2022). However, the accuracy of this assessment can be influenced by the selection of the tumor region having the highest mitotic activity, which may vary due to the uneven distribution of mitotic figures (Aubreville et al. 2020). Three DL-based methods (indirect approach to predict mitotic figure segmentation map, directly estimating mitotic figures, and detecting mitotic figures as objects) were evaluated for their effectiveness in assessing the highest mitotic density. Surprisingly, the predictions made by all models surpassed those of expert pathologists on average (Aubreville et al. 2020). Particularly noteworthy was the two-stage object detector performance that consistently outperformed most human pathologists across the different tumor cases (Aubreville et al. 2020). This underscores the remarkable capabilities of DL algorithms in detecting the most

mitotically active regions within tumors (Aubreville et al. 2020). This suggests the potential for integrating these advanced technologies into clinical practice to enhance the efficiency and accuracy of tumor grading processes.

An AI-based software program (AISP) designed to detect dental issues in dogs and cats was evaluated compared to human evaluators (Nyquist et al. 2024). Pathologies were assessed, including periapical lucency, furcation bone loss, resorptive lesions, retained tooth roots, attachment loss, and tooth fractures. Inter-rater reliability showed good to excellent agreement among all parties, indicating the AISP's comparable performance to human evaluators in detecting specified pathologies (Nyquist et al. 2024). The sensitivity and specificity of the AISP were evaluated. The results showed low sensitivity and high specificity, indicating a tendency for false negatives. This raises concerns about its initial screening tool efficacy (Nyquist et al. 2024). However, the AISP demonstrated a low rate of false positives, indicating utility as a supplementary tool, enhancing diagnostic accuracy rather than serving as a standalone diagnostician (Nyquist et al. 2024). This technology could augment dental radiography utilization and diagnostic capabilities with proper understanding and integration into veterinary practice.

DL in veterinary science represents a promising avenue, mainly in computer-aided detection using CNNs. One such application focuses on detecting abnormalities from cat lateral thoracic radiographs (Dumortier et al. 2022). Thoracic radiography is a fundamental diagnostic tool in small animal medicine, offering valuable insights into pulmonary health through analyzing radiographic pulmonary patterns. Despite significant strides in DL for veterinary imaging, a notable gap remains in developing CNNs tailored to detect radiographic pulmonary patterns from thoracic radiograph images (Dumortier et al. 2022). This represents a crucial area of investigation, given the importance of accurate and timely diagnosis in veterinary medicine, particularly in identifying pulmonary abnormalities in veterinary patients (Dumortier et al. 2022). By leveraging CNNs, we can enhance diagnostic accuracy and efficiency, ultimately improving patient care and outcomes in veterinary practice.

In another study, three DL networks with multiple pretraining strategies aimed to predict different primary thoracic lesions in canine and feline patients from thoracic radiographs (Boissady et al. 2020). Lesions included left atrial enlargement, tracheal collapse, pneumothorax, alveolar patterns, and pulmonary masses. Following pretraining, the algorithms underwent specific training using over 22,000 thoracic veterinary radiographs, each accompanied by an expert veterinary radiologist's report as the standard (Boissady et al. 2020). Error rates for each observer were calculated for the 15 labels and subsequently compared. The network's overall error rate significantly outperformed unaided veterinarians and those aided by the network (10.7% *vs* 16.8% *vs* 17.2%, respectively) (Boissady et al. 2020). Notably, the network performed significantly better detecting cardiac enlargement and bronchial patterns. The evaluated network solely aids lesion detection and does not provide diagnostic conclusions (Boissady et al. 2020). Considering its commendable performance, this technology could serve as a valuable aid for general practitioners while awaiting a radiologist's report, potentially expediting diagnostic processes and improving patient care.

Researchers have also applied a DL and AI technique to analyze thoracic radiographs of dogs for diagnosing left atrial enlargement and compared it to the interpretations of veterinary radiologists (Li et al. 2020). A total of 792 radiographs were utilized to train and test a CNN algorithm (Li et al. 2020). The sensitivity and specificity were subsequently assessed with those determined by the experts. In comparison, sensitivity and specificity obtained by expert veterinary radiologists were identical, standing at 82.71%, 68.42%, and 87.09%, respectively (Li et al. 2020). While the accuracy of both the accuracy-driven CNN algorithm and radiologists was almost similar, their concordance reached an impressive 85.19%, indicating a higher agreement between the two approaches (Li et al. 2020).

Similarly, researchers examined the efficacy of an AI algorithm (Vetology AI®) for identifying pleural effusion in canine thoracic radiographs (Müller et al. 2022). The algorithm classified images into those with and without pleural effusion (Müller et al. 2022). The AI achieved an accuracy rate of 88.7% in detecting pleural effusion, with sensitivity and specificity levels at 90.2% and 81.8%, respectively (Müller et al. 2022). Utilizing this technology in evaluating radiographs presents promising prospects and merits additional exploration and validation through further investigation and testing. The effectiveness of Vetology AI® was also evaluated for identifying pulmonary nodules in canine thoracic radiography (Pomerantz et al. 2023). Positive cases were validated through CT, cytology, or histopathology, confirming pulmonary pathology. Among the confirmed cases, the AI software successfully detected pulmonary nodules or masses in 31 out of 56 instances (Pomerantz et al. 2023). Additionally, it accurately classified 30 out of 32 control cases. The AI model demonstrated an accuracy of 69.3%, balanced accuracy of 74.6%, sensitivity of 55.4%, and specificity of 93.75% (Pomerantz et al. 2023). These results indicate promising potential for the AI software in detecting pulmonary pathology, with notable sensitivity and specificity values.

Likewise, researchers assessed the accuracy of AI-powered software in diagnosing canine cardiogenic pulmonary oedema. Results revealed impressive metrics, with the AI-based software achieving 92.3% accuracy, 91.3% sensitivity, and 92.4% specificity compared to radiologist diagnoses (Kim et al. 2022). These findings advocate for integrating AI software screening in evaluating thoracic radiographs for dogs suspected of having cardiogenic pulmonary oedema. This approach is valuable in

aiding short-term decision-making, particularly when access to a radiologist is limited or unavailable (Kim et al. 2022). A computer-aided detection device employing CNNs was developed to identify cardiomegaly from right lateral chest radiographs in dogs (Burti et al. 2020). The diagnostic accuracy of four distinct CNN models in detecting cardiomegaly was assessed, revealing that all tested models exhibited high diagnostic accuracy (Burti et al. 2020). This suggests that CNNs have the potential to aid veterinarians in the detection of cardiomegaly in dogs from plain radiographs.

Another study has investigated the viability of BOF and CNN for computer-aided detection. It compared their efficacy to differentiate normal from abnormal thoracic radiographic findings of dogs (Yoon et al. 2018). Across testing sets, both models exhibited accuracy ranging from 79.6% to 96.9%. Notably, CNN demonstrated superior accuracy (92.9-96.9%) and sensitivity (92.1-100%) compared to BOF (accuracy: 79.6-96.9%, sensitivity: 74.1-94.8%) (Yoon et al., 2018). BOF and CNN promise to enhance work efficiency through double reading (Yoon et al. 2018). In another study, a deep CNN was trained to identify medial retropharyngeal lymph nodes using a small dataset comprising CT scans of canine heads (Schmid et al. 2022). The findings suggest that these architectures can effectively segment anatomical structures within complex and breed-specific regions like the head, potentially even with limited training sets (Schmid et al. 2022). However, veterinary radiologists exhibited a statistically lower error rate than CNNs (Adrien-Maxence et al. 2022). Some CNNs showed superiority over veterinary radiologists, indicating potential (Adrien-Maxence et al. 2022). This addresses several questions the current study raises to standardize AI and enhance patient care (Joslyn and Alexander 2022; Lungren and Wilson 2022).

In the context of time constraints physicians face during patient consultations, integrating automated systems can significantly expedite diagnostic processes while ensuring accuracy. Presently, many such systems rely on supervised DL methodologies. However, a significant drawback of these approaches is their reliance on extensive datasets with labeled data (Celniak et al. 2023). Acquiring such datasets is often challenging and resource-intensive in terms of time and financial investment. In response to this challenge, a recent study proposed a novel solution to improve classification accuracy while minimizing the dependency on large labelled datasets (Celniak et al. 2023). This methodology leverages knowledge transfer from self-supervised learning methods across species and pathologies. By harnessing inter-species self-supervised learning techniques, this approach facilitated the extraction of valuable insights from diverse datasets, thereby enhancing classification scores (Celniak et al. 2023). This innovative approach addresses the limitations associated with traditional supervised learning methods and offers a more efficient and cost-effective alternative for developing automated diagnostic systems (Celniak et al. 2023). Through knowledge transfer from various sources, our solution has the potential to revolutionize diagnostic processes, enabling clinicians to make faster yet reliable diagnoses (Celniak et al. 2023).

AI algorithms, when applied to predictive modelling, can assist in optimizing treatment plans for individual animals (Zhang et al. 2024). By considering the predicted response to different therapeutic interventions based on historical data and case studies, veterinarians can tailor treatment approaches, improving efficacy and minimizing potential side effects (Lungren and Wilson 2022; Paudyal et al. 2023). AI's predictive modelling extends to population-level health management (Shaban-Nejad et al. 2018). Veterinary authorities can use these tools to assess and address the health needs of entire animal populations, guiding resource allocation, disease prevention strategies, and public health initiatives (Guitian et al. 2023; Olawade et al. 2023). AI-driven predictive models can also factor in environmental variables, assessing how climate or habitat changes may impact animal populations' health. This holistic approach enables a comprehensive understanding of the interconnected factors influencing animal health (Sarker 2022). As more data becomes available and the algorithms encounter new scenarios, they adapt and improve their predictive accuracy, ensuring that the models remain relevant over time (Aldoseri et al. 2023).

The adoption of AI within the realms of veterinary and human medicine are experiencing a swift and widespread surge. This expansion is evident in medical image analysis, where ML methodologies are frequently employed (Hennessey et al. 2022). Among these methodologies, CNNs are a prominent choice in DL classification and regression models, owing to their ability to process and interpret complex medical images effectively (Hespel et al. 2022). CNNs offer a sophisticated approach to analyzing medical images, allowing for detailed and nuanced assessments that contributing to accurate diagnoses and treatment planning. Furthermore, utilizing NLP techniques can streamline the generation of "truth-data," essential for training AI systems in radiation oncology applications (Hespel et al. 2022). By harnessing NLP, annotating and categorizing medical images becomes more efficient and precise, facilitating the development and refinement of AI-driven diagnostic and therapeutic tools (Hespel et al. 2022). As the integration of AI continues to evolve and expand within veterinary and human medicine, a comprehensive understanding of these methodologies, particularly CNNs and NLP, becomes increasingly crucial. Healthcare professionals can enhance their diagnostic capabilities, improve patient outcomes, and optimize care delivery across various medical specialties (Lungren and Wilson 2022).

Therefore, it is clear that the integration of AI into veterinary radiology is transforming diagnostic imaging practices, offering a wide array of advanced tools and techniques to enhance the interpretation and analysis of radiographic images for animals. One

significant application of AI in this field is image interpretation and diagnosis, where AI-powered algorithms analyze radiographic images, CT scans, MRI, and other diagnostic images to assist veterinarians in detecting abnormalities and diagnosing various conditions in animals. These algorithms can identify subtle changes in images that may indicate the presence of tumours, fractures, foreign bodies, or other health issues, thereby aiding in early detection and intervention. Moreover, AI algorithms automate the process of segmentation and annotation within radiographic images, facilitating the visualization and understanding of anatomical structures for precise diagnosis and treatment planning, particularly in complex cases where accurate anatomical localization is essential. Additionally, AI models trained on large datasets of veterinary imaging studies can classify different diseases and predict clinical outcomes based on radiographic findings, providing valuable insights for veterinarians in disease management and prognosis. Furthermore, AI-based techniques can help reduce noise, improve contrast, and reconstruct 3D images from 2D radiographs, offering veterinarians a comprehensive view of complex anatomical structures for surgical planning and intervention.

4 Personalized treatment plans

One area where AI excels is in the analysis of genetic data, which is important in tailoring personalized treatment plans (Johnson et al. 2021). By leveraging AI algorithms to sift through vast genetic datasets, veterinarians can gain valuable insights into the genetic predispositions of animals to certain diseases and conditions (Johnson et al. 2021; Rezayi et al. 2022). Through genetic data analysis, AI can identify genetic markers associated with specific health risks or animal susceptibilities (Vilhekar and Rawekar 2024). This information allows veterinarians to proactively screen for potential health issues and design preventive measures tailored to each animal's unique genetic profile (Johnson et al. 2021; Vilhekar and Rawekar 2024). AI may recommend specific dietary adjustments to mitigate the risk of genetic diseases or enhance overall health and well-being (Johnson et al. 2021; Rezayi et al. 2022).

In addition to genetic data analysis, AI-powered systems can also consider environmental factors when tailoring treatment plans for individual animals (Johnson et al. 2021; Paudyal et al. 2023). Environmental factors such as diet, exercise levels, living conditions, and exposure to toxins or pollutants can significantly impact an animal's health and treatment outcomes. AI algorithms can analyze environmental data from various sources, including wearable sensors, environmental monitoring devices, and EHR (Figure 3)(Aldoseri et al. 2023; Shajari et al. 2023). By correlating environmental factors with health outcomes, AI can identify patterns and trends that may influence an animal's response to treatment. For example, AI may recommend changes in diet or exercise routines based on environmental factors to optimize

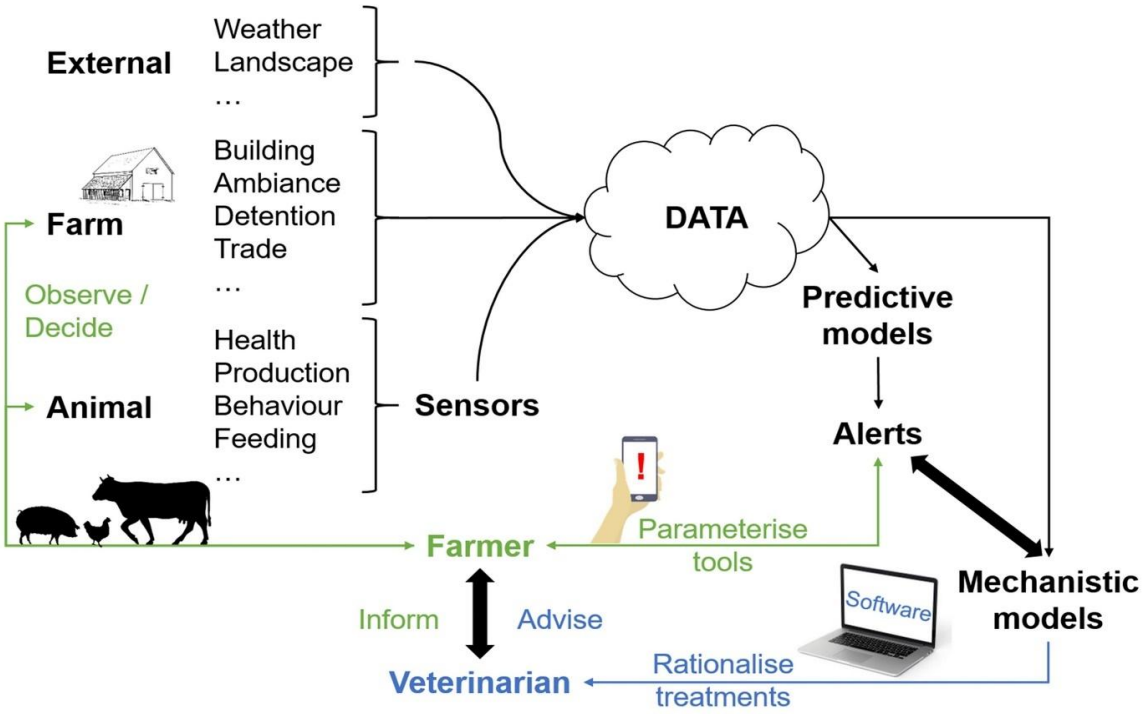

Figure 3 Enhancing animal health monitoring and treatment rationalization through data-driven approaches. Machine learning methods enable the identification of patterns and signals within extensive datasets, such as spatial data or time-series of disease cases. Reproduced from under CC BY license (Ezanno et al. 2021).

treatment efficacy and promote better health outcomes (Anholt et al. 2014).

AI can help to integrate historical data into the treatment planning process, allowing veterinarians to leverage past medical records and treatment outcomes to inform future decisions (Anholt et al. 2014; Paynter et al. 2021; Aldoseri et al. 2023). By analyzing historical data, AI can identify trends, patterns, and treatment responses that may guide personalized treatment strategies for individual animals (Paynter et al. 2021). Historical data integration enables veterinarians to track the progression of diseases over time, monitor the effectiveness of previous interventions, and identify factors associated with treatment success or failure (Cravero et al. 2022). This valuable information empowers veterinarians to make evidence-based decisions and adjust treatment plans in real-time based on each animal's unique medical history and response to therapy.

Using image registration, advanced contouring, and treatment optimization software is standard practice for clinical care in veterinary radiation oncology. However, significant progress has been made over the years due to developments in computing power and the rapid evolution of open-source software packages, neural networks, and data science. These developments have ushered in a new era of AI systems in radiation oncology, revolutionizing research and clinical applications (Bouhali et al. 2022; Leary and Basran 2022). Unlike conventional software, AI technologies exhibit greater complexity and can learn from representative and localized data. In human radiation oncology, these AI systems have already demonstrated their potential across various stages of patient care, including deformable registration, treatment simulation, adaptive radiotherapy, auto-segmentation, quality assurance, and modelling (Leary and Basran 2022).

While the veterinary field benefits significantly from these technologies in terms of time and cost savings, caution is warranted in their adoption due to the limited understanding of their full range of applications (Guitian et al. 2023). Nevertheless, several practical applications in veterinary radiation oncology are anticipated in the coming years, including deformable registration, automated segmentation, and adaptive radiotherapy (Leary and Basran 2022).

5 Remote monitoring and telemedicine

Remote monitoring, facilitated by AI technology, enables real-time tracking of an animal's health status (Shaik et al. 2023). By using wearable devices, sensors, and other monitoring tools, veterinarians can remotely monitor vital signs and activity levels (Shajari et al. 2023). AI algorithms analyze the data from such devices to detect abnormalities (Shaik et al. 2023; Shajari et al. 2023). Continuous health monitoring allows veterinarians to identify signs of potential health issues, enabling timely interventions and preventive measures (Shaik et al. 2023). For example, AI algorithms can detect subtle changes in an animal's behavior or physiological parameters that may indicate pain, distress, or the onset of illness (Akinsulie et al. 2024; Shaik et al. 2023). Remote monitoring facilitates prompt diagnosis and treatment by alerting veterinarians to these changes, ultimately improving animal health outcomes (Jiang et al. 2024).

Telemedicine, powered by AI technology, extends the reach of veterinary care beyond traditional clinic settings, enabling remote consultations, diagnoses, and treatment planning (Rezaei et al. 2023). Through telemedicine platforms, veterinarians can communicate with pet owners, assess symptoms, review medical histories, and provide guidance on managing health concerns (Huang and Chueh 2021; Rezaei et al. 2023). AI-driven telemedicine applications leverage advanced algorithms to assist veterinarians in diagnosing and triaging cases remotely (Huang and Chueh 2021). For example, AI-based diagnostic tools can analyze medical images, laboratory results, and other diagnostic data to help veterinarians make accurate assessments and recommendations (Burrell 2023; Huang and Chueh 2021; Rezaei et al. 2023). Telemedicine also facilitates follow-up appointments and ongoing monitoring, allowing veterinarians to track treatment progress and adjust interventions as needed (Huang and Chueh 2021; Rezaei et al. 2023).

The key benefits of AI-enabled remote monitoring and telemedicine are overcoming geographical constraints and providing access to veterinary care in underserved or remote areas (Burrell 2023; Huang and Chueh 2021). By leveraging digital communication technologies, veterinarians can reach clients and patients in isolated regions where traditional veterinary services may be limited (Burrell 2023). AI-based telemedicine platforms enable virtual consultations and remote diagnostic evaluations, removing the need for pet owners to travel long distances to access veterinary care (Sharma et al. 2023). This improves convenience for pet owners and ensures that animals receive timely and appropriate medical attention, regardless of their location (Burrell 2023; Sharma et al. 2023). By breaking down geographical barriers, AI-driven telemedicine expands access to veterinary services, promoting the health and well-being of animals worldwide (Sharma et al. 2023).

6 AI in research and drug development

The advent of AI has revolutionized the pharmaceutical realm to a great extent. Conventional techniques in pharmaceutical research are limited by their dependence on trial-and-error experimentation and their difficulty in precisely predicting the behaviour of novel bioactive compounds (Xu et al. 2021). AI-based algorithms assist in identifying new targets for drug development, such as specific

biochemical or genetic pathways involved in diseases (You et al. 2019). ML precisely predicts small molecules' physical and chemical properties at a level comparable to quantum mechanics. AI is proficient in finding correlations between molecular representations and biological or toxicological activities (Alowais et al. 2023). The synthetic pathways of new drug candidates are efficiently explored using AI-based algorithms. In conjunction with AI, robotics probes the chemical space for novel reactions through automated analysis of reaction feasibility. AI enables rapidly screening a virtual compound library containing billions of molecules within a few days(Álvarez-Machancoses and Fernández-Martínez 2019). Identifying preclinical candidates through an AI-based computational pipeline can be accomplished in a significantly shorter time. Moreover, DL algorithms are currently used to predict native protein folding and analyze protein structures quickly. It also contributes to the novel drug design from the databases of existing therapeutic compounds.

The drug discovery process is a complex, laborious, and time-consuming endeavour. It demands significant capital investments and, in some instances, ends up in failure in the final stages of drug development, leading to significant loss (Blanco-González et al. 2023). AI methodologies, such as ML and NLP, empower and accelerate drug discovery through highly accurate and efficient analysis of extensive databases. The effective application of DL accurately predicts drug compounds' efficacy. The drug discovery process consists of four phases: (i) identification and validation of targets, (ii) screening and refinement of compounds for lead optimization, (iii) preclinical investigations, and (iv) clinical trials (Chan et al. 2019). AI-driven methods are actively employed across various stages of the process to enhance efficiency in terms of time and cost. Real-time image-based cell sorting, cell classification, quantum mechanics calculations for compound properties, computer-aided organic synthesis, molecular design, and prediction of 3D structures for target proteins are the various platforms that utilize AI applications (Nitta et al. 2018; von Lilienfeld 2018). These procedures can be automated and optimized using AI to accelerate the research and development process for drug discovery significantly. AI is highly efficient in sorting and classifying cells based on image analysis, replacing traditional visual inspection due to its inefficiency in handling extensive datasets. The least-squares SVM method is an explicit AI-based approach to categorizing various cell types (Samui and Kothari 2011). Modern Image-Activated Cell Sorting devices depend on electrical, optical, and mechanical cell properties to automate cell sorting at scale. These devices achieve high-speed digital image processing and decision-making by implementing AI-based convoluted DNN algorithms (Ho et al. 2019). AI is pivotal in predicting the physical properties for effective drug design, particularly concerning bioavailability, toxicity, and bioactivity (Lynch et al. 2007).

The molecular representations employed in AI drug design algorithms encompass various inputs, such as molecular fingerprints, molecular graphs, simplified molecular-input line-entry system (SMILES) strings, potential energy measurements, Coulomb matrices, etc., undergoing a DNN training phase for accurate processing (Sanchez-Lengeling and Aspuru-Guzik 2018). The algorithm, such as molecular fingerprints and coulomb matrices, assesses biomolecules' physico-chemical and toxicological properties when selecting lead compounds. AI-based QSAR approaches, including linear discriminant analysis, random forest, and SVMs, are employed to identify potential drug candidates to expedite the process (Zhang et al. 2017). The prediction of drug-target binding affinity is crucial for anticipating drug-target interactions (Öztürk et al. 2018).

One notable area where AI has made significant advancements is in veterinary drug development (Blanco-González et al. 2023). AI-driven technologies are revolutionizing the process of discovering, designing, and developing new drugs for treating and managing various diseases in animals (Niazi 2023). AI offers various applications in veterinary drug development, revolutionizing traditional drug discovery and development processes (Paul et al. 2021). One significant application lies in virtual screening, where AI algorithms analyze extensive databases of chemical compounds to pinpoint potential drug candidates with therapeutic efficacy against particular diseases (Álvarez-Machancoses and Fernández-Martínez 2019; Han et al. 2023). AI-driven predictive modelling techniques, including ML and DL, empower the swift screening of millions of compounds, markedly expediting the drug discovery process (You et al. 2019). AI algorithms analyze complex biological data to identify molecular targets associated with animal disease pathways (Vora et al. 2023). By unraveling the fundamental mechanisms of diseases, AI plays a pivotal role in identifying novel drug targets and validating existing ones. This process paves the way for developing more precise and effective therapies, ultimately improving patient outcomes (Niazi 2023).

Furthermore, AI has a role in pharmacokinetic and pharmacodynamic modelling, optimizing dosing and predicting animal efficacy and safety profiles (Vora et al. 2023; Wu et al. 2024). AI-driven predictive modelling techniques analyze physiological and pharmacological data to simulate distribution, metabolism, and elimination processes in animal species, guiding the design of optimal drug formulations and dosing strategies (Wu et al. 2024). AI integration into veterinary drug development offers several benefits to researchers, pharmaceutical companies, veterinarians, and animal patients (Lungren and Wilson 2022). AI-driven virtual screening helps to identify potential drug candidates, saving time and cost compared to traditional high-throughput screening methods (Qureshi et al. 2023). Additionally, AI enhances the efficiency and accuracy of identification and

validation processes, leading to the development of targeted and personalized animal therapies (Akinsulie et al. 2024; Vora et al. 2023). By leveraging large-scale biological datasets and advanced computational algorithms, AI enables researchers to gain deeper insights into the molecular mechanisms of diseases, facilitating the discovery of innovative drug targets (Visan and Negut 2024; Vora et al. 2023). Moreover, AI-driven predictive modelling techniques improve the prediction of drug efficacy and safety profiles in animals, minimizing the risks associated with drug development. By optimizing dosing regimens and predicting adverse drug reactions (Paul et al. 2021), AI helps pharmaceutical companies streamline preclinical testing and accelerate the translation of promising drug candidates from the laboratory to clinical trials (Paul et al. 2021; Yang and Kar 2023).

AI is revolutionizing veterinary medicine by offering innovative solutions to address AMR. AI algorithms analyze vast proteomic, genomic, and metabolomic data datasets to identify potential drug targets for combating AMR. By deciphering complex microbial interactions and host-pathogen dynamics, AI aids in pinpointing vulnerabilities in pathogenic microorganisms, facilitating the development of targeted therapeutic interventions (Akinsulie et al. 2024). AI-driven approaches, such as ML and computational modelling, streamline the antibiotic discovery process by generating virtual libraries of candidates based on chemical structures and pharmacological properties. These candidates undergo further optimization and testing to identify promising leads for drug development (Akinsulie et al. 2024). Moreover, AI-powered decision support systems can help users optimize antibiotic use by analyzing patient data and microbial susceptibility patterns in real-time, enabling personalized treatment recommendations tailored to individual patients. AI models trained on large datasets predict the likelihood of AMR in clinical isolates, allowing for early detection of emerging resistance trends and proactive intervention strategies (Akinsulie et al. 2024). AI offers a transformative approach to accelerate drug discovery, optimize antibiotic use, and combat AMR, safeguarding animal and human health.

An NLP system can automate the extraction of essential data on proper antimicrobial use, including clinical indications, antimicrobial selection, dosage, and therapy duration. It analyzed over 4.4 million animal patient clinical records in Australia, focusing on consultations involving antimicrobial use (Hur et al. 2022). The primary objective was to gain insights into antibiotic usage patterns and the underlying reasons for their administration at a population level. However, the analysis revealed a significant limitation: only around 40% of the records contained comprehensive information regarding the rationale for prescribing antimicrobials, along with details on dosage and treatment duration (Hur et al. 2022). This gap poses a substantial challenge for data extraction, even with advanced NLP and DL techniques (Hur et al. 2022). While NLP and DL hold promise for overcoming such obstacles by extracting valuable insights from free-text clinical records, their efficacy depends on the availability of essential data within the records themselves. In cases where critical information is inadequately recorded, these technologies face limitations in fully capturing the nuances of antimicrobial prescribing practices. Thus, addressing the issue of incomplete data recording in clinical settings remains a crucial aspect to enable the effective utilization of advanced data extraction techniques in veterinary medicine (Hur et al. 2022).

Establishing MRLs for veterinary medicines is critical in safeguarding the human food supply (Pratiwi et al. 2023). Regulatory authorities provide guidelines for setting MRLs, which are adhered to by drug sponsors in each jurisdiction (Pratiwi et al. 2023; Zad et al. 2023). In the typical drug approval steps, residue limits are customized for particular species and matrices. Consequently, MRLs are often lacking for species other than those specifically targeted during approval. One of the study has evaluated the feasibility of predicting MRLs reliably for under-represented groups using ML techniques (Zad et al. 2023). By leveraging ML algorithms, we can accurately forecast and estimate MRLs even in cases where they have not been formally established. This has the potential to significantly reduce the necessity for live animal use, lower associated costs, and alleviate the overall research burden involved in determining new MRLs (Zad et al. 2023). The utilization of ML in predicting MRLs for diverse food commodity groups holds promise for streamlining regulatory processes, enhancing efficiency, and promoting more sustainable practices in veterinary medicine (Zad et al. 2023).

Despite several issues, the future of AI in veterinary drug development is promising (Zhang et al. 2017). Advances in AI technologies, coupled with the increasing availability of large-scale biological datasets and collaborative research initiatives, are poised to accelerate innovation in animal healthcare (Bohr and Memarzadeh 2020). By harnessing the power of AI-driven predictive modelling, virtual screening, and target identification techniques, researchers can develop safer, more efficacious, and personalized therapies for animals, addressing unmet medical needs and improving the quality of veterinary care worldwide (Chan et al. 2019; Pratiwi et al. 2023). As AI continues to evolve and integrate into veterinary drug development pipelines, it can revolutionize animal healthcare and transform the lives of countless animal patients.

7 Challenges and ethical considerations

Along with the potential benefits, several issues must be considered to realize the full potential of AI in veterinary practice (Lustgarten et al. 2020). One of the foremost challenges in leveraging AI in veterinary medicine is the quality of available

data (Anholt et al. 2014; Nie et al. 2018). Large and diverse datasets are required for training AI algorithms effectively (Lustgarten et al. 2020). Unlike human healthcare, where EHRs are more standardized and readily available, veterinary medical data are often fragmented, heterogeneous, and stored in various formats (Anholt et al. 2014; Santamaria and Zimmerman 2011). This lack of standardized data poses a significant obstacle to developing robust AI models that accurately predict and diagnose veterinary conditions. Veterinary patient records predominantly contain free-text entries lacking standardized clinical coding or fixed vocabulary (Anholt et al. 2014; Nie et al. 2018). Text-mining techniques enable the identification of pertinent cases within the unstructured data and facilitate the introduction of organization and structure to the records (Anholt et al. 2014). Text miners have previously demonstrated a sensitivity of 87.6% and a specificity of 99.3% in retrieving cases with enteric signs compared to the assessments made by human reviewers (Anholt et al. 2014). This indicates that the text-mining tool exhibited high accuracy in correctly identifying cases of enteric syndrome, with a low rate of false positives and negatives.

Moreover, the cost associated with developing and implementing AI-powered technologies presents another significant challenge for veterinary practices (Santamaria and Zimmerman 2011; Zhang et al. 2024). The initial investment required to acquire AI infrastructure, develop custom algorithms, and integrate AI systems into existing workflows can be substantial (Zhang et al. 2024). For many small and medium-sized veterinary clinics, the financial burden of adopting AI technology may be prohibitive, limiting access to advanced diagnostic and treatment capabilities. Ethical considerations also play a crucial role in adopting AI in veterinary medicine (Gerke et al. 2020; Cohen and Gordon 2022). As AI algorithms become highly influential in clinical decision-making, critical issues such as transparency and accountability come to the forefront of concern (Naik et al. 2022). Biases inherent in training data can result in discriminatory outcomes, potentially affecting the quality of care delivered to animal patients (Zhang et al. 2024).

Additionally, concerns have been raised about the displacement of veterinary professionals due to AI-based technologies, raising questions about the ethical implications of automation in the veterinary workforce (Bouchemla et al. 2023). Furthermore, the lack of regulatory systems and oversight mechanisms for AI in veterinary medicine presents a significant challenge. Unlike human healthcare, where regulatory bodies such as the FDA oversee the approval and monitoring of medical devices and AI algorithms, veterinary medicine lacks similar regulatory structures (Benjamens et al. 2020; Cohen and Gordon 2022). Without clear guidelines for AI systems in veterinary care, there is a risk of inadequate quality control, patient safety concerns, and legal liabilities (Hooper et al. 2023). Addressing such challenges requires collaboration between stakeholders, including veterinary professionals, researchers, policymakers, and industry leaders.

Initiatives to improve data sharing and interoperability, such as developing standardized veterinary medical ontologies and EHR systems, can help overcome data-related challenges and facilitate the development of AI-driven solutions (Lustgarten et al. 2020). Moreover, innovative financing models and partnerships between veterinary clinics, research institutions, and technology companies can help alleviate the financial barriers to AI adoption in veterinary practice (Hangl et al. 2023). By pooling resources and sharing costs, veterinary practices can access AI technologies and expertise that would otherwise be out of reach (Bouchemla et al. 2023; Hangl et al. 2023). Measures such as algorithmic auditing, bias detection, and explainable AI can help mitigate ethical risks and ensure that AI systems align with professional standards and ethical principles in veterinary care (de Manuel et al. 2023; Lungren and Wilson 2022). Lastly, establishing regulatory frameworks tailored to the unique needs and challenges of veterinary AI is essential for ensuring these technologies' safe and responsible use (Coleman and Moore 2024). Regulatory agencies, professional associations, and industry consortia should collaborate to develop guidelines, standards, and certification programs to govern AI systems' development, evaluation, and deployment in veterinary medicine (Hooper et al., 2023).

Our comprehension of the capabilities of AI models like ChatGPT in veterinary fields is currently in its infancy (Dave et al. 2023; Coleman and Moore 2024). We urgently need to deepen our understanding of these models to unlock their full potential, encourage responsible utilization, and ensure alignment with educational objectives (Abani et al. 2023; Jiang et al. 2024). One study assessed the knowledge and response consistency of ChatGPT by administering true/false and multiple-choice questions from fifteen courses of third-year veterinary students (Coleman and Moore 2024). The study revealed a lower overall performance score, indicating the need for caution among veterinarians when retrieving data from such AI-based platforms (Coleman and Moore 2024).

8 Balancing automation and professional expertise

Striking a balance between AI-driven automation and the expertise of veterinary professionals is crucial to ensuring the highest standards of care (Appleby and Basran 2022). This collaborative model acknowledges that while AI can enhance efficiency and accuracy in diagnosis and treatment, it cannot replace the nuanced judgment and clinical acumen of experienced veterinarians (Jiang et al. 2024). In this collaborative approach, AI will act as an essential tool that complements the skills and knowledge of veterinary professionals (Bouchemla et al. 2023). AI algorithms

can uncover patterns that may elude human observation, thereby aiding veterinarians in making well-informed decisions and delivering better patient care (Schofield et al. 2021; Alowais et al. 2023). For example, AI-powered diagnostic tools can help veterinarians interpret imaging studies, detect subtle changes in laboratory values, and predict disease outcomes (Burti et al. 2020; Celniak et al. 2023; Joslyn and Alexander 2022). However, it is necessary to recognize that AI algorithms are not infallible and may have limitations, particularly in complex and nuanced cases (Bouchemla et al. 2023). Therefore, the collaborative approach involves veterinarians working alongside AI systems, critically evaluating their recommendations, and providing context-specific insights that AI may lack. By combining the strengths of both humans and machines, veterinary professionals can ensure that patients receive the most accurate diagnoses and effective treatments (Bouchemla et al. 2023).

The concept of a veterinarian-AI partnership emphasizes the symbiotic relationship between veterinary professionals and AI technologies (Appleby and Basran 2022). In this partnership model, AI is not a replacement for veterinarians but rather a complementary tool that enhances their capabilities and extends their reach (Currie et al. 2023). The key benefit of the veterinarian-AI partnership is the potential to leverage AI's computational power to process extensive data quickly and efficiently (Currie et al. 2023). This enables veterinarians to make evidence-based decisions in real-time, resulting in accurate diagnoses and personalized treatment plans (Bouchemla et al. 2023). Moreover, AI algorithms learn and improve continuously, adapting to new information and refining their diagnostic accuracy. By incorporating feedback from veterinary professionals, AI systems can become increasingly sophisticated and effective in assisting with clinical decision-making (Currie et al. 2023). However, it is necessary to identify the limitations of AI and the importance of human oversight in the veterinarian-AI partnership. Veterinary professionals play a crucial role in validating AI-generated recommendations, ensuring their clinical relevance, and providing context-specific insights that AI may overlook (Currie et al. 2023). Additionally, veterinarians communicate effectively with clients, interpret AI-generated findings, and integrate them into comprehensive patient care plans.

As the integration of AI technologies advances, there is a pressing need to address regulatory and ethical issues to ensure the safe and effective use of AI systems in animal health (Bouchemla et al. 2023). One of the primary challenges is overcoming regulatory deficits that may hinder the widespread adoption of AI technologies in veterinary medicine (Bellamy 2023). Regulatory bodies should generate frameworks tailored explicitly to the use of AI systems in veterinary practice (Hooper et al. 2023). The potential for incorrect decisions by AI algorithms and the ambiguity surrounding liability for erroneous AI decisions raise concerns within the veterinary community (Cohen and Gordon 2022). Veterinary professionals, policymakers, and legal experts must collaborate to establish clear guidelines for accountability and liability in cases involving AI systems in diagnostic or treatment decisions (Hooper et al. 2023). This may involve defining the responsibilities of veterinarians, AI developers, and pet owners in case of adverse outcomes resulting from AI-assisted procedures. Developing a robust regulatory structure for AI systems in veterinary practice is essential to ensure patient safety, uphold professional standards, and mitigate potential risks (Bellamy 2023).

Furthermore, the veterinary profession may need to revise its ethical guidelines for integrating AI technologies (Bellamy 2023). Ethical considerations surrounding issues such as patient autonomy, informed consent, and the veterinarian-client-patient relationship may need to be reexamined in AI-assisted veterinary care (Hooper et al. 2023). Veterinarians must uphold the highest ethical standards while leveraging AI technologies to enhance patient care (Marks 2024). In addition to regulatory and ethical considerations, education and training will prepare veterinary professionals to use AI systems in practice (Currie et al. 2023). Continuing education programs should incorporate training on AI technologies and critical appraisal of AI-generated recommendations to ensure veterinarians can access knowledge and skills to effectively integrate AI into their clinical workflows (Marks 2024).

Conclusion and prospects

AI accelerates the pace of veterinary research and drug development. ML algorithms can analyze complex veterinary data and identify potential drug candidates. This expedites the discovery process and contributes to developing novel therapies for various veterinary conditions. The prospects of AI in veterinary science are undeniably transformative, redefining animal healthcare. AI offers a spectrum of benefits from rapid and precise diagnostics to personalized treatment plans and proactive disease management. However, embracing this future requires careful navigation of ethical considerations and a collaborative synergy between human expertise and AI capabilities.

AI-driven diagnostic tools can analyze massive datasets with unprecedented speed and accuracy. This accelerates the diagnostic process and enhances precision, allowing veterinarians to make informed decisions swiftly. AI algorithms excel at identifying patterns and trends. In veterinary science, predictive models can anticipate disease outbreaks, track zoonotic disease spread, and even predict individual health risks for animals. This proactive approach enables preventive measures and early interventions, ultimately improving animal health. Tailoring treatment plans to individual animals becomes more feasible with AI. AI can recommend personalized medications, dietary plans, and

rehabilitation strategies by analyzing genetic, environmental, and historical data. This individualized approach optimizes treatment outcomes and enhances the well-being of each animal. AI facilitates remote monitoring, allowing veterinarians to track animals' vital signs, detect anomalies, and provide timely interventions, even from a distance. AI-powered telemedicine enables consultations and follow-ups, overcoming geographical barriers and ensuring that animals receive continuous care.

Addressing regulatory deficits and ethical issues surrounding AI use in veterinary practice is essential while safeguarding animal health and welfare. By developing clear regulatory frameworks, fostering ethical discussions, and providing comprehensive education and training, the veterinary profession can embrace AI as an important tool in advancing veterinary care. AI can reform how we diagnose and treat animals, with applications ranging from analyzing medical images for faster and more accurate diagnoses to developing personalized treatment plans. AI-powered tools can also predict disease outbreaks, improve surgical precision through robotic assistance, and accelerate the discovery of new medications. These advancements promise significant benefits, including improved accuracy in veterinary care, earlier disease detection, and faster animal recovery times. However, challenges remain. Large datasets of veterinary medical data are required to train AI models effectively, and the cost of implementing these technologies can be a hurdle for veterinary practices. Ethical considerations surrounding bias in algorithms and potential job displacement for veterinarians need careful attention. Additionally, regulatory frameworks must be established for AI's ethical and safe use in animal healthcare. By acknowledging these issues and working towards solutions, we can tap the transformative potential of AI and ensure a future of improved animal health and well-being. As we unleash AI in veterinary science, a new era of compassionate, data-driven, and efficient animal care emerges, promising a healthier future for our animal companions.

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Ethical Approval

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CRediT authorship contribution statement

Khan Sharun: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization; S. Amitha Banu: Writing – review & editing, Writing – original draft; Merlin Mamachan: Writing – review & editing, Writing – original draft; Laith Abualigah: Writing – review & editing, Writing – original draft; A. M. Pawde: Writing – review & editing, Writing – original draft; Kuldeep Dhama: Writing – review & editing, Writing – original draft.

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Biosynthesis of secondary metabolites in aromatic and medicinal plants in response to abiotic stresses: A review

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ABSTRACT

Climate change has massive consequences on non-living factors in the environment, resulting in irregular precipitation, fluctuating atmospheric temperature, and variations in humidity. These changes cause biotic and abiotic stresses; plants must have defense mechanisms to survive. Therefore, plants divert some synthesized energy towards producing numerous plant secondary metabolites (PSMs), *viz*., flavonoids, alkaloids, and essential oils. These compounds act as protections for the plants, helping them to survive under stressful conditions. Medicinal and aromatic plants (MAPs) are sessile organisms that are not immune to harmful consequences of various abiotic stresses in which the PSMs have an important role in acting against the adverse effects. In this regard, the MAPs have a coherent defense mechanism for abiotic stresses. The secondary metabolites produced by these plants are useful as medicines and aromatic products for humans. However, not all stresses produce high secondary metabolites, as their production is highly specific to certain stresses. This review provides a comprehensive understanding of secondary metabolite production under various stressful conditions, including extreme temperature, drought, water logging, salinity, harmful radiation, elevated levels of ozone and CO_2, heavy metals, and agrochemicals on MAPs. Additionally, the production of these compounds can be modified by subjecting plants to various stressors. Many authors have reported on PSMs in MAPs, which need to be well documented and exploited for humankind.

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1 Introduction

Since time immemorial, plants have provided food, medicine and shelter for mankind. These generate a diverse kind of valuable primary and secondary metabolites. The primary metabolites have a vital role in the growth and development of plants, while the secondary metabolites participate in defense mechanisms against biotic and abiotic stresses. Climate change mainly, water stress, and heat stresses are found to affect plant morphology and physiology (Rodrigues et al. 2021), where the medicinal and aromatic plants are much more vulnerable to adverse conditions as per their biodiversity (Qaderi et al. 2023; Rahman et al. 2023). Human anthropogenic activities like rapid urbanization, industrialization, deforestation, automobiles, etc., lead to extreme changes in climatic conditions, resulting in the prevalence of unseasonal rainfall, extreme temperature, high wind, heavy snowfall, inundation of sea water, flood, drought, and rising pests population posing a significant risk for many plant species on earth including medicinal and aromatic plants (Figure 1). A defense response is evolved in plants by producing secondary metabolites (PSMs) to avoid cell and tissue injury (Yeshi et al. 2022). PSMs like terpenoids, alkaloids, and phenolic biochemicals form the major PSM group. It is reported that the medicinal and aromatic plants, as the sessile group of plants, are most evolved with the production of PSMs under even mild abiotic stresses without adversely impacting growth and development, while in some cases, the plant's performance may improve (Jampílek and Kráľová 2023). PSMs evolution may increase or decrease as per the intensity of environmental stresses (Liu et al. 2023). Secondary metabolite production was increased in *Thymus vulgaris*, *Rosmarinus officinalis*, and *Mentha pulégium* with the increase in temperature and water stress up to 50%, while further extreme temperature and water pressure at 70% the levels of the compound depleted in plants (Laftouhi et al. 2023). The findings explained that the MAPs generate secondary metabolites (SMs) under non-living stresses to avoid interruption in the physiological process and damage to cells and tissues, though the extreme environment is quite detrimental. The growth and productivity of MAPs are strongly interrelated with the change in external climatic factors. Various researchers reported that plants have various responses like gene expression, physiological manipulation, architectural modification, and production of SMs to tackle the harmful effects of biotic and abiotic stresses (Mareri et al. 2022; Balfagón et al. 2022). The MAPs produce diverse secondary metabolites in low molecular weight such as alkaloids, flavonoids, phenolic compounds, steroids, terpens, and anthocyanins, which are important for adaption, protection, and environment adjustment (Wink 2003). The production of these biochemical compounds is the main way they adapt to their environment (Figure 2) (Verma and Shukla 2015).

Medicinal and aromatic plants are cultivated for roots, leaves, bark, seeds, flowers, and stems, which are rich in secondary metabolites used for medicine, neutraceutical, perfumes, food flavour, soap, or cosmetics. As per the Food and Agriculture Organization (FAO), 60% of the world's population depends on these herbs for wellbeing and beauty (Mahajan et al. 2020), leading to huge exploitation. In addition, the alarming climate change has a dangerous impact on plant morphology and physiology of MAPs, which may cause their extinction. So, it is critical to study the impact of abiotic stressors on MAPs and to draw the attention of research personnel for its sustainable use in the future. This article elaborates on critical secondary metabolites in medicinal and aromatic plants and their synthesis under different abiotic stresses.

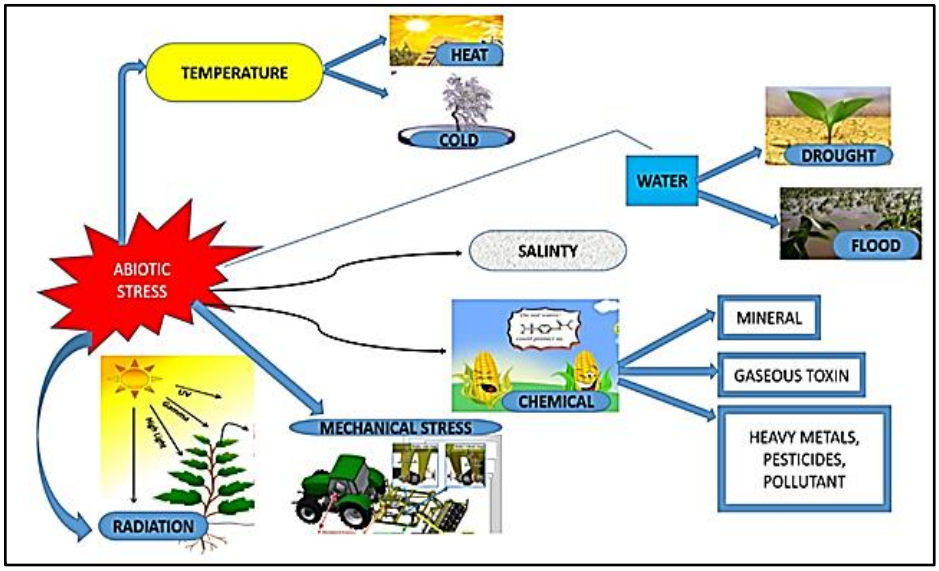

Figure 1 Various abiotic stresses affecting the growth and development of the plant

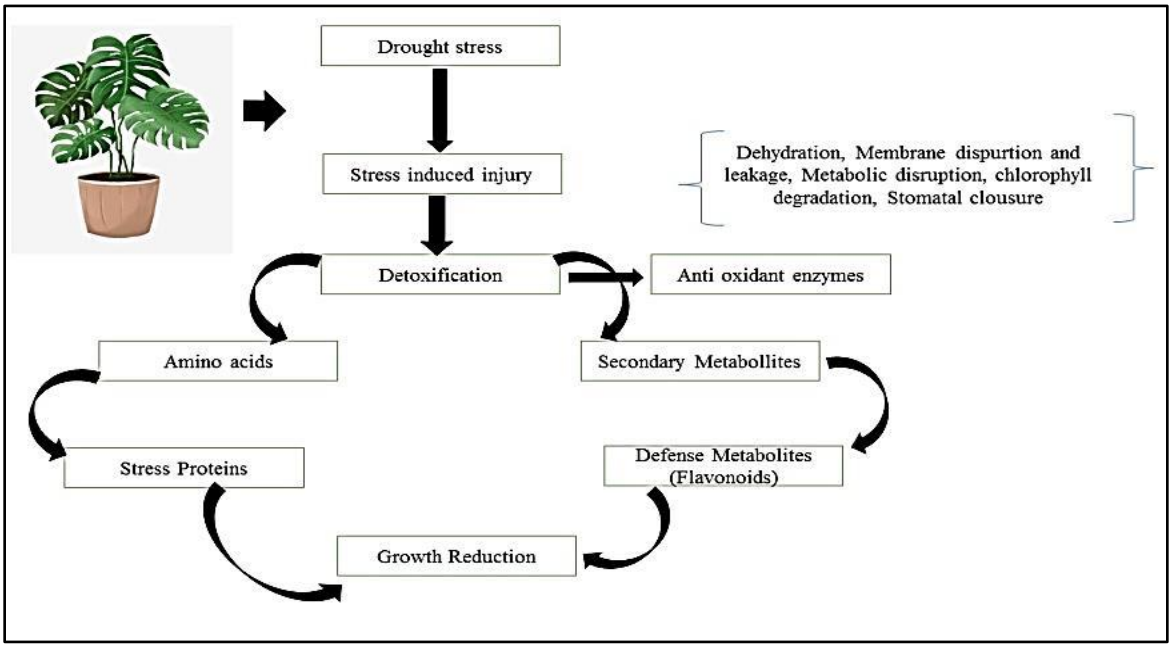

Figure 2 Illustrations of the effect of stress on plants growth and development

2 Secondary Metabolites and their Importance in Plants

Secondary metabolites (SMs) present in plants are among the most extensively studied chemicals due to their significant role in health, food, and beauty. The SMs have a direct correlation with morphological and physiological processes in plants. The plant is stunted under adverse conditions, which can be recovered by secondary metabolites generated in the plant body (Punetha et al. 2022). Several primary metabolites are produced in plants and can be easily extracted and crystallized, while the secondary metabolites are generated in extremely small quantities, and their extraction is complex and energy-intensive. Antibiotics and hormones are examples of secondary metabolites that are crucial for plant survival and growth. Plant secondary metabolites play more than one role in the plant metabolism process (Pagare et al. 2015). These metabolites impart a protection system when plants suffer biotic and abiotic stressors. Phenyl amides are formed, and polyamines are accumulated in beans and tobacco under abiotic stresses, explained the phytochemicals act as antioxidants to protect plants (Edreva et al. 2000). Likewise, accumulation of anthocyanin is observed in plants under the influence of drought, high light intensity, UV, wounds, disease attack, blue light expose, sugar and nutrient deficiencies (Winkel-Shirley 2001). The SMs are also crucial in plant pollination, chemical defense activities, molecule signaling, adaptation, seed dispersal, protection from insects, diseases, herbivores, and allelopathic injury (Pandita and Pandita 2021). The production and concentration of SMs are significantly affected by biotic factors, including insect and disease attacks (Taiz and Zeiger 2006). Plant secondary metabolites can also protect against various organisms, including fungi, bacteria, viruses, nematodes, and insects (Tak and Kumar 2020). Examples of antimicrobial secondary metabolites plants use to defend against invaders include phytoalexins and phytoanticipins.

3 Use of SMs as medicine

In the present-day scenario, a large percentage of the global population still uses traditional medicine practices based on herbal medicines, which rely on the therapeutic qualities of plants (WHO 2013; Hosseinzadeh et al. 2015). Many plant-derived polyphenolic nutraceuticals or pharmaceuticals undergo initial transformations in the intestine via microbiota and enterocyte enzymes before being absorbed at the level of colonocyte and enterocyte. This process confers a broad range of consumer benefits, such as substantial protection against various pathogens, including bacteria and protozoa (Marin-Bruzos and Grayston 2019; Marin et al. 2015). This shows that the intricate relationship between secondary metabolites and human health underscores the continued importance of exploring their multifaceted roles in plant biology and therapeutic applications.

One of the untouched potent natural sources of antibacterial drugs is secondary plant metabolites. However, merely an insignificant fraction (< 1%) of tropical plant species on the planet has been subjected to phytochemical and pharmacological screening (Keita et al. 2022). Several bioactive secondary metabolites and their derivatives are produced by plants which have immense potential to be used as medications for the treatment of many disorders, such as cancer and neurological conditions, bacterial, fungal, and viral infections, and showed encouraging outcomes in the battle against

the spread of antibiotic-resistant bacteria (Lahlou 2013). Many of these metabolites have already been released onto the market or are currently being evaluated in clinical trials. Berberine, a natural compound obtained from plants, including *Rhizoma coptidis*, is effective against methicillin-resistant *Staphylococcus aureus* (MRSA) by preventing the formation of biofilms. Additionally, berberine has beneficial synergistic effects when taken with other antibiotics (Zuo et al. 2012; Chu et al. 2016; Zhang et al. 2020). Sulfur-containing allicin has been discovered to have wide antibacterial activity in contrast to gram +ve and -ve bacteria, *Streptococcus* sp., *E. coli*, MRSA, and *Salmonella enterica* (Barbieri et al. 2017). It is a derivative of raw garlic (*Allium sativum*). S-allylmercap to alteration of thiol-containing proteins by allicin reasons a reduction in glutathione levels, the progress of protein aggregation, and the deactivation of critical enzymes in bacteria (Wallock-Richards et al. 2014; Müller et al. 2016; Nakamoto et al. 2020). Piperine is an alkaloid found in the Piper species, such as *Piper longum* and *P. nigrum*, and has strong antimicrobial activity against both Gram+ve (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-ve bacteria (*Escherichia coli* and *Salmonella* sp.). It is an efflux pump inhibitor in Staphylococcus aureus when administered with ciprofloxacin (Hikal 2018). Ajoene, an organo-sulfur compound found in oil-macerated garlic, demonstrates antibacterial activity against various gram +ve and -ve bacteria (Bhatwalkar et al. 2021), and its mechanism of action is like allicin (Han et al. 2011). Eugenol, a hydroxyphenyl propene found in essential oils from the Lauraceae, Lamiaceae, Myristicaceae, and Myrtaceae families, exhibits various modes of action, such as disrupting the cell membrane of *Salmonella typhi*, inhibiting biofilm formation and enterotoxin production in *Streptococci*, and decreasing gene expression related to *S. aureus* contamination (Yadav et al. 2015). Moreover, eugenol hinders the synthesis of bacterial virulence agents like pyocyanin, violacein, and elastase (Marchese et al. 2017; Mak et al. 2019). Several plant species, including bananas, groundnuts, grapevines, pines, beans, pomegranates, and soybeans, contain resveratrol, a naturally occurring polyphenolic antioxidant with antibacterial properties against various Gram-negative and Gram-positive foodborne bacteria (Keita et al. 2022). Resveratrol inhibits toxin production, suppresses gene expression, impedes biofilm formation, interferes with motility, and disrupts quorum sensing in various fungal, bacterial, and viral species (Ma et al. 2018). Continued research into these natural compounds could lead to new treatments for infectious diseases and help address challenges posed by antimicrobial resistance.

4 Influence of Abiotic Stresses on Secondary Metabolites (SMs) Production

The ability of plants to produce and aggregate (i.e., accumulate) phytochemicals is profoundly influenced by abiotic stresses such as temperature, soil, light intensity, and humidity (Radušienė et al. 2012). In addition, the production of plant secondary metabolites (SMs) can be influenced by minerals, radiation, heavy metals, and gaseous toxins (Akula and Ravishankar 2011). In order to adapt to varying environmental conditions, plants require acclimatization and adaptation, which leads to molecular, biochemical, physiological, and morphological responses. These responses may alter plant metabolic activity to decrease or repair damage caused by stress. This mechanism aims to safeguard the species' continual survival against certain growth conditions (Kapoor et al. 2020). In response to adverse environmental conditions, plants commonly produce reactive oxygen species (ROS), which are the final products of all stresses, such as superoxide (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) (Sharma et al. 2021). These can lead to cell damage by initiating an oxidative chain reaction called oxidative stress. As a countermeasure, plants employ secondary metabolism and other enzymatic and non-enzymatic processes to produce and store defensive chemicals. In nature, the intricate strategies employed by plants in response to environmental stresses highlight their remarkable capacity to adapt and survive through the synthesis and accumulation of protective phytochemicals.

Plants have limited mobility and a weak defense system, so they either change their orientation by moving or producing chemicals to defend themselves against environmental stress. When plants are under various environmental stresses, they typically produce and use more enzymes that help protect themselves, synthesizing secondary metabolite compounds. Chalcone synthase (CHS) and phenylalanine ammonia-lyase (PAL) are the enzymes that are essential in the synthesis of flavonoids. PAL (EC 4.1.3.5) plays a pivotal function in the defensive mechanisms of plants by producing phenol and lignin, while CHS (EC 2.3.1.74) is primarily responsible for the synthesis of flavonoids (Blanco-Ulate et al. 2015) (Figure 3).

Polyphenolic compounds, including flavonoids, proanthocyanidins, phenolic acids, and anthocyanins, can effectively reduce the harmful effects of salinity (Hichem and Mounir, 2009). This is because phenolic compounds possess antioxidant properties and function as ROS hunters. The production of these compounds typically occurs in response to biotic or abiotic stresses (D'Souza and Devaraj 2010). The synthesis and deposition of secondary metabolites are controlled by specific genes activated during the transcription stage. Certain transcription factors regulate the production and accumulation of these metabolites, with the total quantity depending on the expression of these genes. This process can produce numerous secondary metabolites through bioregulators and elicitors (Verma and Shukla 2015). This understanding enhances our knowledge of plant adaptation strategies and holds promise for applications in biotechnology and agriculture through controlled metabolite production (Table 1).

Table 1 Compilation of certain secondary metabolites and their respective roles

TERPENES		
Quinone	Helps in oxidation-reduction reaction	Yang et al. 2022
Pyrethroids	Highly toxic to insects	Meijar et al. 2024
β- pinene, α-pinene, limonene and myrcene	Toxic to numerous insects and serious pests of conifers	Nikolić et al. 2024
Gossypol	Repellent to herbivores in cotton	Xie et al. 2024
Abscisic acid	A PGR, Stomatal closure, dormancy, abscission	Kumar et al. 2024
Abietic acid	Powerful new anticancer drug	Ahmad et al. 2024
Gibberellins	One of the major groups of phytohormones, role in seed germination	Hussain et al. 2024
Phorbol	Toxic to herbivorous mammal	Medina-Rodelo et al. 2024
Sterols	Components of cell membrane; Retard the permeability of small molecules by retarding the motion of fatty-acid chain	Samanta et al. 2024
Limonoids	Anti-herbivore compounds	Rzyska et al. 2024
Azadirachtin	Highly toxic to insects	Sarkar et al. 2024
Cardenolites	Used in the treatment of heart diseases	Akanmu et al. 2021
Saponins	Act as fungicide	Morcia et al. 2022
Yamogenin	Used in making birth control pills	Vishwakarma et al. 2022
Carotene and xanthophylls	Significant role in light-harvesting and shielding chlorophyll molecules against photo-oxidation	Sachdev et al. 2021
Rubber	It is a polyterpene from the latex of *Hevea brasiliensis*	Tran et al. 2023
PHENOLIC COMPOUNDS		
Protocatechuic acid and catechol	Protect onion bulbs against Smudge disease	Nag et al. 2024
Coumarins	Inhibit the growth of micro-organisms; with scopoletin are inhibitors of seed germination and cell elongation; stimulate the IAA oxidase	Wang et al. 2023
Lignin	Most abundant organic substance in plants, distributed in cell walls, tracheid, and vessels elements of the xylem, provides tensile strength and cementing of cell walls	Ghorbani et al. 2024
Anthocyanin	Get dissolved in the cell sap of epidermal cells and impart a bright color to flowers	Yoshida 2024
Quercetin	Bright yellow color of lemon juice	Tahosin et al. 2024
Flavonoids (flavones and flavanols)	Distributed in epidermal cells of green leaves and stems, they serve as UV-absorbing pigments, i.e., harmful to cells; legumes and nitrogen-fixing symbionts interact through substances excreted into the soil by legume roots	Guo et al. 2024
Tannins	A second large category of plant phenolics and astringent in taste, distributed in the cell sap, cell walls, barks, and leaves, accumulate in dead tissues; rich in unripe fruits; protect the plant against desiccation, decay, and injury by animals and microbes attack	Hameed et al. 2020
ALKALOIDS		
Anabasine	Synthesized in shoot of *Nicotiana glauca*	Zenkner et al. 2019
Ricine	Found in castor seeds	Yadav et al. 2022
Cocaine- atropine	Natural cocaine	Zamarripa et al. 2024
Reserpine	Collected from *Rauwolfia serpentine* and used for curing hypertension	Bankar et al. 2024
Strychnine	Obtained from *Strychnousnux-vomica;* used as a poison for rats	Sadhunavar et al. 2015
Cinchonine and quinine	Extracted from the bark of Cinchona and used as a drug against malaria	Parveen et al. 2024
Colchicines	Inhibit the formation of spindle fiber formation during cell division; used in polyploidy formation	Ramirez-Castillo et al. 2024
Glycosinolates	Characteristic smell and taste of the members of the family Brassicaceae;	Raffo et al. 2024
Porphyrins	Constituent of chlorophyll and phytochrome	Ko et al. 2024

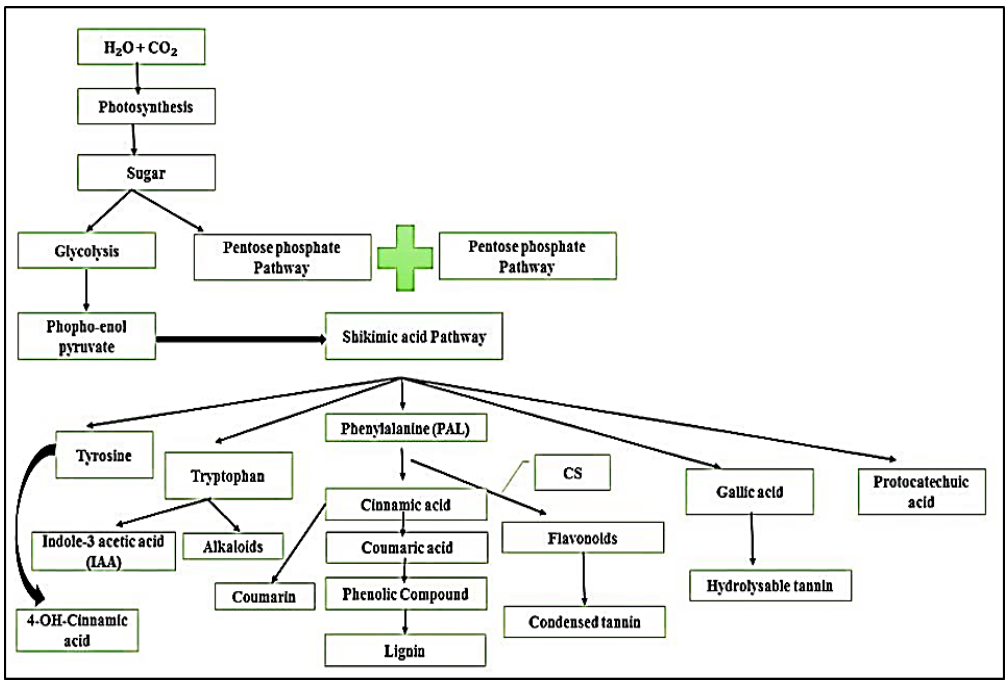

Figure 3 The concept of the Shikimic acid pathway

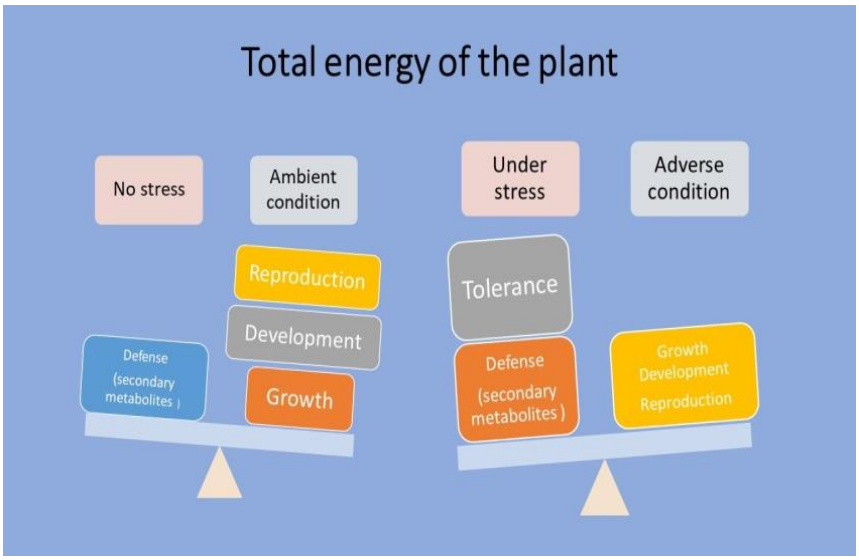

Figure 4 Secondary metabolites and the source sink balance hypothesis

4.1 CO_2 concentration and its influence on SMs

The concentration of CO_2 in the air has a substantial influence on how plants utilize energy and develop. The equilibrium of carbon and nutrients, especially nitrogen, influences multiple growth, development, and differentiation processes. The alterations influence the relationship between the source-sink systems in the proportion of carbon allocated to growth, carbon-based secondary or structural components, and total non-structural carbohydrates (Figure 4). In the current climate change scenario, greenhouse gases, mainly CO_2, are rising sharply. Consequently, the C:N ratio is inappropriate, harming growth over time. Depiction of Figure 4 revealed that the allocation of resources between sources and sinks is influenced by the rise in CO_2 levels and the limited availability of nitrogen. Further, CO_2 influences source strength more than sink strength, while nutrient stress affects sink strength more. However, both are anticipated to boost the quantity of carbon-based secondary products in plant tissue. Some studies suggest that CO_2 can directly or indirectly influence *Taxus bacatta*, *H. perforatum*, and *Echinacea purpurea* to produce more secondary metabolites

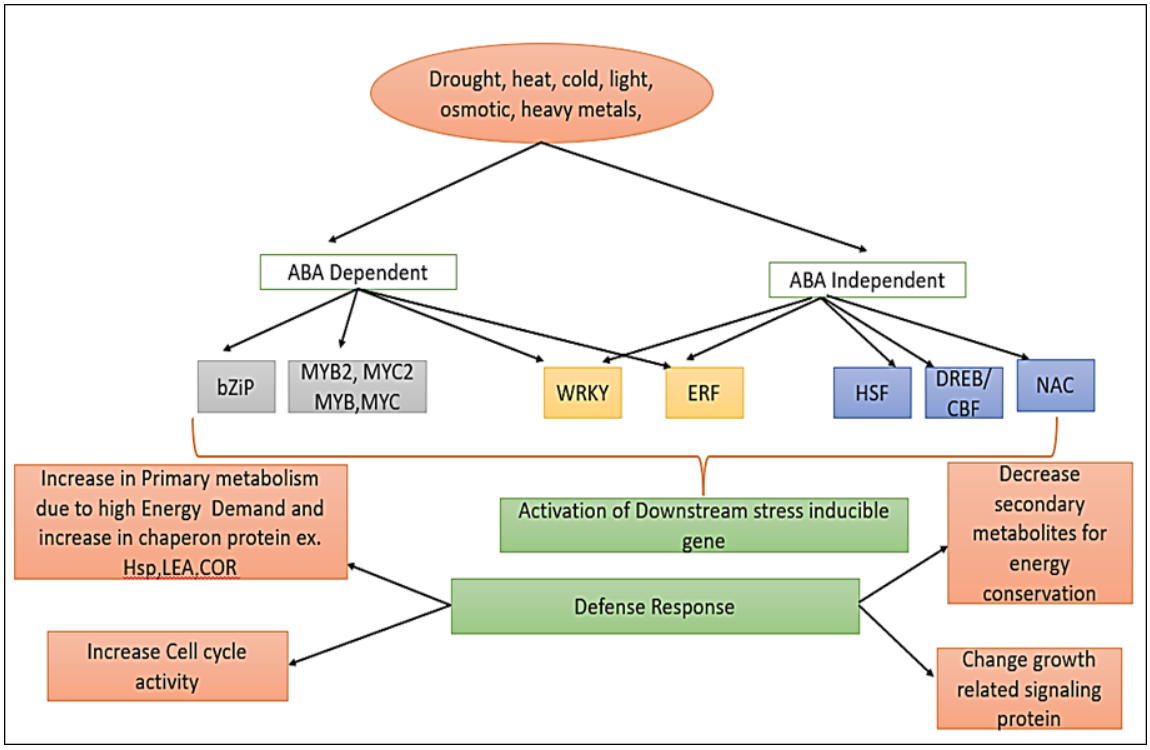

Figure 5 Effect of drought, heat, cold, light, osmotic, and heavy metal stress on secondary metabolite production

like phenols, flavonoids, lignins, nicotine, and coumarins (Savé et al. 2007). Further, Ibrahim et al. (2017) reported that elevated CO_2 levels increase phenol and flavonoid content in *Labisia pumila* plants. Similarly, lignins, nicotine, coumarins, and phenylpropanoids accumulate in tobacco plants after elevated CO_2 treatment (Li et al. 2018; Matros et al. 2006). Rising atmospheric CO_2 levels significantly influence how plants utilize energy and develop, impacting their carbon and nutrient balance. These changes highlight the intricate relationship between environmental shifts and plant biochemistry, with implications for ecological systems and agriculture.

4.2 Impacts of Drought on SM Production

Plant morphology is directly affected by soil water content. A rain or soil water deficiency leads to drought stress, which decreases the plant's water potential and leaf turgidity. This, in turn, triggers or adjusts various biochemical and morpho-physiological components and wide-ranging genetic feedback based on the cultivar or species (Zhou et al. 2017). In order to survive, water-limited vegetation may postpone glucose accumulation and use carbon for secondary metabolism (Herms and Mattson 1992). Furthermore, drought increases the levels of isoprinoid abscisic acid in the leaf apoplastic area (Liu et al. 2005). Studies have shown that abscisic acid (ABA) treatment induces *Orthosiphon stamineus* to produce reactive oxygen species and secondary metabolites as a defence mechanism (Khan et al. 2011).

Plants generally produce bioactive chemicals such as phenolic compounds in response to water scarcity (Khan et al., 2011). Sampaio et al. (2016) found that water deprivation decreases the photosynthesis rate and increases ROS production and accumulation, resulting in increased phenolic compound production as a plant defence mechanism (Figure 5). Under mild water stress, *Labisia pumila* has more total flavonoids, anthocyanins, and phenolics than under severe stress (Jaafar et al. 2012). *Pisum sativum* cv. *meteor* under water deficit stress had higher anthocyanin and flavonoid content than well-watered controls (Nogués et al. 1998). Jaleel et al. (2007) observed that drought-induced oxidative stress enhanced the total alkaloid content in both the shoot and root of *Catharanthus roseus*. Further, drought stress also raised glycine betaine content in the *C. roseus* plant.

4.3 Impacts of Salinity Stress on SMs Production

Salt stress causes plant ionic and osmotic stressors, which increase or reduce secondary metabolites. Antioxidant secondary metabolites help plants balance their oxidative state. Salt stress causes many metabolic changes in plants. Plants produce more suitable osmolytes (neutral, soluble organic compounds) to deal with salt stress (Nelson et al. 1998). Salinity stress raises sodium levels and causes cytoplasmic potassium imbalances. It creates ROS and inactivates and unsaturates numerous enzymes (Luo et al. 2005). Further, salt stress also increases proline aggregation

Table 2 Secondary Metabolite Production in Response to Salinity Stress in a Range of Species

S. N.	Plant species	Compounds	References
1.	*M. chamomilla*	Increase in phenolic acids, including chlorogenic, caffeic, and protocatechuic acid	Said-Al Ahl and Omer (2011); Abd El-Azim and Ahmed (2009)
2.	*Nigella sativa*	Increase in Phenols	Bourgou et al. (2008)
3.	*Mentha pulegium*	Increase in Phenols	Oueslati et al. (2010)
4.	*Matricaria reutita* *Satureja hartensis* *Salvia officinalis*	Increase in essential oil	Said-Al Ahl and Omer (2011)
5.	*Thymus maroccanus* *Origanum vulgare* *Mentha piperita* *Majorana hartensis* *Salvia officinalis* *M. chamomilla*	Decrease in essential oil	Said-Al Ahl and Omer (2011)
6.	*Plantago ovata*	Increase in proline, flavonoids, and saponins	Haghighi et al. (2012)

(Cardoso et al. 2019). Similarly, Akula and Ravishankar (2011) also suggested that salt stress can increase or reduce plant secondary metabolite levels through osmotic and ionic stress. Adaptation to stress involves alteration in proline metabolism. Proline dehydrogenase and 1-pyrroline-5-carboxylate dehydrogenase catalyze two dehydrogenation processes in proline catabolism. Mitochondrial matrix NAD+-dependent dehydrogenase raises NAD+ PAL and converts this into polyphenols and antioxidants. Benjamin et al. (2019) found that NaCl increases flavonoids and other phenolic compounds in *S. brachiate* but decreases them in *S. portulacastrum*, which accumulates carotenoids, fighting ROS and aiding photosynthesis. Usually, shoots have more ricinine alkaloid content than roots and underground stems. Said-Al Ahl and Omer (2011) found that salt stress increases *Rouvolfia tetraphylla* reserpine and *Catharanthus roseus* shoot vincristine alkaloids. Numerous investigations are supported by current reviews and outlined in Table 2. Salt stress significantly impacts plant metabolism and secondary metabolite production through ionic and osmotic stress mechanisms. However, there remains a research gap in understanding the specific regulatory pathways and genetic mechanisms governing the differential responses of plants to salt stress, particularly to secondary metabolite synthesis under varying environmental conditions.

4.4 Impact of Temperature on SMs Production

Global warming and disruptive seasonal events may reduce secondary metabolite production in medicinal and aromatic plants (MAPs), affecting livelihoods and biodiversity. Timely interventions are needed to avert biodiversity loss (Das et al. 2016). Low and high temperatures alter plant cell proteins, enzymes, and lipids, affecting membrane integrity. Thakur et al. (2019) suggested low and high temperatures affect plant secondary metabolite synthesis. Human activity has raised the average global temperature by 0.74°C, and the temperature is anticipated to increase by 0.2°C each decade. As a supplementary defence against high temperatures, plants manufacture SMs (Isah 2019). Wahid and Close (2007) state that ROS from temperature stress harms plant cells.

Plants produce flavonoids and phenylpropanoids to deal with high temperatures. Table 3 illustrates the effects of elevated and reduced temperature stresses on producing various secondary metabolites in plants. Global warming and seasonal disruptions threaten secondary metabolite production in medicinal and aromatic plants (MAPs), impacting biodiversity and livelihoods. Extreme temperature alters plant cellular processes and membrane integrity, affecting secondary metabolite synthesis. As temperatures rise, plants increasingly rely on secondary metabolites like flavonoids and phenylpropanoids as a defence. However, further research is needed to fully understand and mitigate the effects of climate change on these vital plant compounds. Global warming and seasonal disruptions threaten secondary metabolite production in medicinal and aromatic plants (MAPs), impacting biodiversity and livelihoods. Extremes temperature may also alter plant cellular processes and membrane integrity, affecting secondary metabolite synthesis. As temperatures rise, plants increasingly rely on secondary metabolites like flavonoids and phenylpropanoids as a defence. However, further research is needed to fully understand and mitigate the effects of climate change on these vital plant compounds.

4.5 Effect of Light Irradiation on SMs Production

Growth and metabolism in plants are significantly impacted by solar radiation. Research has shown that fluctuations in solar radiation levels can cause plants to produce and accumulate secondary metabolites to adapt to their environment (Yang et al. 2018). The length of the light period is one of the critical factors

Table 3 Secondary Metabolite Production in Response to Temperature Stress in a Range of Species

S. N.	Temperature	Plant species	Compounds	References
1	Low	*Melastoma malabathricum*	Anthocyanin	Chan et al. (2010)
2	Low	*Rhodiola crenulata*	Melatonin	Zhao et al. (2011)
3	High	*Ribes nigrum*	Delphinidin-3-O- glucoside, Delphinidin-3-O-rutiniside, Myricetin-3-O-glucoside	Zheng et al. (2012)
4	Low	*Glycine max*	Genistein, Diazein, Genistin	Janas et al. (2002)
5	High	*C. accuminata*	10- hydroxycampothecin	Zu et al. (2003)
6	High	*Lupinus angustifolius*	Alkaloids	Jansen et al. (2009)
7	High	*C. roseus*	Vindoline, Catharanthine, Vinblastine	Guo et al. (2007)
8	High	*Picea abies*	Piperidine	Virjamo et al.(2014)
9	High	*Betula pendula* *Populus tremula*	Terpenoid	Ibrahim et al.(2010)

associated with irradiation that can impact the levels of secondary metabolites in plants. Studies have demonstrated that longer photoperiods can increase the levels of secondary metabolites, which can help plants resist the effects of light exposure. Conversely, shorter day-length conditions have been shown to reduce coumarin content in stems and leaves, while longer day-length periods have significantly increased coumarin content (de-Castro et al. 2007).

Light quality is a critical factor that significantly impacts the levels of secondary metabolites in plants. The escalation of ultraviolet radiation has been attributed to the diminution of the ozone layer, which had a detrimental effect on living organisms. Consequently, plant cells produce reactive oxygen species and accelerate the production of secondary metabolites that can absorb UV radiation (dos Santos Nascimento et al. 2015). Furthermore, it promotes antioxidant activity to minimize and correct oxidative harm (Takshak and Agrawal 2014). Park et al. (2007) demonstrated that the biosynthesis of anthocyanins is enhanced by the increased expression of genes responsible for producing proteins and enzymes, including dihydroflavonol reductase (DFR), flavanone 3-hydroxylase (F3H) and chalcone synthase. UV-B radiation leads to heightened activity of enzymes like cinnamyl alcohol dehydrogenase, phenylalanine ammonia-lyase (PAL), chalcone-flavanone isomerase, dihydroflavonol reductase (DFR), and 4-coumarate CoA ligase, along with increased levels of flavonoids, anthocyanins, and tannins in *Withania somnifera* (Takshak and Agrawal 2014). According to Ma et al. (2016), the effects of UV-B radiation on the phytochemical constituents of *Chrysanthemum* flowers, specifically chlorogenic acid and flavonoids, were investigated. These phytochemicals are the primary components that impart the healing properties of the flowers.

Solar radiation significantly influences plant metabolism and secondary metabolite production. Fluctuations in radiation levels prompt plants to adjust secondary metabolite synthesis, with longer photoperiods enhancing their production. Additionally, UV radiation induces reactive oxygen species in plants, stimulating the biosynthesis of protective secondary metabolites like flavonoids and anthocyanins. Heavy metals such as chromium and cadmium disrupt plant metabolism, triggering oxidative stress and altering secondary metabolite profiles. Understanding these environmental impacts is crucial for optimizing plant-based bioactive compound production.

4.6 Effect of Heavy Metal on SMs Production

The term "heavy metal" describes metallic elements with a high-density level that can inflict harm even at low concentrations. Heavy metals, including chromium, cadmium, iron, zinc, and manganese, can potentially elevate ROS generation, causing an imbalance between ROS generation and detoxification. These metals can have a detrimental impact on plants by either directly binding to proteins, owing to their affinities for histidyl-, thioyl-, and carboxyl-groups that mark catalytic, structural, or transport locations in cells or eliciting the production of reactive oxygen species, potentially leading to oxidative stress (Table 4) (Kaczor-Kamińska et al. 2020). In addition, the presence of heavy metals can affect plants by displacing essential cations from specific binding sites (Sharma and Dietz 2009). Kovacik and Klejdus (2008) found that varying copper doses, like 120 and 60 µM, significantly boosted PAL activity, increasing lignin content and phenolic compound the day after treatment. This result reflects the response of the defence mechanism to metal entry. Reports are also available that higher artemisinin levels in *A. annua* were achieved by the application of arsenic-induced stress through two methods: converting dihydroartemisinic acid (monocarboxylic acid) to artemisinin via ROS breakage and promoting genes involved in artemisinin production (Rai et al. 2011a; Khare et al. 2020).

Table 4 Impact of heavy metal stress on the production of secondary metabolites by different species

S. N.	Heavy metal	Plant species	Compounds	References
1	$AgNO_3$	*Perovskia abrotanoides*	Tanshinone	Zaker et al. (2015)
2	Ag	*Salvia castanea*	Tanshinone	Li et al. (2016)
3	$AgNO_3$	*Datura metel*	Atropine	Shakeran et al. (2015)
4	Cd, Co, Ag	*Vitis vinifera*	Resveratrol	Cai et al. (2013)
5	Cu	*Bacopa monnieri*	Bacoside	Sharma et al. (2015)
6	Pb	*Mentha crispa*	Carvone	Sá et al. (2015)
7	Cu and Zn	*Mentha pulegium*	Pulegone Cineol Thymol	Lajayer et al. (2017)
8	Cd and Co	*Trigonella rogusum*	Diosgenin	De and De (2011)
9	Cr, Cd, Pb and Ni	*Ocimum basilicum*	Chavicol Cinalol	Prasad et al. (2011)
10	As	*Artemisia annua*	Artemisinin	Rai et al. (2011b)

The research gap lies in understanding the precise mechanisms by which different heavy metals individually and collectively influence secondary metabolite production in plants. Current studies often focus on specific metals like copper and arsenic, but comprehensive comparative analyses are limited across a broader spectrum of heavy metals. Additionally, there is a need to explore how varying concentrations and combinations of heavy metals affect different plant species' secondary metabolite profiles under realistic environmental conditions. This knowledge is crucial for developing strategies to mitigate heavy metal-induced stress and sustainably optimize plant-based bioactive compound production.

Conclusion

Plant cells are recognized as a significant source of biochemical compounds, encompassing primary metabolites like sugars, fatty acids, and amino acids alongside a diverse array of secondary metabolites, including alkaloids, terpenoids, phenols, and sulfur-containing complexes. These secondary metabolites serve various functions, such as providing defence protection or engaging in offensive/invasive tactics in response to environmental factors, including microbes, insect pests, herbivorous predators, and insects. Various environmental factors, including sunlight, temperature, soil fertility, soil water, and acidity/salinity largely impact the generation and accumulation of secondary metabolites in plants. To cope with these conditions, plants adjust their metabolism towards producing numerous secondary metabolites. During environmental challenges, the orientation of secondary metabolism in plants involves complex signal transduction pathways. The current review presents comprehensive and reliable reasons for the variation in the composition of secondary metabolites in different ecological circumstances. Acquiring knowledge about secondary metabolism and its instability in plants may benefit both agriculturalists and geneticists. As advancements in modern techniques continue to emerge, the significance of secondary metabolism in plant adaptation cannot be overstated. Furthermore, conducting extensive research into the physiological, molecular, and biochemical responses of plants and the primary genetic mechanisms involved can provide valuable insights and enhance adaptation to various environmental influences. By doing so, scientists may be able to strategically apply stress factors to increase the production of various secondary metabolites, ultimately benefiting humanity.

However, gaps remain in understanding the intricate signal transduction pathways and regulatory networks that govern secondary metabolism in response to diverse environmental challenges. Future research should unravel these complexities across various plant species and environmental conditions. Advancements in modern techniques, including omics technologies and gene editing tools, offer promising avenues to deepen our understanding of secondary metabolism. This knowledge could potentially enable agriculturalists and geneticists to manipulate plant metabolism strategically. By harnessing stress factors or enhancing genetic pathways, scientists may enhance the production of valuable secondary metabolites for medicinal, agricultural, and industrial applications, benefiting human health and sustainable agriculture practices.

Conflict of interest

The authors declare no conflicts of interest

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Plant growth promotion activities of *Bacillus* spp. isolated from Jakrem hot water spring of Meghalaya, North East India

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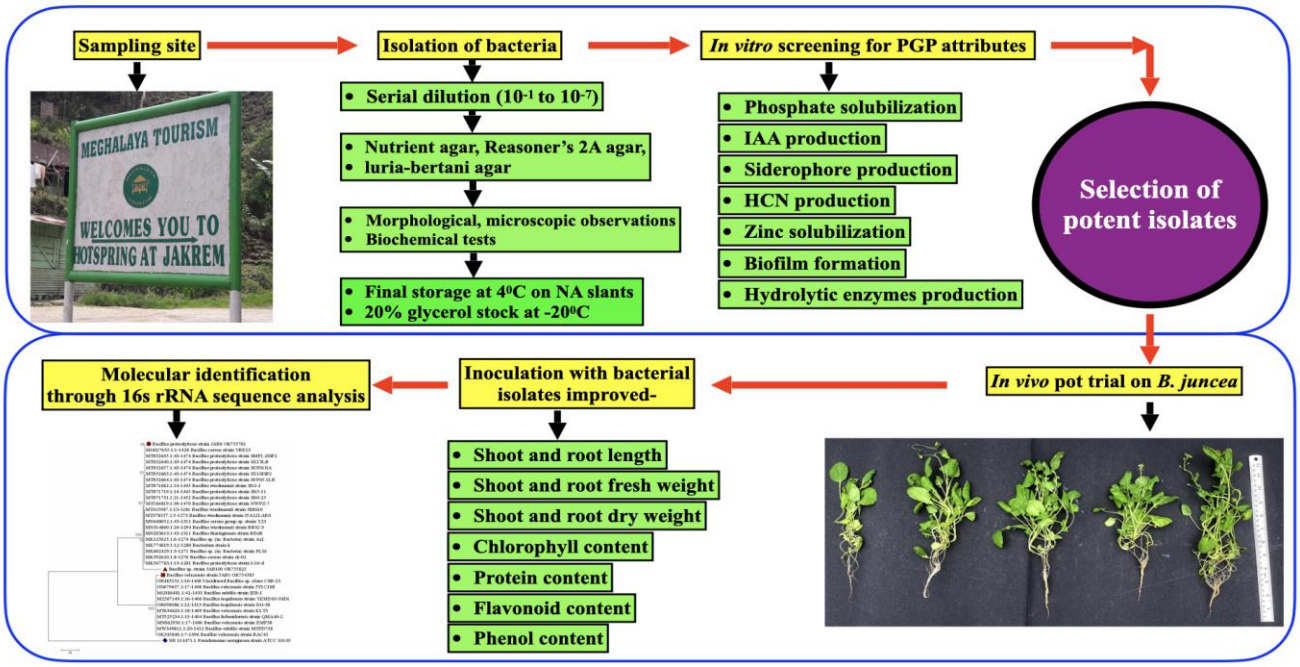
GRAPHICAL ABSTRACT



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Growth promotion

ABSTRACT

The study aims to investigate plant growth promotion (PGP) activities of thermophilic bacteria isolated from the Jakrem hot spring in Meghalaya, North-East India, and determine their effect on *Brassica juncea's* growth. The bacteria were isolated by a culture-dependent approach following a serial dilution method in a nutrient agar medium. All the isolates were determined for PGP attributes such as indole acetic acid, phosphate solubilization, hydrolytic enzymes, and siderophore production. The potent bacterial isolates were characterized by 16S rDNA sequencing and phylogenetic analysis. Altogether, 53 bacterial isolates were obtained, most belonging to the genus *Bacillus*. Of the total isolates, 37.7% exhibited both PGP and hydrolytic enzyme activities. Three isolates, namely JAB1, JAB8, and JAB100, showed promising PGP and were identified as *Bacillus velezensis*, *B. proteolyticus*, and *Bacillus* sp., respectively. The PGP attributes of these isolates were determined *in vivo* on *B. juncea*, and their effects were measured in terms of shoot and root length biomass and biochemical contents. It was observed that combined inoculation of all three isolates significantly enhanced the growth and development of *B. juncea*, evident by increased shoot and root length, fresh and dry weight, and higher levels of protein, phenol, flavonoid, and chlorophyll content compared to the control. In conclusion, the study highlights the potential application of thermophilic *Bacillus* spp. from hot springs as bioinoculants to enhance crop productivity in sustainable agricultural practices.

1 Introduction

Substantial demand for food has increased in recent years due to limited land resources and the rise of the global population. Food production has increased significantly through chemical fertilizers in conventional agricultural systems to feed the growing population (Mishra and Dash 2014). However, indiscriminate and excessive use of chemical fertilizers to increase crop productivity has severely adverse effects on living organisms and the environment. Therefore, a sustainable agricultural system is urgently needed to overcome this problem and achieve food security for the growing global population (Glick 2018). Organic farming using beneficial microorganisms has gained tremendous attention as an alternative to agrochemicals. These microbes are used as bioinoculants to increase soil fertility and improve plant growth and soil health by increasing the supply of readily available nutrients or protecting them from biotic and abiotic stresses (Lugtenberg and Kamilova 2009).

Hot springs represent a unique ecological niche for microorganisms thriving under extreme environmental conditions. Recently, microbes, especially bacterial communities isolated from such environments, appeared to be of utmost importance due to producing industrially significant enzymes and secondary metabolites with multiple applications (Verma et al. 2018). Bacterial genera belonging to such extreme conditions have been reported to possess higher metabolic rates and stability when compared with their mesophilic counterpart (Verma et al. 2018). Moreover, enzymes produced by various bacterial genera, such as *Bacillus* sp. isolated from hot springs, remained stable and active at higher temperatures (Panda et al. 2013). Due to this property, thermophilic microorganisms have gained considerable attention for large-scale production of enzymes, sugars, and a wide array of secondary metabolites (Satyanarayana et al. 2005; Lele and Deshmukh 2016; Mohammad et al. 2017). Besides, thermophilic microorganisms have also shown their ability to increase plant growth, resistance to salinity in crops of agricultural interest, and biocontrol against phytopathogens (Verma et al. 2018; Shilev 2020). Many instances have demonstrated that hot springs microorganisms, especially *Bacillus* spp., are potent agents for inducing the growth of plants through various plant growth-promoting attributes (Saharan and Verma, 2014; Verma et al. 2018). Production of different phytohormones viz. indole-3-acetic acid (IAA), cytokinins, gibberellins along with phosphate solubilization, iron chelation, production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and nitrogen-fixing properties that aid in plant growth promotion had also been previously reported from different species of *Bacillus* isolated from extreme environmental condition (Stein 2005; Yang et al. 2009; Arguelles-Arias et al. 2009; Rahman et al. 2016; Kaki et al. 2017; Verma et al. 2018; Kumar et al. 2021). Therefore, isolating beneficial microorganisms, particularly bacteria, from pristine environments, such as hot springs, would be paramount for bioinoculants for plant growth and health (Podile and Kishore 2006; Saharan and Nehra 2011).

Meghalaya is situated in India's North-Eastern Region (NER) and is rich in various natural resources. The state is endowed with several high-altitude lakes and hot springs. One such hot spring is Jakrem, which is less explored and located in one of the mega biodiversity-rich zones of the world in the Himalayan geothermal belt (Rakshak et al. 2013; Panda et al. 2016). Preliminary studies on microbial diversity of this hot spring by culture-independent approach had revealed a rich diversity of

microorganisms dominated by Firmicutes, Cloroflexi, unclassified bacteria, and a large number of sequences reads from bacterial taxa Arthronema which may represent novel species (Panda et al. 2016). However, a culture-dependent approach has not yet been undertaken to investigate the microbes for biotechnological applications. Therefore, the present study aimed to isolate and characterize the bacterial species from this hot spring by a culture-dependent approach, evaluate the isolates for plant growth promotion potential, and observe their effect on the growth and development of *B. juncea*, a commonly used leafy vegetable of North East India.

2 Materials and Methods

2.1 Study site and collection of sample

The hot spring of Jakrem is located in the West Khasi hill district of the Meghalaya state, which is situated at 25°24.463′ N and 91°32.409 ′E and an altitude of 1439 m above mean sea level (MSL). Geographical positioning data and temperature of the water sample were recorded at the sampling site using a GPS locator system (Garmin eTrex 10) and portable thermometer (HICKS, Oval) respectively. The pH of the collected samples was recorded in the laboratory using a digital pH meter (Cyberscan, EUTECH)**.** For collection of water samples, plastic containers (500 ml) were first cleaned with 20% sodium hypochlorite solution, washed thoroughly with several steps of sterile distilled water, and autoclaved at 121℃ for 15 min. The containers were dried in a hot air oven at 50°C for 1 h and sterilized further under UV light in a laminar airflow cabinet for 1 h. Water samples were randomly collected in triplicates from hot springs in these plastic containers, stored in a dry ice box, and transported immediately to the laboratory (Kambura et al. 2016).

2.2 Isolation and identification of bacteria

Bacteria were isolated following serial dilution technique (10^{-1} to 10^{-7}) using three different growth mediums viz. nutrient agar (NA), Reasoner's 2A agar (R2A), and Luria-Bertani agar (LBA) medium. Briefly, 100 µl aliquot of each dilution was spread on growth media and sealed with paraffin strips. These plates were then incubated at 44±1^{0}C for 72-96 h. After the incubation period, the dilution factor, at which the number of colonies ranged between 30-300, was further selected to calculate the colony format unit (CFU)/ml of the water sample. Distinct colonies on the growth medium were purified by repeated sub-culturing, and finally, purified bacterial isolates were stored at 4^{0}C on NA slants and 20% glycerol stock at -20^{0}C (Fasina et al. 2020; Kumar et al. 2021). Microscopic examinations were carried out at 1000X magnification using immersion oil in the compound microscope (LYNX, Lawrence & Mayo), and Cell morphology, viz. shape, size, elevation, pigmentation, and colour were recorded. Different biochemical tests viz. catalase, oxidase, gram staining, and citrate utilization were carried out for identification up to genus level (Brenner et al. 2005; Yazdani et al. 2009; Kumar et al. 2012; Islam et al. 2017; Fasina et al. 2020; Tripathi and Sapra 2021).

2.2.1 Thermo-tolerance profile of isolated bacterial strains

The thermo-tolerant property of the bacterial isolates was determined by growing the isolates in freshly prepared NA medium and then incubating them at different temperatures: 30±1°C, 40±1°C, 50±1°C and 60±1°C for a period of 24 to 72 hours. The visible appearance of bacterial growth at specific thermal intervals was recorded as positive and was classified according to binary code viz. 0 (no growth) and 1 (appearance of growth on the medium), as suggested by Moreno et al. (2021) and López et al. (2021).

2.3 Physico-chemical analysis of water sample

Samples were analyzed for turbidity, Iron, chloride, fluoride, total hardness, calcium, magnesium, sulphate, nitrate, lead, nitrite, alkalinity, sodium, potassium, sulphide, cadmium, chromium, phosphorous, total organic carbon, total nitrogen, total suspended solids (TSS), volatile suspended solids (VSS) and total dissolved solids (TDS) as per the standard methodology (Indian Standard "IS 3025" 2009; APHA 2017; Kalsait et al. 2018; Agarwal et al. 2019).

2.4 Screening of the isolated bacterial isolates for plant growth-promoting traits

2.4.1 Solubilization of phosphate

The isolated bacterial isolates were screened for their inorganic phosphate solubilization potential using Pikovskaya's agar medium. A loopful of bacterial culture was spot inoculated onto the agar plates and incubated at 30±1°C for 72-96 hours. The ability of the bacteria to solubilize inorganic phosphate was determined by forming a clear halo zone surrounding the bacterial colony, indicating phosphate solubilization activity (Syiemiong and Jha 2019). The diameter of the halo zone was measured to quantify the solubilization efficiency of each isolate. Phosphate solubilization was quantified in Pikovskaya's broth medium, which contained 0.5% tri-calcium phosphate as a sole source of inorganic phosphorous. The broth medium was inoculated with 100 µl of overnight grown bacterial strains (10^6 cells/ml) and incubated in continuous shaking (120 rpm) for 144 h at 30±1^{0}C. After incubation, a cell-free supernatant was obtained by centrifugation for 5 minutes at 10000 rpm. The obtained cell-free supernatant (0.5 ml) was mixed with 10% trichloroacetic acid (0.5 ml) and color reagent (4 ml) [1 (3M H_2SO_4): 1 (2.5% ammonium molybdate): 1 (10% ascorbic acid): 2 (distilled water)] (Syiemiong and Jha 2019). The resulting mixture was incubated at 30±1^{0}C for 15 min, and absorbance of the resulting mixture was read at 820 nm

wavelength against control using a spectrophotometer. Solutions of KH_2PO_4 was used at different concentrations to prepare the standard curve, and the amount of phosphorous solubilized by the bacterial isolates was calculated and expressed as µg/ml (Syiemiong and Jha 2019).

2.4.2 Indole-3-acetic acid (IAA) production

IAA production was quantified by supplementing L-tryptophan (0.2%) as the precursor for IAA synthesis in the Luria Bertani broth medium. The broth medium was inoculated with overnight grown bacterial strains (100 µl; 10^6 cells/ml) and incubated at 30 ± 1^0C for 96 h in shaking condition (120 rpm). Following incubation, cell-free supernatant (1 ml) was obtained by centrifugation at 10000 rpm for 10 min and mixed with Salkowsky reagent (3 ml). The reaction mixture was incubated in the dark for 15 min, and the absorbance of the developing color was read at 530 nm wavelength using a spectrophotometer against control (3 ml of Salkowskyreagent + 1 ml Luria-Bertani broth medium supplemented with 0.2% L-tryptophan). For estimation of IAA produced by bacterial strains, a standard curve was prepared from different concentrations viz. 5, 10, 20, 50 and 100 µg/ml of pure IAA was used and expressed as µg/ml (Syiemiong and Jha 2019).

2.4.3 Siderophore production

A qualitative assay for Iron chelating activity by the bacterial isolates was performed on CAS (Chrome azurol S) agar medium. For this assay, solution A (60.5 mg of CAS in 50 ml of sterile distilled water was mixed with 10 ml of 1 mM $FeCI_3.6H_2O$ prepared in 10 mM HCl) was mixed with solution B (72.9 mg of hexadecyl tri-methyl ammonium bromide in 40 ml of sterile distilled water) (Kumar et al. 2021). A solid agar medium (Kings medium B base; 42.23 gm/l) was prepared separately, and both the stock solution and the agar medium were autoclaved and allowed to cool inside the laminar airflow cabinet. When the temperature lowered to 40–50^0C, both the solutions were mixed and poured onto petri plates and allowed to solidify. Bacterial strains were spot-inoculated onto petri plates and incubated for 96 h at 30 ± 1^0C. The development of the orange-yellow zone surrounding the bacterial colony was recorded as positive for siderophore production (Kumar et al. 2021).

2.4.4 Hydrogen cyanide (HCN) production

A qualitative assay for HCN production was carried out on Tryptic soy broth (30 g/l) amended with glycine (4.4 g/l). Briefly, overnight grown bacterial strains (100 µl, 10^6 cells/ml) were inoculated onto broth medium, and sterilized filter paper strips of uniform size (5 cm×0.5 cm) soaked into a solution of 0.5 % picric acid in 2% Na_2CO_3 was placed inside the tube in hanging position. The tubes were air-tight with parafilm and incubated at 30 ± 1^0C for 96 h. Following incubation, the filter paper was observed for color change from yellow to orange-brown for positive HCN production by the bacterial strains (Wani and Khan 2013).

2.4.5 Zinc solubilization

Zinc solubilization was estimated qualitatively using zinc oxide (0.1%) amended in a minimal agar medium (MM9). A loopfull culture of bacterial isolate was spot inoculated onto the agar plates and incubated at 30 ± 1^0C for 48 h. Following the incubation period, the development of a clear zone surrounding the colony was recorded as positive for zinc solubilization (Goteti et al. 2013).

2.4.6 Biofilm formation

Quantitative detection of biofilm activity was carried out by inoculating bacterial strains (100 µl, 10^6 cells/ml) in a 5 ml broth medium. Following inoculation, the broth medium was incubated for 72 h at 30 ± 1^0C under shaking conditions (120 rpm). After an incubation period, the cultured medium containing the bacterial cells was discarded completely and washed with phosphate buffer saline thrice (1X PBS pH 7.2) to remove adherent cells. The tubes were dried inside the laminar air flow cabinet and stained with crystal violet solution (0.2%) for 5 min. The tubes were again rinsed with phosphate buffer saline to remove excess stain, followed by air-drying. The absorbance of the stain adhered was read at 630nm wavelength by adding 2 ml of 95% ethanol in each tube against blank (Lotfi et al. 2014). The degree of adhesión was recorded as:

ODs <ODc: non-adherent

ODc< ODs < 2XODc: weakly-adherent

2x ODc< ODs < 4XODc: moderately-adherent

4xODc <ODs: strongly-adherent

Where ODc- control; ODs-sample, respectively.

2.5 Screening for the production of extracellular hydrolytic enzymes

2.5.1 Cellulase

For this assay, bacterial isolates were spot inoculated onto solid agar plates amended with 0.1% carboxymethyl cellulose (CMC) as substrate. Following incubation, for 48 h at $30\neq1^0$C, the solid agar plates were flooded with 0.1% congo red solution for 5 min and decanted. The plates were again flooded with 1M sodium chloride for 15 min. The development of a clear zone surrounding the bacterial colony was recorded as positive for extracellular cellulase activity (Kumar et al. 2021).

2.5.2 Amylase

For this assay, bacterial isolates were spot inoculated onto solid agar plates amended with 1% soluble starch as substrate. Following incubation, for 48 h at 30±1^{0}C, the plates were flooded with grams iodine solution for 5 min and decanted. Development of the clear zone surrounding the bacterial colony was recorded as positive for extracellular amylase activity (Kumar et al. 2021).

2.5.3 Lipase

For this assay, a loopfull culture of bacterial strain was spot inoculated onto Tributyrin agar (without Tributyrin) supplemented with Tributyrin (10 ml/ 1000 ml) as substrate and incubated at 30±1^{0}C for 48 h. Following incubation, the development of the clear zone surrounding the bacterial colony was recorded as positive for extracellular lipase activity (Berg 2009).

2.5.4 Protease

For the hydrolysis of protease, 10% skim milk was used as a substrate onto a solid agar medium (MM9). This medium was autoclaved and cooled to 40–50°C, then poured onto petri plates to solidify. Bacterial isolates were then spot-inoculated onto agar plates, following incubation at 30±1^{0}C for 48 h. The development of a clear zone surrounding the bacterial colony was recorded as positive for extracellular protease activity (Kumar et al. 2021).

2.5.5 Laccase

A solid agar medium was amended with 0.1% guaiacol as a substrate for laccase activity. Bacterial isolates were spot inoculated onto agar plates and incubated at 30±1^{0}C for 96 h. Following incubation, grams iodine was flooded onto petri plates for 5 min. Development of the clear zone surrounding the bacterial colony was recorded as positive for laccase activity (Kumar et al. 2021).

2.6 Mass multiplication of bacterial isolates

Based on the results from various *in-vitro* evaluations for plant growth promotion activities, the potent bacterial isolates were selected for *in-vivo* pot experimental trials. Mass multiplication of bacterial isolates was carried out by inoculating a loopfull culture of bacterial isolates in a 150 ml nutrient broth medium and incubating under continuous shaking conditions for 24 h at 30±1^{0}C. Following incubation, cells were harvested by centrifugation at 10000 rpm for 10 min and suspended in sterile distilled water. Bacterial density was further adjusted to 10^6 cells/ml using a haemocytometer count and stored at 4^{0}C for field application (Kumar et al. 2021).

2.7 Pot experimental trial of potent bacterial isolates

A pot experimental trial on *B. juncea* evaluated the in-vivo plant growth-promoting potential of the potent bacterial isolates. Briefly, seeds of *B. juncea* were obtained from the National Bureau of Plant Genetic Resources (NBPGR), Umiam, Meghalaya, with cultivar number-IC 597866 and surface sterilized with a solution of sodium hypochlorite (2.5%) for 10 min. After surface sterilization, seeds were thoroughly washed with several steps of sterile distilled water (until traces of sodium hypochlorite was removed). The surface sterilized seeds were dried on sterile filter paper inside a laminar airflow cabinet (Gupta et al. 2020; Goswami and Deka 2020; Kumar et al. 2021). Ten seeds were transferred to each pot containing 200 g of double autoclaved sterilized soil and 20 ml of bacterial cell suspension (10^6 cells/ml) was inoculated in pot with following sets of treatment: T1- Control (seeds without bacterial inoculation); T2 - seeds inoculated with bacterial isolate JAB1; T3 seeds inoculated with isolate JAB8; T4- seeds inoculated with isolate JAB100; T5- seeds inoculated with isolates JAB1+ JAB8; T6- seeds inoculated with isolates JAB8+ JAB100; T7- seeds inoculated with isolates JAB1+ JAB100; T8- seeds inoculated with isolates JAB1+ JAB8+ JAB100.

2.8 Growth and biochemical parameters studied

2.8.1 Evaluation of length (shoot and root), fresh weight and dry weight

After 45 days of seed sowing, different growth parameters, like shoot and root length, were determined using a standard scale. Fresh weight (root and shoot) was determined by harvesting the mustard plant and then washing it with running water to remove adhering particles. Excess moisture was removed using filter papers, and the fresh weight (root and shoot) was measured using an electronic weighing balance. Plant samples were then dried in an oven at 70^{0}C and measured consecutively after 24 h until constant weight was attained for dry weight determination (Huang et al. 2017).

2.8.2 Chlorophyll content

The chlorophyll content was estimated in mustard leaves with 80% acetone. Briefly, mustard leaves (0.5 g) were finely ground using a mortar and pestle in 20 ml of acetone (80%). Supernatant from the resulting mixture was collected following centrifugation at 10000 rpm for 10 min, and the absorbance was recorded at 645 nm and 663 nm wavelength in a spectrophotometer against blank (only 80% acetone). Chlorophyll content was recorded as milligrams (mg) per gram (g) of fresh weight (FW) using the following formula (Arnon 1949; Sarkar and Kalita 2022).

Chlorophyll a (mg/g FW): {12.7× (Absorbance at 663nm)- 2.69× (Absorbance at 645nm)} ×V/1000×W

Chlorophyll b(mg/g FW): {22.9 × (Absorbance at 645nm)- 4.68× (Absorbance at 663nm)} ×V/1000×W

Total chlorophyll (mg/g FW): {20.2× (Absorbance at 645nm) +8.02× (Absorbance at 663nm)} ×V/1000×W

Where, V: Final volume of 80% acetone; W: Sample weight

2.8.3 Protein content

Estimating total protein content in leaves was conducted per the methodology described by Lowry et al. (1951) with some modifications. Briefly, fresh leaves (0.2 g) were crushed in 10 ml of 0.1M phosphate buffer using mortar and pestle. The resulting mixture was centrifuged for 10 min at 10000 rpm, and the obtained supernatant (1ml) was mixed with alkaline copper reagent (5 ml) [48 ml of 2% Na_2CO_3 prepared in 0.1N NaOH (48 ml)+ 1% $KNaC_4H_4O_6{\cdot}4H_2O$ (1 ml)+ 0.5% $CuSO_4$ (1 ml)]. The final mixture was incubated for 15 min, and then 2N freshly prepared Folin-Ciocalteau reagent (1 ml) [Folin-Ciocalteau reagent (1): water (1)] was further added, followed by additional incubation in the dark for 30 min. After incubation, the absorbance of the developing mixture was read at 660 nm wavelength against control using a spectrophotometer. The amount of soluble protein was calculated from a bovine serum albumin (BSA) standard and expressed as mg BSAE/ g of fresh weight (Sarkar et al. 2020).

2.8.4 Phenol content

Total phenolic content was estimated according to the methodology described by Sarkar and Kalita (2022) with some modifications. Dried leaves (0.5g) were crushed using a mortar and pestle in chilled methanol and centrifuged at 10000 rpm for 10 min. Estimation of total phenol was carried out by adding 2.5 ml of Folin- Ciocalteau reagent (10% in water) and 2.5 ml of 7.5% Na_2CO_3 to 500 µl of the obtained supernatant, followed by incubation in the dark for 40 min at 45±1^{0}C. The absorbance of the mixture was read at 765 nm wavelength using a spectrophotometer. The total phenol content in the sample was expressed as mg of gallic acid equivalent (GAE) g $^{-1}$ DW using gallic acid as the standard.

2.8.5 Flavonoid content

Flavonoid content was estimated as per the methodology described by Sarkar and Kalita (2022). Briefly, supernatant extracted in methanol (100 µl) was mixed with distilled water (400 µl) and 5% $NaNO_2$ (30 µl) and incubated for 5 min at 30±1^{0}C. 10% AlCl3 (30 µl) was added to the mixture and incubated for 6 min at 30±1^{0}C. After incubation, 1M NaOH (200 µl) was added to the mixture, and the final volume was adjusted to 1 ml with distilled water. The mixture was vortexed and incubated again for 5 min at 30±1^{0}C. After incubation, the absorbance of the sample was read at 510 nm wavelength using a spectrophotometer and expressed as mg of quercetin equivalent (QE) g $^{-1}$ FW of the sample using quercetin as standard.

2.9 Molecular identification of potent bacterial isolates

Genomic DNA of the potent bacterial isolates was extracted using a Genomic DNA extraction kit as per manufacturer instructions. The extracted DNA was subjected to PCR amplification using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) in a thermal cycler. The reaction mixture contained dATP (0.2 mM), dCTP (0.2 mM), dGTP (0.2 mM), and dTTP (0.2 mM), 27F (1 µM), 1492R (1 µM), $MgCl_2$ (2 mM), Taq DNA polymerase (1.25 U), genomic DNA (1 µl) with following reaction condition, 95^{0}C (2 min), followed by 35 cycles of 95^{0}C (30 s), 53^{0}C (30 s) and 72^{0}C (1.30 min) and final extension at 72^{0}C (7 min). The amplified products were resolved through electrophoresis containing 1.2% agarose and were imaged in a gel documentation system. Purification and 16S rRNA gene sequencing of the amplified PCR products were performed at Unigenome (Unipath Specialty Laboratory). Using 16S rRNA gene sequences of the isolates, the phylogenetic tree was constructed using the neighbour-joining method to know their phylogenetic affiliations. The sequences were submitted to the GenBank NCBI database, and accession numbers were obtained (Agarwal et al. 2020; Kumar et al. 2021).

2.9.1 Statistical analysis

All the experiments were conducted in triplicates and expressed as a mean of three replicates with standard deviation. For data analysis of plant growth, ANOVA was conducted using *R* software (Version-1.2.1335) and significant differences were determined using the least significant differences test (LSD) at a significance level of $P \leq 0.05$*.

3 Results and Discussion

3.1 Bacteria isolated from hot water spring

The pH of water samples obtained from the Jakrem hot spring was alkaline (8.54±0.02) with a recorded temperature of 44.96±0.2^{0}C at the sampling site. The temperature range of the hot water spring of Jakrem was classified as moderate thermophilic, and in many instances, the temperature of hot water springs located in different parts of India has also been reported to be moderately thermophilic (Yadav et al. 2015; Poddar and Das 2018; Narsing Rao et al. 2021). Our result corroborates with many previous studies where the pH of hot water springs was reportedly alkaline, and the temperature was often higher than 40^{0}C (Mohammad et al. 2017; Sahay et al. 2017). Following serial dilutions, 53 distinct bacterial isolates were isolated in different media compositions. The water samples' colony forming unit (CFU) was 1.36 x 10^{-1} CFU/ml.

Based on morphological, microscopic and biochemical characterization, the isolates were rod-shaped and gram-positive bacteria belonging to the genus *Bacillus*. Worldwide geothermal sites are favourable habitats for thermophilic bacteria, which have developed unique survival strategies to adapt to such an environment. The dominant isolated bacteria belong to the genus Bacillus in the present study. The prevalence of the genus *Bacillus* and related genera as the dominant members have been previously reported from different hot springs (Adiguzel et al. 2009; Yadav et al. 2015; Priya et al. 2016). The occurrence of *Bacillus* species in different hot springs worldwide may be attributed to their high GC content and endospore formation, which might have favoured better adaptability for surviving in such extreme environmental conditions (Panda et al. 2013).

3.1.1 Thermo-tolerance profile of bacterial isolates

In the present study, thermo-tolerance profiles of the 53 bacterial isolates were determined at 4 different thermal intervals viz. 30, 40, 50 and 60°C. The results showed a score of 0 (no growth) for only 2 bacterial isolates at 30°C and 1 (visible growth) for 51 bacterial isolates at 30°C. In the present study, all 53 bacterial isolates were recorded positive and showed visible growth on NA medium with a score of 1 at 40 and 50°C. However, when the incubation temperature was increased to 60°C, only 29 bacterial isolates were recorded positive with a score of 1 (visible growth); therefore, these isolates can be considered thermophilic. The three potent bacterial isolates identified as *B. velezensis*, *B. proteolyticus*, and *Bacillus* sp. were thermophilic. Depending on the temperature range required for growth, bacteria can be classified as mesophilic, psychrophilic, thermophilic or thermotolerant (Moreno et al. 2021). Microorganisms that grow in wide temperature ranges offer better adaptability for survival in extreme environments (Kumar et al. 2013; Pandey et al. 2015; Khan et al. 2020). Isolation of thermo-tolerant strains of *Bacillus* and subsequent application as plant growth-promoting organic amendment have been cited in numerous literature (Kumar et al. 2013; Pandey et al. 2015; Khan et al. 2020). In this study, combined inoculation of the selected Bacillus species enhanced the growth and development of *B. juncea*. A similar result was observed in which inoculation with thermotolerant *Bacillus* sp. increased plant growth and development in soybean (Khan et al. 2020).

3.2 Physico-chemical properties of the water sample

The collected water sample was subjected to a comprehensive physico-chemical analysis. The physicochemical analysis of the water samples recorded 14 different mineral elements (Table 1).

The major elements consisted of sodium (20.76±1.45 mg/l), sulphate (14.53±0.35 mg/l) and chloride (14.47±0.67 mg/l). The chemical composition of hot water is predominantly influenced by the interactions it undergoes with reservoir rocks and the minerals present in those rocks during its ascent, potentially leading to the spring water exhibiting either acidity or alkalinity (Chan et al. 2017). Notably, sodium content was found to be considerably low, which was agreed upon compared to a similar study on Jakrem conducted by Panda et al. (2016). Such low sodium content is often a characteristic of medium-low temperature geothermal systems (Mao et al. 2022). The physicochemical properties of the water samples followed the result obtained by Singh et al. (2018), who studied water samples from 9 different hot springs with increased concentrations of these elements. In this study, the sulphur content of the water sample was found to be high. Poddar and Das (2018) have also reported high sulphur content in water

Table 1 Physico-chemical properties of water samples from Jakrem hot spring

Parameter	Values
Temperature	44.96±0.2
pH	8.54±0.02
Turbidity (NTU)	0.49±0.37
Iron (mg/l)	0.02±0.005
Chloride (mg/l)	14.47±0.67
Fluoride (mg/l)	0.06±0.01
Calcium (mg/l)	4.73±1.44
Magnesium (mg/l)	0.51±0.16
Sulphate (mg/l)	14.53±0.35
Nitrate (mg/l)	10.73±0.15
Lead (mg/l)	0.003±0.001
Nitrite (mg/l)	0.001±0.0005
Alkalinity (mg/l)	8.56±1.25
Sodium (mg/l)	20.76±1.45
Potassium (mg/l)	2.83±0.65
Sulphide (mg/l)	0.004±0.001
Chromium (mg/l)	0.001±0.0005
Cadmium (mg/l)	Not Detected
Phosphorous (mg/l)	0.27±0.21
Total Organic Carbon (TOC) (mg/l)	0.42±0.03
Total nitrogen (mg/l)	0.04±0.011
Total hardness (mg/l)	16.9±6.45
Total Dissolved Solids (TDS) (mg/l)	141±27.22
Total Suspended Solids (TSS) (mg/l)	0.44±0.02
Volatile Suspended Solids (VSS) (mg/l)	0.85±0.54

Values are the mean of 3 replicates (n-3); ± standard deviation

samples of different hot springs in India. As suggested by earlier studies, the abundance of sulfate-reducing bacterial forms in the water sample could be the reason for this observation (Panda et al. 2016). In addition, trace amounts of heavy metals, specifically chromium (0.001±0.0005 mg/l) and lead (0.003±0.001 mg/l), were also detected, with their low concentrations unlikely to be toxic to the microbial population. Tekere et al. (2012) have also reported the presence of trace amounts of chromium and lead in water samples collected from different hot springs. Gram-positive bacteria identified in this study indicate a potential tolerance to the existing concentrations of these heavy metals, as they are generally more resilient to chromium than gram-negative counterparts (Fathima and Rao 2018). The sample also revealed 141±27.22 mg/l of total dissolved solids (TDS), 0.44±0.02 mg/l of total suspended solids (TSS) and 0.85±0.54 mg/l of volatile suspended solids (VSS). A lower TDS level usually favors microbial growth, unlike a high TDS level, which can create a hostile environment for certain microbes, potentially affecting their survival and growth (Hanson et al. 2019). Similarly, the total dissolved solids (TDS) were in accordance with the water samples of different hot springs reported by many previous workers (Tekere et al. 2011; Kumar and Sharma 2019). Additionally, total organic carbon (TOC) and total nitrogen were found to be 0.42±0.03 mg/l and 0.04±0.011 mg/l, respectively, providing insights into the water's organic matter and nutrient content (Badhai et al. 2015). It was interesting to note that the local communities used the water sample of Jakrem hot spring for their curative properties against various skin ailments. This may be because sulphur from such natural hot springs has been known to alleviate the symptoms and have curative properties of an array of diseases, such as skin, high blood pressure and arthritis (Das et al. 2016; Jena et al. 2018). These results contributed to our understanding of the physico-chemical characteristics of Jakrem hot spring water and highlighted the potential implications for microbial communities in similar environments.

3.3 Plant growth promotion properties of the bacterial isolates

3.3.1 production of extracellular hydrolytic enzymes

In the present study, out of 53 bacterial isolates, qualitative analysis revealed that isolate JAB100 showed 19 bacterial isolates positive for proteolytic activity and maximum activity. Similarly, 19 isolates showed positive results for cellulolytic activity and amongst the positive isolates, maximum halozone formation was shown by the isolate JAB1. Again, 20 isolates showed positive results for lipolytic activity with maximum halozone formation, as shown by the isolate JAK9. For amylolytic activity, 16 bacterial isolates showed positive results and maximum halozone formation was observed in the isolate JAK17. Screening for laccase enzyme also revealed 19 isolates with positive activity, and maximum halozone formation was shown by the isolate JK54 (Table 2). The result indicated that thermophilic bacterial isolates could produce different hydrolytic enzymes. Similarly, Lele and Deshmukh (2016) reported that bacterial isolates isolated from Indian hot springs produced considerable hydrolytic enzymes. Production of different hydrolytic enzymes is considered an important attribute towards induction of plant growth promotion and biocontrol against numerous phytopathogens (Villarreal-Delgado et al. 2018; Morales-Cedeno et al. 2021). In addition, enzymes produced by thermophilic bacteria have an inherent ability to remain active at higher temperatures and can withstand harsh environments; therefore, they have potential industrial applications (Sahay et al. 2017). Since the isolates producing these enzymes are *Bacillus* species, the findings corroborate with Aanniz et al. (2015), who reported cellulase, amylase and protease enzyme production by thermophilic *Bacillus* spp. These enzymes have been known to play an important role in the biological transformation of wastes. Therefore, it can be assumed that producing hydrolytic enzymes by the thermophilic bacterial isolates might help decompose organic matter, thereby determining the physical and chemical properties of the hot springs water.

3.3.2 Indole-3-acetic acid (IAA) production

Altogether, 20 isolates showed positive results for IAA activity in qualitative analysis both in the presence and absence of L-tryptophan (Table 3). Quantitative analysis revealed that the bacterial isolate JAB100 showed the highest IAA production. The isolate produced 2.751±0.078 µg/ml of IAA in the presence of L-tryptophan, followed by the isolates JAB1 and JAB8, which produced 2.717±0.138 µg/ml and 1.916±0.047 µg/ml of IAA respectively. However, in the absence of L-tryptophan, the highest IAA production was shown by the isolate JAB1, which produced 1.849±0.048 µg/ml of IAA, followed by the isolates JAB8 and JAB100 with IAA production of 1.458±0.074 µg/ml and 1.097±0.054 µg/ml respectively. Similarly, Verma et al. (2018) have also reported the production of IAA by thermophilic *Bacillus* strains isolated from the hot springs of the Leh and Ladakh regions of India. However, very few reports on the IAA production by thermophilic bacteria isolated from hot springs water are available. In recent years, global warming and climate change have severely affected the agricultural system, threatening food security. In this context, the production of IAA by thermophilic bacteria is of great significance, and such isolates are applicable as bioinoculants that withstand drought and abiotic stresses. Among various phytohormones, IAA is pivotal in enhancing cell division, yield, and plant growth (Verma et al. 2018). It was observed that the isolates could produce considerable IAA in the growth medium. However, adding L-tryptophan in the growth medium significantly enhanced IAA production. Ahmad et al. (2005) obtained a similar result and observed that *Azotobacter* and *Pseudomonas* enhanced IAA production when the medium was added with L-tryptophan as a precursor.

Table 2 Extracellular enzyme activities of the bacterial isolates isolated from Jakrem hot spring

Bacterial isolates	Enzymes activity (in mm)				
	Cellulase	Amylase	Lipase	Protease	Laccase
JAK1	9.2±0.36	7.32±0.3	20.79±1.7	25.13±0.82	10.26±0.58
JAK3	5.2±0.26	12.44±0.41	28.63±0.43	16.35±1.15	6.15±0.59
JAK6	19.98±1.25	11.68±0.69	14.16±0.3	11.33±0.55	7.64±1.08
JAK7	11.44±0.64	13.79±0.82	20.18±1.25	26.74±1.91	7.82±0.31
JAK9	26.26±1.15	14.61±0.53	39.92±1.66	22.08±0.91	10.02±0.72
JAK10	-	-	21.08±1.69	-	-
JAK17	16.77±1.81	15.72±0.43	27.3±1.95	26.42±1.33	8.55±0.26
JAK18	17.91±0.84	9.29±0.52	21.59±1.59	29.79±1.6	5.09±0.94
JAK22	13.2±1.4	10.42±0.56	28.22±1.91	26.35±1.18	8.34±0.46
JK24	27.13±1.66	18.06±0.3	16.18±1.29	23.52±1.12	15.03±0.16
JAK31	10.99±1.07	8.66±0.57	10.64±0.56	8.57±0.5	2.79±0.46
JK54	9.72±1.39	12.16±0.28	18.03±0.21	31.23±1.18	15.39±1.13
JAK251	16.71±0.43	7.45±0.38	21.04±1.74	27.39±0.91	13.84±0.95
JAW1	10.78±1.03	8.65±0.29	5.99±0.27	9.3±0.51	10.03±0.09
JAB1	38.63±0.88	-	14.91±0.5	20.21±1.01	8.91±0.18
JAB8	9.06±0.83	10.06±0.13	15.17±0.48	15.56±0.6	13.11±0.86
JAB9	11.81±0.81	15.31±0.48	13.16±0.99	20.64±1.49	10.16±0.2
JAB11	10.57±0.38	-	8.9±0.2	4.01±0.5	9.59±0.28
JAB12	12.96±0.76	4.88±0.45	16.09±0.87	9.51±0.69	3.66±0.38
JAB100	11.04±1.67	-	9.32±0.29	36.6±0.89	7.56±0.37

Values are the mean of 3 replicates (n-3); ± standard deviation

3.3.3 Solubilization of phosphate

The positive isolates for IAA production were further determined for qualitative and quantitative phosphate solubilization. Qualitative analysis showed 18 bacterial isolates as potential phosphate solubilizers that formed distinct halo zones surrounding bacterial colonies on Pikovskaya's agar medium (Table 3). The quantitative analysis of the strains revealed that the isolate JAB100 showed the highest Solubilization of inorganic phosphorous with a solubilization value of 19.4±0.73 µg/ml**.** The other isolates that showed considerable phosphate solubilization were JAB1 and JAB8, with a solubilization value of 14.28±0.65 µg/ml and 11.23±0.23 µg/ml, respectively. Amongst macro elements, phosphorus is essential for plant growth and health. However, this element is considered a limiting factor in plants as most available phosphorus is insoluble. In this context, the Solubilization of inorganic phosphorous by phosphate solubilizing bacteria would be beneficial in improving plant growth and soil health and the maintenance of regular biogeochemical cycles (Gamalero and Glick 2011). In the present study, most bacterial isolates showed good phosphate solubilization activity. Similarly, different bacterial genera such as *Pseudomonas*, *Rhizobium*, *Burkholderia*, *Enterobacter* and several *Bacillus* species have been reported with phosphate solubilization activity increasing productivity in agricultural crops (Erman et al. 2010; Rajput et al. 2013; Pereira and Castro 2014). Studies conducted by Rodriguez and Fraga (1999) believed that applying phosphate-solubilizing bacteria could effectively reduce the application of synthetic phosphorous fertilizers by 50% without affecting crop productivity.

3.3.4 Siderophore, hydrogen cyanide, zinc solubilization and biofilm activities

The isolates that showed positive for phosphate solubilization were further determined to produce siderophore, hydrogen cyanide (HCN), Solubilization of zinc and biofilm activities. Out of the 18

Table 3 *In vitro* plant growth promoting (PGP) activities of bacterial isolates of Jakrem hot spring

Bacterial isolates	IAA production with Trp (µg/ml)	IAA production without Trp (µg/ml)	Phosphate solubilization (µg/ml)	Siderophore production	Zinc solubilization
JAK1	1.394±0.054	0.745±0.037	4.29±0.07	-	-
JAK3	0.575±0.032	0.494±0.013	-	-	-
JAK6	0.85±0.029	0.775±0.026	4.38±0.14	-	-
JAK7	1.319±0.054	0.618±0.040	-	-	-
JAK9	1.488±0.051	0.55±0.016	6.33±0.15	-	-
JAK10	0.672±0.009	0.485±0.036	3.27±0.09	-	+
JAK17	0.698±0.032	0.683±0.017	3.14±0.11	-	-
JAK18	1.222±0.064	0.605±0.023	7.98±0.15	-	+
JAK22	0.913±0.036	0.88±0.037	7.41±0.08	-	-
JK24	1.147±0.061	0.292±0.013	2.73±0.02	+	+
JAK31	1.336±0.016	0.941±0.038	4.005±0.21	-	+
JK54	0.709±0.042	0.532±0.048	2.73±0.02	+	+
JAK251	1.241±0.003	1.04±0.019	5.94±0.17	-	+
JAW1	0.457±0.006	0.253±0.013	5.03±0.08	-	-
JAB1	2.717±0.138	1.849±0.048	14.28±0.65	+	+
JAB8	1.916±0.047	1.458±0.074	11.23±0.23	+	+
JAB9	0.502±0.038	0.406±0.030	4.94±0.14	+	+
JAB11	0.715±0.019	0.588±0.026	1.18±0.08	-	-
JAB12	0.999±0.023	0.61±0.019	4.83±0.05	-	-
JAB100	2.751±0.078	1.097±0.054	19.4±0.73	+	+

Values are mean of 3 replicates (n=3); ± standard deviation; + indicate positive activity; - indicate no activity; Trp- Tryptophan

bacterial isolates, 6 were recorded positive for siderophore production, which was indicated by forming a distinct orange-yellow halo zone surrounding the bacterial colony on CAS blue agar medium. However, qualitative analysis revealed that all the bacterial isolates were negative for HCN production. Again, 10 bacterial isolates were recorded positive for the Solubilization of zinc and formed distinct halo zones surrounding the bacterial colonies under qualitative analysis. Only 11 bacterial isolates were recorded positive for biofilm-forming activity (Table 3). Plants require Iron as an important micronutrient for different metabolic processes, such as nitrogen fixation, respiration, photosynthesis, etc. (Slatni et al. 2008). However, the availability of Iron in the soil is generally much lower than required for the normal functioning of plants. Numerous bacteria of the genus *Bacillus* and *Pseudomonas* have been reported to maintain the bioavailability of Iron through the production of low molecular weight siderophore molecules, which chelate Fe^{3+} with high affinity and transport the labelled Iron, i.e. siderophore-Fe^{3+}complex directly to the plants (Sudisha et al. 2006; Beneduzi et al. 2012; Gupta et al. 2015). In the present study, the bacterial isolates have shown siderophore activity that might be useful for promoting plant growth and health. Yu et al. (2011) reported the biocontrol efficiency of siderophore-producing *B. subtilis* CAS15 on fusarium wilt disease. Further, increased plant growth with reduced disease intensity has been reported from rhizospheric bacteria *B. megaterium* mediated through siderophore production (Sivasakthi et al. 2014). Zinc is also an essential micronutrient for plant growth and health. Its deficiency affects various metabolic processes like nitrogen metabolism, photosynthesis, flowering, fruit formation and maturity, reducing crop plants' nutritional status and overall productivity (Kushwaha et al. 2021). In the present study, some bacterial isolates also showed zinc solubilization activity. Similarly, various workers have reported zinc solubilization and plant growth promotion of *Bacillus* spp. in numerous agricultural crops (Ramesh et al. 2014; Hussain et al. 2015; Khande et al. 2017; Zaheer et al. 2019). The bacterial isolates have also been reported to form biofilm in *in-vitro* studies. Biofilm formation enables the bacteria to survive in adverse environmental conditions mediated through quorum sensing by different bacterial species (Camele et al. 2019).

Table 4 Effect of potent bacterial isolates on growth and development of *B. juncea* under different treatments

Treatment	Shoot length (cm)	Root length (cm)	Shoot fresh weight (gm)	Shoot dry weight (gm)	Root fresh weight (gm)	Root dry weight (gm)
Control	18.8±0.65 ^{h}	8.46±0.15 ^{g}	5.56±0.43 ^{f}	0.542±0.03 ^{fg}	0.053±0.008 ^{efg}	0.035±0.003 ^{de}
JAB1	22.13±1.05 ^{fg}	10.9±0.2 ^{ef}	6.58±0.32 ^{e}	0.621±0.02 ^{def}	0.068±0.004 ^{d}	0.04±0.002 ^{cd}
JAB8	23.7±1.22 ^{def}	10.46±0.66 ^{f}	6.16±0.06 ^{e}	0.681±0.06 ^{de}	0.081±0.007 ^{b}	0.042±0.003 ^{bc}
JAB100	23.8±1.15 ^{def}	11.1±0.26 ^{def}	7.15±0.24 ^{d}	0.803±0.08 ^{c}	0.077±0.006 ^{c}	0.042±0.003 ^{bc}
JAB1+JAB8	25.3±1.32 ^{bcd}	11.63±0.61 ^{cde}	8.08±0.2 ^{c}	0.936±0.06 ^{b}	0.083±0.006 ^{bc}	0.062±0.001 ^{a}
JAB8+JAB100	26.16±0.9 ^{bc}	12.26±0.81 ^{bc}	8.15±0.21 ^{c}	0.822±0.03 ^{c}	0.09±0.009 ^{bc}	0.025±0.005 ^{gh}
JAB1+JAB100	24.23±1.59 ^{cde}	11.76±0.61 ^{cd}	8.83±0.2 ^{b}	0.938±0.06 ^{b}	0.084±0.006 ^{bc}	0.047±0.004 ^{b}
JAB1+JAB8+JAB100	31.3±1.8 ^{a}	13.76±0.45 ^{a}	10.87±0.47 ^{a}	1.131±0.12 ^{a}	0.116±0.007 ^{a}	0.064±0.005 ^{a}

Data were calculated after 45 days of inoculation and are mean of 3 replicates ± standard deviation; Different letters indicate significantly different values; *≤0.05; LSD test

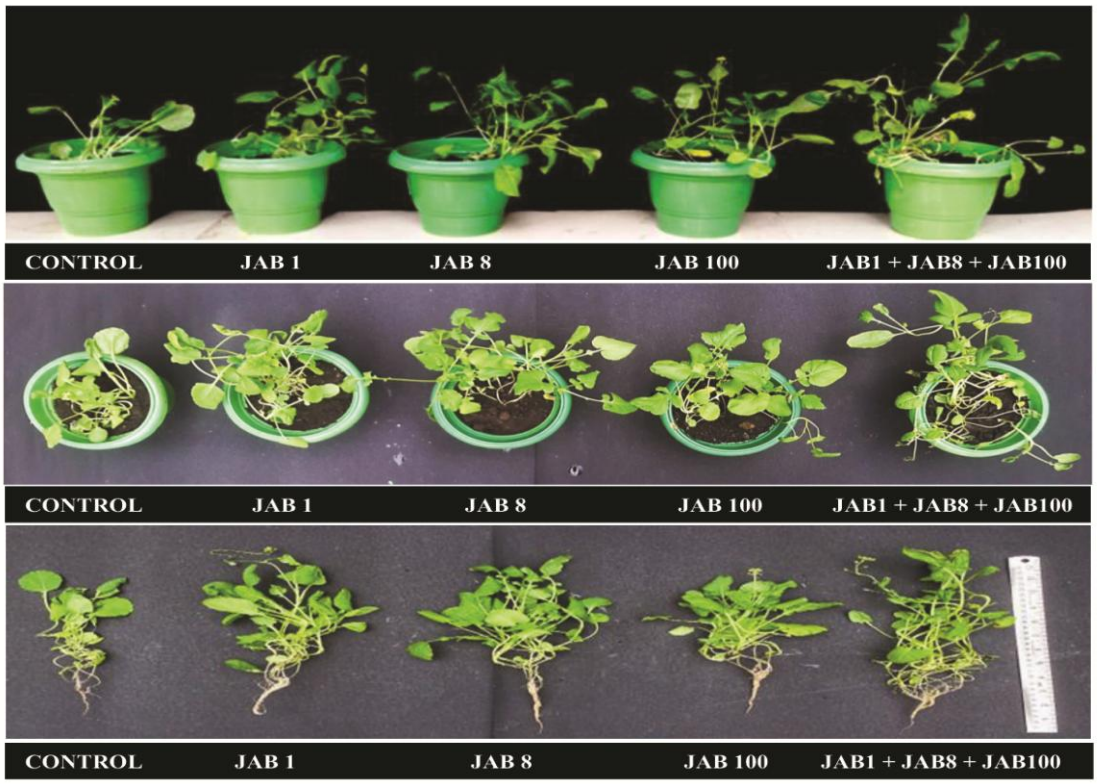

Figure 1 Root and shoot length of *B. juncea* after different treatments using potent bacterial isolates

3.4 Effect of bacterial isolates on growth promotion of *B. juncea*

3.4.1Plant length (shoot and root) and weight (fresh and dry)

Based on *in vitro* plant growth-promoting activities, 3 bacterial isolates, JAB1, JAB8 and JAB 100 were selected for *in vivo* pot experimental trials. The experiment was evaluated following inoculation of the bacterial isolate alone and in combination with each other. After that, the growth promotion of the experimental plant was determined in terms of length, fresh weight, and dry weight. The result revealed that the highest shoots and root length were observed in the treatment sets where all three bacterial isolates (JAB1, JAB8 and JAB100) were co-inoculated in the rhizosphere region (Table 4, Figure 1). It was observed that the shoot length increased by 66.48%, whereas the root length increased by 62.64% as compared to the control (without bacterial inoculation). Similarly, fresh weight and dry weight were also

recorded as being highest in the same treatment set where shoot fresh weight increased by 95.5% and root fresh weight increased by 118.86% compared to control. Again, it was observed that the shoot dry weight increased by 108.67%, and the root dry weight increased by 82.85%**.** A similar study conducted by Sun et al. (2016) reported increased height, fresh weight, and dry weight of *B. napus* when co-inoculated with two strains of *B. subtilis* (LHS11 + FX2). In another study, the tomato's increased height and dry weight were observed after co-inoculation with *B. subtilis* (Singh et al. 2012). Therefore, our present study corroborates with many previous studies which showed that co-inoculation of bacterial isolates with plant growth-promoting activity indicated better enhancement in plant growth-promotion abilities, and such strains can be used as bioinoculants for application in crop productivity without the requirement of any chemicals.

3.4.2 Chlorophyll and protein content

Highest chlorophyll a, b, total chlorophyll and protein content were recorded in the treatment set with co-inoculation of all the selected bacterial isolates (JAB1+JAB8+JAB100) in the rhizospheric region (Table 5). It was observed that the treatment produced 2.13±0.003 mg/g FW of chlorophyll a, 0.917±0.006mg/g FW of chlorophyll b, and 3.04±0.006mg/g FW of total chlorophyll. There was also a gradual increase in total soluble protein content, which was 76.62±4.11 µg/ml, as compared to the control, which showed only 33.54±1.44µg/ml of total soluble protein. Our study was similar to the findings of Cui et al. (2019), who reported increased chlorophyll a and b content in maize plants after treatment with *B. amyloliquefaciens* T B9601-Y2. The bio-based application of *Bacillus* for promoting plant growth with increased biochemical constituents has been cited in numerous literature (Goswami and Deka 2020; Kumar et al. 2021). The exogenous application of *B. methylotrophicus* KE2 as a plant growth promoter has been reported to increase protein content in lettuce leaves (Radhakrishnan and Lee 2016).

3.4.3 Phenol and flavonoid contents

The total phenolic (TPC) and flavonoid content were recorded highest again in the treatment set, which was co-inoculated with all the potent bacterial isolates (JAB1+JAB8+JAB100) (Table 5). The result showed that such a treatment set recorded 1.25±0.005 mgGAE/gram dried plant tissue compared to the control, which recorded 0.71±0.048 mg GAE/gram dried plant tissue. The flavonoid content was observed to be 0.486±0.016 QE/gm plant tissue in the combined inoculated set compared to the control, which showed 0.265±0.009 QE/gm plant tissue. These phenolic derivatives act as a precursor for lignin biosynthesis, and the fungi's toxic nature mediates the defense responses induction in plants and inhibits the growth of the fungal pathogen (Nakkeeran et al. 2006; Dutta et al. 2008). Increased flavonoid content after applying *B. subtilis* CBR05 has been reported in tomatoes (Chandrasekaran et al. 2019). Similarly, inoculation of maize plants with *B. thuringiensis* PM25 under salt stress has reported a 9.24% increase in total flavonoid content (Ali et al. 2022). Thus, the present study agreed with many previous reports on increased phenolic content in plant tissue upon inoculation with plant growth-promoting bacteria.

3.5 Molecular identification of potent bacterial isolates

The molecular identification of all three potent bacterial isolates (JAB1, JAB8 and JAB100) was confirmed by 16S rRNA gene sequences analysis (Figure 2). Based on the NCBI BLAST search, the isolate JAB1 showed the closest homology to *B. velezensis* (similarity 100%) with accession number ON679657, and the isolate JAB8 showed the closest homology with *B. proteolyticus* (similarity 100%) with accession number MT184819. The isolate JAB100 also showed homology with *Bacillus* sp.; however, the percentage of nucleotide sequence similarity was found to be less than 97%, which is below the minimum percentage similarity required for species-level identification (Drancourt et al. 2000).

Table 5 Effect of potent bacterial isolates on biochemical parameters of *Brassica juncea* under different treatments

Treatment	Protein content (µg/ml)	Phenol content (mg/gm DW)	Flavonoid content (mg/gm FW)	Chlorophyll a (mg/gm FW)	Chlorophyll b (mg/gm FW)	Total chlorophyll (mg/gm FW)
Control	33.54±1.44 ^{j}	0.71±0.048 ^{k}	0.265±0.009 ^{cd}	1.36±0.007 ^{k}	0.591±0.004 ^{i}	1.95±0.003 ^{m}
JAB1	58.46±3.25 ^{de}	0.9±0.009 ^{ij}	0.297±0.009 ^{c}	1.89±0.011 ^{c}	0.781±0.007 ^{d}	2.67±0.008 ^{d}
JAB8	53.79±0.94 ^{ef}	0.91±0.015 ^{ij}	0.281±0.009 ^{cd}	1.81±0.015 ^{f}	0.671±0.008 ^{f}	2.48±0.011 ^{h}
JAB100	59.79±0.38 ^{d}	0.93±0.006 ^{hi}	0.303±0.024 ^{c}	1.45±0.006 ^{j}	0.586±0.006 ^{i}	2.03±0.013 ^{l}
JAB1+JAB8	66.37±0.14 ^{bc}	0.95±0.005 ^{h}	0.297±0.008 ^{c}	1.68±0.025 ^{e}	0.84±0.005 ^{b}	2.52±0.027 ^{g}
JAB8+JAB100	70.37±4.75 ^{b}	1.18±0.04 ^{f}	0.382±0.022 ^{b}	1.85±0.006 ^{d}	0.741±0.004 ^{e}	2.59±0.003 ^{f}
JAB1+JAB100	62.46±8.59 ^{cd}	0.89±0.01 ^{j}	0.378±0.018 ^{b}	1.89±0.008 ^{c}	0.812±0.009 ^{c}	2.7±0.019 ^{c}
JAB1+JAB8+JAB100	76.62±4.11 ^{a}	1.25±0.005 ^{e}	0.486±0.016 ^{a}	2.13±0.003 ^{a}	0.917±0.006 ^{a}	3.04±0.006 ^{a}

Data were calculated after 45 days of inoculation and are mean of 3 replicates ± standard deviation. Different letters indicate significantly different values; *≤0.05; LSD test).

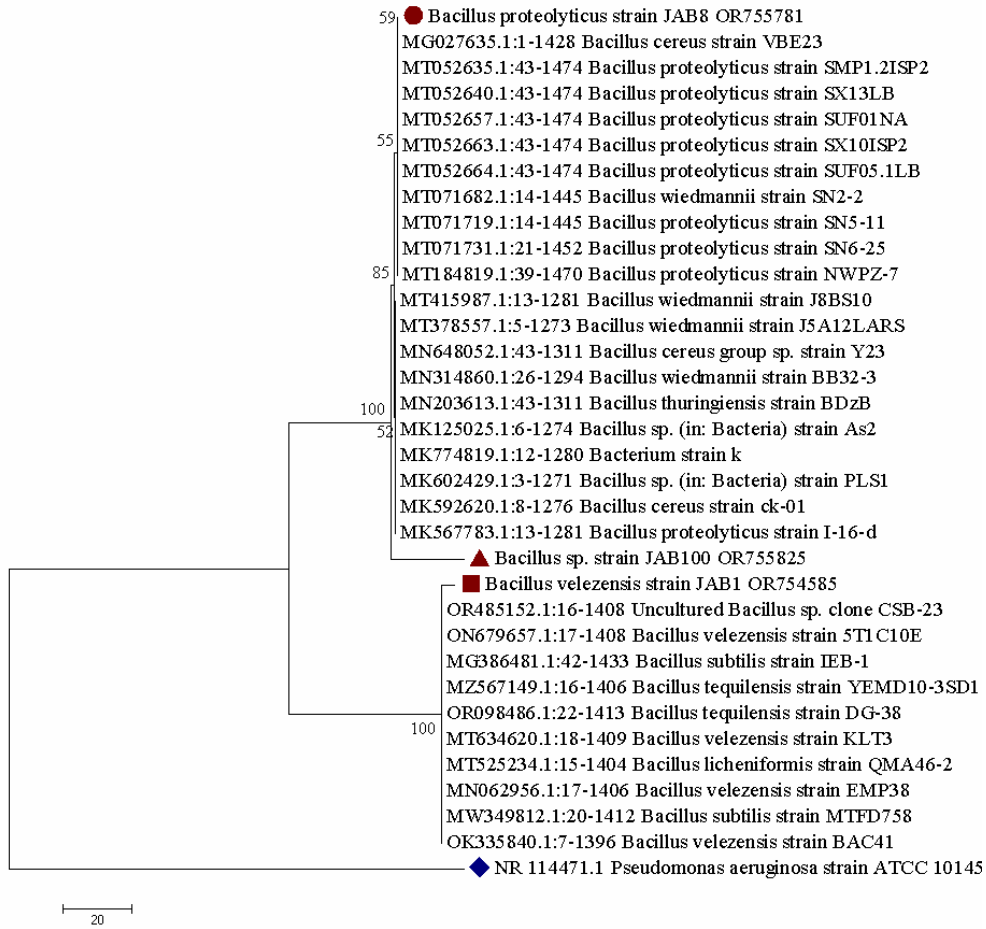

Figure 2 Phylogenetic trees showing the evolutionary relationship of three potent bacterial isolates (JAB1, JAB8 and JAB100) inferred using the Neighbour-joining method considering 16S rDNA sequences.

Therefore, JAB100 could not be conclusively identified at the species level and requires further analysis for accurate taxonomic classification. The phylogenetic tree generated by the neighbor-joining method also validated the identity of the bacterial isolates. The sequences of the isolates JAB1, JAB8 and JAB100 were deposited in NCBI, GenBank database with accession numbers OR754585, OR755781 and OR755825, respectively. Many previous workers have reported the occurrence and dominance of Bacillus species in various hot springs (Adiguzel et al. 2009; Nazari and Mehrabi 2019). The isolation of *B. velezensis* strain MRC 5958f has recently been reported from the Bakra hot spring (Sarangthem et al. 2023). Such findings indicated that members of the genus *Bacillus* might be common inhabitants and well-adapted to survive in extreme environments like hot springs.

Conclusion

The study revealed that Jakrem hot water springs are a rich source of thermophilic bacteria that are dominant by *Bacillus* spp., capable of producing hydrolytic enzymes, and possess plant growth-promoting potential. Characterization of the potent *Bacillus* isolates by morphological, biochemical and 16S rRNA sequencing identified the isolates as *B. velezensis*, *B. proteolyticus*, and *Bacillus* sp. Combined inoculation of these *Bacillus* strains demonstrated significant improvements in plant growth parameters and biochemical constituents of *B. juncea* in *in-vivo* experiments. The findings highlight the ecological significance and biotechnological potential of hot spring bacteria for application as bioinoculants for sustainable agriculture. Harnessing their capabilities can offer promising solutions to mitigate agricultural challenges, promote soil health, and contribute to enhanced crop productivity in the face of evolving climate change and environmental degradation.

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Authors contribution

All the authors contributed to research conception, data analysis and interpretation. AK Collected the sample, performed the experiment and drafting. JR-Participated in the drafting and critical revisions of the article. KT- Writing, review, editing, supervision and validation. All the authors consented and approved to submit the work in the present journal.

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Conflict of interest

The authors have no conflict of interest.

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Role of Probiotic Microorganisms in the Brain Plasticity Development

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ABSTRACT

Probiotics are defined as beneficial microorganisms that are responsible for the maintenance of homeostasis mechanisms within the host system, especially in humans. Other than homeostasis, it is also used to improve a host system's cognition, immune functions, and antioxidant levels. Over the past decades, probiotic microorganisms have been used most commonly as traditional fermented foods in our country and some parts of southeast asia. These fermented food products majorly consist of *Lactobacillus* species, including *Lactobacillus acidophilus*, *L. fermentum*, and *L. plantarum*. The present study explored the potential role of three different lactobacillus strains (*L. acidophilus*, *L. fermentum*, and *L. Plantarum*) in forming brain plasticity changes (BPC) with the help of a cue-based learning paradigm (CBLP). Two staged behavioral studies were conducted for all behavioral analysis groups (BAG) before (without probiotic infusions - WiPI) and after probiotic infusions (with probiotic infusions - WPI) in RBLP. Behavioral responses of the WiPI & WPI phases showed the effect of a stress-free habituated environment in developing BPC and strengthening of BPC by oral infusions of probiotic microorganisms (PM). WiPI and WPI behavioral analysis were used in this study to validate BPC in a laboratory-controlled environment. Infusion of probiotic microorganisms through oral passage may have a more significant impact on the synthesis, production, and transmission of neurotransmitter precursor compounds (NPC) from the gut to the central nervous system (CNS) through the blood-brain barrier (BBB). Increased transmission of the NPC strengthens the formed plasticity changes, which results in the formation of cognitive memory functions. Thus, the present study proved that probiotic microorganisms may play a major role in cognition development through the BPC.

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1 Introduction

Synaptic plasticity is a unique adaptive feature of our central nervous system that uses activity-dependent mechanisms to restructure/alter the strength of neuronal connections based on stimulus exposure (Chaudhury et al. 2016; Kim et al. 2018; Abraham et al. 2019; Appelbaum et al. 2023; Mukilan et al. 2024a). It was already reported that synaptic plasticity plays an unavoidable role in incorporating beneficial/harmful experiences in the form of long-lasting information in different brain regions during the formation of learning and memory (LM) (Ramirez and Arbuckle 2016; Appelbaum et al. 2023; Mukilan 2023). This formed LM follows two distinct molecular mechanisms for the acquaintance and retrieval of learned information in the brain. These two molecular mechanisms result in the formation of short-term (ST) memory and long-term (LT) memory. ST and LT memory use existing proteins and RNA-dependent-protein synthesis mechanisms to form memory. Formed ST and LT memory will last for 2-6 hours (ST) until the end of life (LT). However, the time needed to form LTM is high compared to STM (Norris 2017; Abraham et al. 2019; Ashok et al. 2019; Evans et al. 2021; Luis and Ryan 2022; Mukilan et al. 2024b). For memory formation, LTM uses specific neuronal signaling pathways like cAMP response element binding protein (CREB)–mediated neuronal signaling pathway (MNSP). This CREB-MNSP is initiated by releasing specific neurotransmitters like 5-HT from the presynaptic neuron into the synaptic cleft (SC). In the SC, released neurotransmitter binds to the specific postsynaptic neuronal receptors (PNR)(Ganesh et al. 2010; Ganesh et al. 2012; Mukilan et al. 2015; Ortega-Martínez 2015; Rajan 2021). Binding neurotransmitters with the PNR results in calcium influx (CI) induction. CI influx later activates adenyl cyclase (AC), cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and enzyme-regulated kinase – 1 (ERK – 1). Upregulation of AC, cAMP, PKA, and ERK – 1 later results in the activation and phosphorylation of CREB, which induces immediate-early genes (IEG) and postsynaptic density (PSD) protein expressions. Reliable expressions of these neuronal signaling molecules are involved in the formation of LT memory in the different brain regions (Peng et al. 2010; Mukilan et al. 2018a, 2018b; Bai and Suzuki 2020; Lin et al. 2021; Mukilan 2022; Mukilan 2023).

Reported research findings showed that oral/gut-beneficial microflora are responsible for the maintenance of the brain homeostasis mechanisms of a host (Appleton 2018; Gentile and Weir 2018; Suganya and Koo 2020). In a healthy state, some oral/gut bacterial strains are unavoidable in synthesizing and producing neurotransmitter precursor compounds (NPCs) in the gut. Produced NPCs are transported from the gut to the CNS through the BBB (Misiak et al. 2020; Mukilan 2023). Transmitted NPCs are involved in the production of a wide range of neurotransmitters [serotonin (5-HT), γ-aminobutyric acid (GABA), dopamine (DA), and noradrenaline (NA)] from the presynaptic neurons for the induction of neuronal signaling pathways (NSP) (Chen et al. 2017; O'Donnell et al. 2020; Sengupta 2020; Dicks 2022). Induction of NSP forms the cognitive memory in the olfactory bulb, hippocampus, and amygdala. Thus, the formed cognitive memory inter-relates the oral-gut-brain axis with the CNS. It also proved that oral/gut-beneficial flora are important in forming cognitive functions (Mukilan et al. 2018a; Salami and Soheili 2022; Mukilan 2023). In normal conditions, existing beneficial flora is affected by various conditions like food habits, alcohol consumption, changes in circadian rhythm, alcohol consumption, diet, smoking, and other environmental conditions. To overcome the depletion of beneficial flora, nowadays's varying range of probiotic microorganisms are taken along with the diet to maintain oral/gut probiotic health (Hillemacher et al. 2018; Savin et al. 2018; Yang et al. 2020; Kumar et al. 2024). In the present work, we tried to elucidate the importance of probiotic microorganisms in strengthening brain plasticity changes during cognitive learning and memory formation with the help of a cue-based learning paradigm (CBLP).

2 Materials and Methods

2.1 Experimental Fishes

Commercially available adult goldfish (*Carassius auratus*) with body lengths (6-8 cm) and weights (8 – 15 g) were obtained from a local market. Obtained fishes were maintained with their respective groups (n = 6/group) in the home aquarium throughout the experimental period. Rectangular glass tanks, 42 X 30 X 21 (length, breadth, and height - LBH) inches, served as home aquariums. In the home aquarium, continuous air circulation, photoperiod (12 hours of light:12 hours of dark), and a standard temperature of 26 ± 2 ºC were provided for all experimental fishes. Commercial dry food pellets (Taiyo Pet Products India Pvt. Ltd) were provided thrice a day @ 8.00, 14.00, and 19.00 hours to meet the energy needs of all experimental animals. The home tank was adequately cleaned and replaced with fresh water on alternative days to maintain a debris-free environment. Experimental design follows the institutional ethical regulatory guidelines of Sri Ramakrishna Institutions, Coimbatore, Tamil Nadu, India.

2.2 Experimental Design

A rectangular glass tank (RGT) having an LBH of 42 X 30 X 21 inches was custom-designed and used in the behavioral analysis. The designed RGT was divided into three different compartments based on the study's needs. These three chambers include two feeding compartments (FC) and one central compartment (CC). FCs and CC vary in their LBH of 6 X 30 X 21 and 30 X 30 X 21 inches. In the two FCs, one FC acts as a positive reward chamber with blue colored cues, and another one acts as a negative chamber

with red colored cues. Both the FCs have a central opening for fish movement from CC to FCs.

2.3 Infusion Mixture Preparation

All three probiotic strains *L. acidophilus* (MTCC No. 10307), *L. fermentum* (MTCC No. 9748), and *L. plantarum* (MTCC No. 12921) were acquired from the MTCC (Microbial Type Culture Collection and Gene Bank), IMTECH (Institute of Microbial Technology), Sector 39, Chandigarh, Punjab, India. Acquired probiotic strains were streaked on *Lactobacillus* MRS Agar to confirm their purity. After purity confirmation, acquired probiotic cultures were used to prepare an oral probiotic mixture in the ratio of 50:50 (as a single dose in a pure form), and 20:20:20:40 (as a single dose in mixed form). Prepared oral mixtures were used for the oral administrations into specific experimental groups after the primary phase of behavioral analysis (PPBA).

2.4 Behavioral Analysis

2.4.1 Experimental Groups

Fishes were randomly separated into four different groups. These are Group -1 (infused with *L. acidophilus*), Group – 2 (infused with *L. fermentum*), Group – 3 (infused with *L. plantarum*), and Group – 4 (infused with *L. acidophilus*, *L. fermentum*, and *L. Plantarum*).

2.4.2 Cue-based Learning Paradigm

The cue-based learning paradigm (CBLP) was used to understand the effect of a controlled environment and probiotic infusions on learning and memory formation (LMF). During LMF, two different color cues (blue, and red) were used for the behavioral studies in the FCs of the experimental apparatus. Blue and red colored cues act as positive and negative rewards with/ without food rewards. Behavioral responses were calculated based on the amount of time spent in the left chamber (LC), central chamber (CC), and right chamber (RC).

2.4.3 Predator Exposure Test

A predator exposure test (PET) was performed to identify whether probiotic oral infusions develop stress or not. In this PET, RGT has a size of 42 X 30 X 21 inches (LBH) and was used as an exposure chamber (EC). The EC was divided into three equal-sized zones (LBH of 14 X 10 X 21 inches), including a no-fear zone (NFZ), mid-fear zone (MFZ), and complete-fear zone (CFZ) with the help of two transparent plexi sheets with central openings. During PET, goldfish (*C. auratus*) and its predator (bluegills) were introduced into NFZ, & isolated chamber in CFZ for 15 minutes. Behavioral responses of all experimental groups were recorded in terms of time spent in NFZ, MFZ, and CFZ.

2.4.4 Open Field Test

Followed by PET, an open field test (OFT) is employed in this study to identify the presence/absence of anxiety-like behavior. OFT was carried out in RGT, which was 42 X 30 X 21 inches (LBH) and had 36 square boxes (5 X 5 cm/each). All 36 square boxes were divided into two different zones, i.e. the outer and inner zones. The outer and inner zone consists of 18 square boxes. Behavioural responses of experimental groups were analyzed in terms of time spent in an inner compartment (TSI) and time spent in an outer compartment (TSO).

2.5 Statistical Representation

Behavioral responses of all three behavioral tests were plotted as a bar diagram with the help of a Microsoft Excel program. An online tool (MedCalc statistical software) was used to calculate the p-value with the help of mean average values and standard error.

3 Results

3.1 Role of stress-free assimilated environment in the formation of cognitive functions

The current study initially uses the primary phase of behavioral analysis (PPBA) to understand the effect of a stress-free assimilated environment on cognitive memory formation using a CBLP in a controlled environment. Initially, animals of all experimental groups underwent learning and memory retention tests in the experimental apparatus after completing the assimilation and exploration phases. During the assimilation process, animals were maintained in the home tank for five days (Days 1-5) for their adaptations to the laboratory-controlled conditions. Following the habituation process, all experimental animals were allowed to explore the experimental apparatus without the color cue in FCs during the exploration phase (Days 6-8). Behavioral responses showed that initially, animals spent more time in CC than LC and RC. Later, time spent in CC was reduced in the subsequent days, showing that animals explored the FCs on the opposite sides of CC. After the exploration, all experimental groups were trained on CBLP based on color cues with/without food rewards between days 9-11. During the training phase, all experimental animals learned about the positive/negative stimuli based on food reward learning. Every visit to RC was awarded a food pellet, and vice versa, in LC. Obtained behavioral scores showed that the initial day (day 9) had fewer visits to RC than other days (days 10 and 11). It showed that animals spend more time in LC and CC on the first training day than in RC. Only a few animals grasped the information on the first day and were rewarded for their effort. Later, all animals gradually increased their visits to RC in the subsequent days due to training (Figure 1).

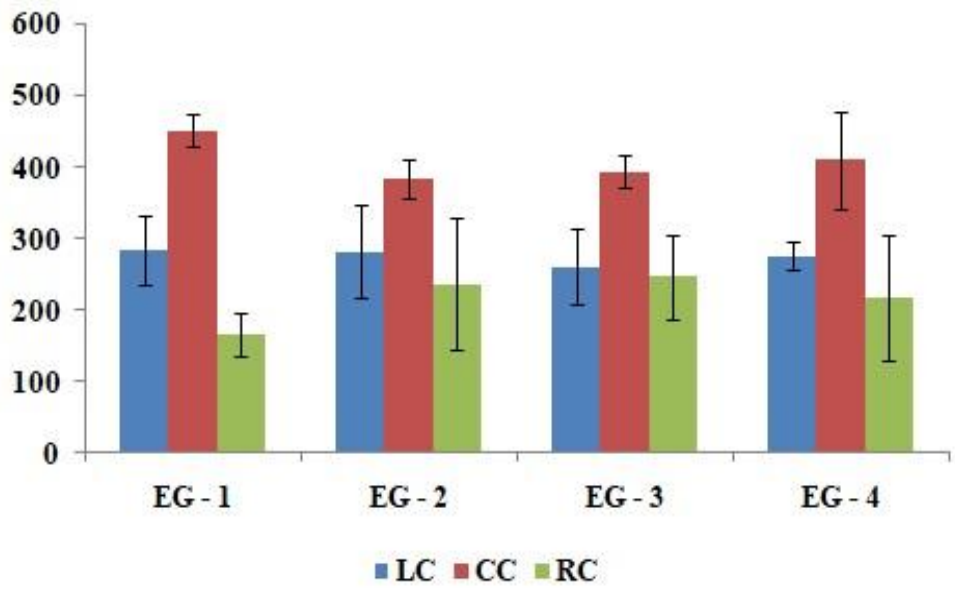

Figure 1 The first phase of behavioral training (without probiotic infusions - WiPI) showed that all experimental animals were trained in the experimental setup with the help of color cues based on reward learning. Initially, the number of visits to the right chamber (RC) was low on day 9 and gradually increased on days 10, and 11 which showed the animal's learning ability was associated with a reward. (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

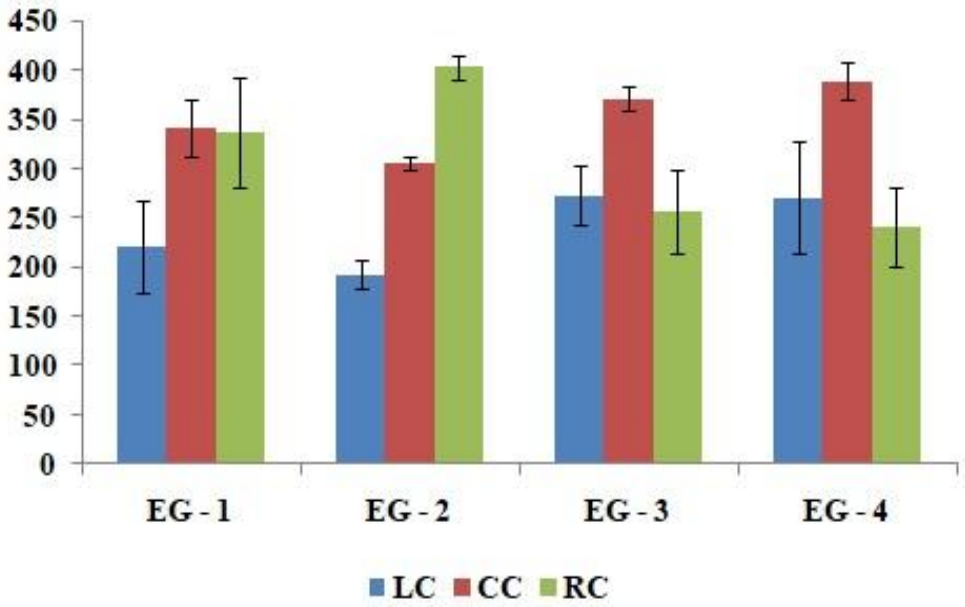

Figure 2 The first phase of behavioral testing (without probiotic infusions - WiPI) proved that experimental groups acquired information about the positive/negative reward with efficient retrieval of learned information. (Y axis denotes the time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

Following training, a five-day time gap (days 12-16) was given to consolidate learned information in the brain. Formed consolidated memory was tested for all experimental groups between days 17-20 in the experimental apparatus using positive and negative color cues. Behavioral scores proved that the training phase is important in retrieving learned information with an increased response to the positive chamber in the CBLP (Figure 2). Consolidated behavioral scores showed that a habituated stress-free controlled environment may involved in the formation of cognitive memory through the enrichment of formed neuronal plasticity (Figure 3). After completion of PPBA, all experimental groups are maintained in the home aquarium between days 21-23 for memory reconsolidation.

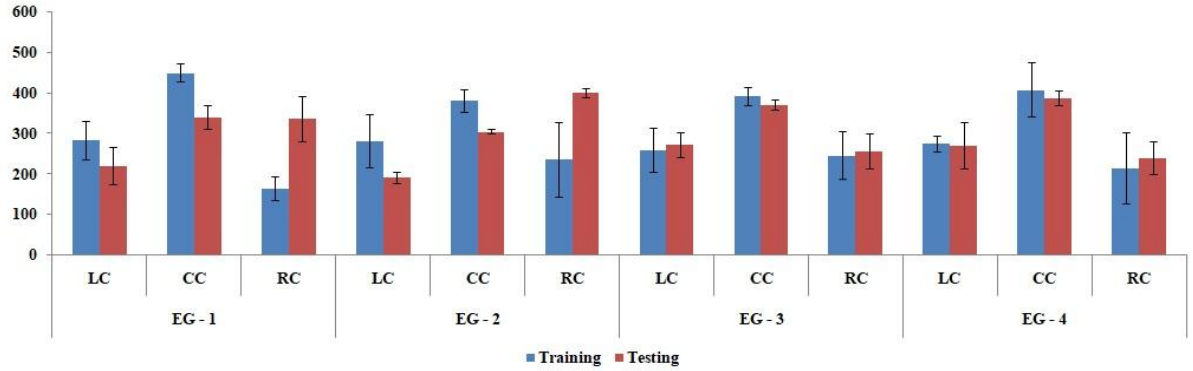

Figure 3 Comparative analysis of the primary phase of behavioral training and testing (without probiotic infusions - WiPI) showed that all four experimental groups learned about the provided positive/negative color stimuli during the process of training and retrieved stored information in an increased manner during the testing phase (Days 21 – 23). (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

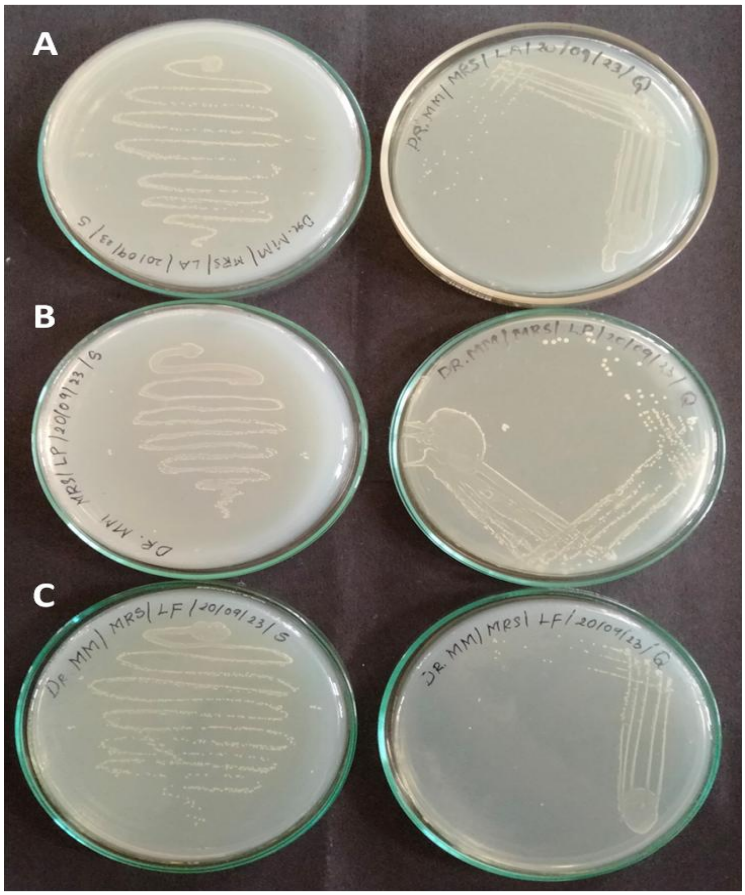

Figure 4 Representative plate pictures (A – *Lactobacillus acidophilus*, B - *Lactobacillus Plantarum*, and C - *Lactobacillus fermentum*) showing the purity confirmation of acquired *Lactobacillus* cultures with the help of the quadrant streak plate method.

3.2 Efficiency of probiotic infusions in the strengthening of developed cognitive functions

Following PPBA, the purity of acquired *Lactobacillus* cultures was identified using the quadrant streak plate method (Figure 4). Obtained individual colonies of all three probiotic microorganisms were inoculated in 5 ml of *Lactobacillus* MRS broth medium and incubated at 37 ºC for 24-36 hours. The prepared overnight culture was used for the preparation of the oral microbial mixture along with phosphate buffer saline (PBS) in a ratio of 50:50 (pure form)

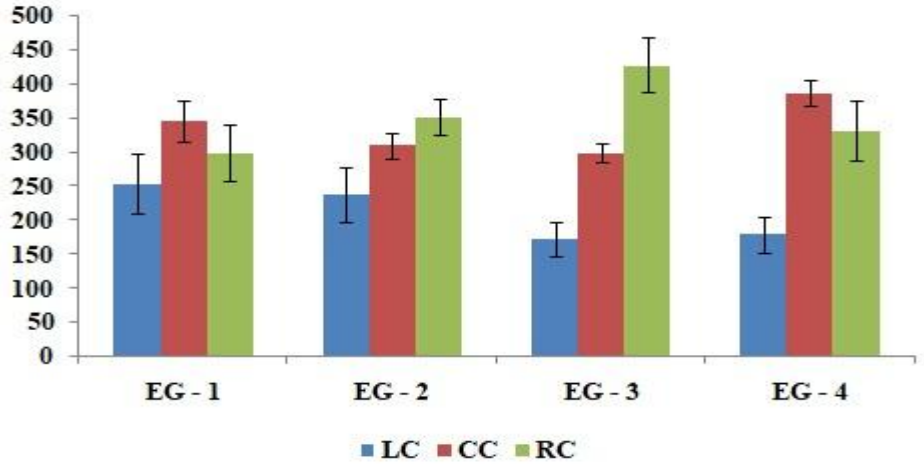

Figure 5 Behavioral scores of second phase training showed that probiotic oral infusions may take part in the strengthening of formed synaptic plasticity during the first phase of behavioral training. (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

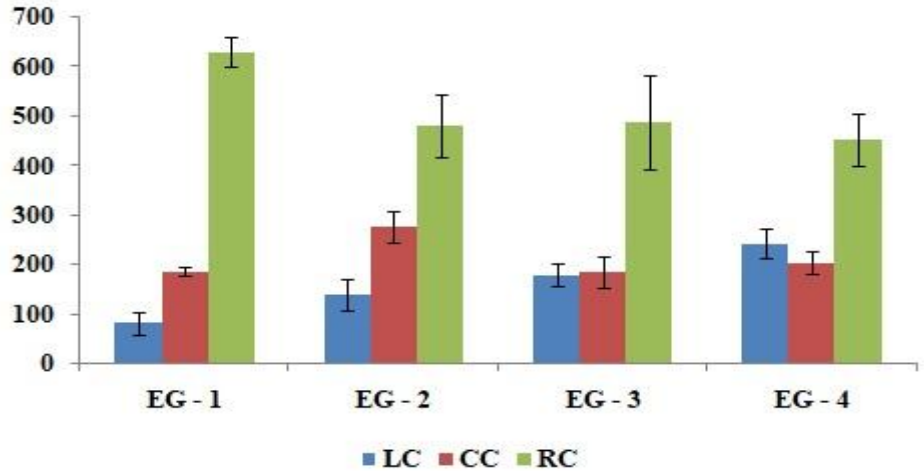

Figure 6 Behavioral scores of the second phase of testing showed that probiotic oral infusions also enhanced information retrieval compared to the first phase of behavioral testing. (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

and 20:20:20:40 (mixed form). According to the study, the prepared infused mixtures were infused into experimental groups in pure and mixed doses. Before the secondary phase of behavioral analysis (SPBA), experimental groups – 1, 2, and 3 (received pure oral infusions) and experimental group – 4 are infused with mixed probiotic cultures. Prepared oral infusion mixtures were orally administrated into the experimental groups on day 24. Following the three-day time intervals (days 25-27), the SPBA training phase was carried out to identify the impact of probiotic oral microbial infusions (POMI) on strengthening synaptic connections during learning and memory formation. The training and testing phases were carried out between two different time intervals. In SPBA, training was carried out for three days between days 28-30. Behavioral responses of the SPBA training phase showed that all experimental groups actively learned about the reward provided in the RC compared to the FPBA training phase (Figure 5). SPBA training scores also showed that POMI may induce increased secretion of NPCs and neurotransmitters responsible for increased information acquisition. Followed by training, testing was carried out after 72 hours (3 days) of SPBA training on days 34-36. Testing behavioral scores showed that all animals spent more time/a higher number of visits to the positive-reward chamber than other chambers (Figure 6). Comparative analysis of training and testing phases proved that POMI played a major role in strengthening formed plasticity changes in the brain (Figure 7).

Comparative analysis of two different training phases, PPBA (without probiotic infusions – WiPI) and SPBA (with probiotic infusions – WPI), proved that a controlled habituated laboratory environment plays an unavoidable role in the formation of stress-free cognitive memory

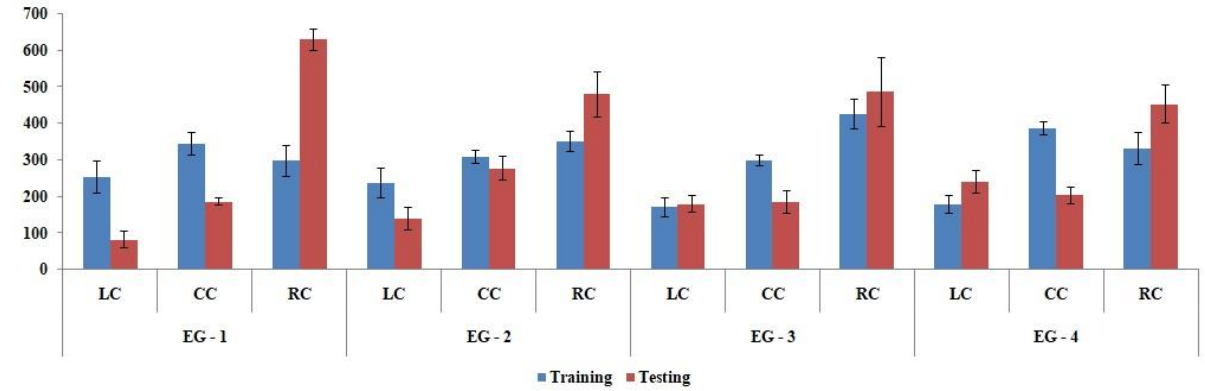

Figure 7 Comparative analysis of behavioral training and testing (with probiotic infusions) showed that all four experimental groups showed enhanced learning abilities during the process of training and efficient retrieval of stored information compared to the without probiotic infusive training and testing. (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

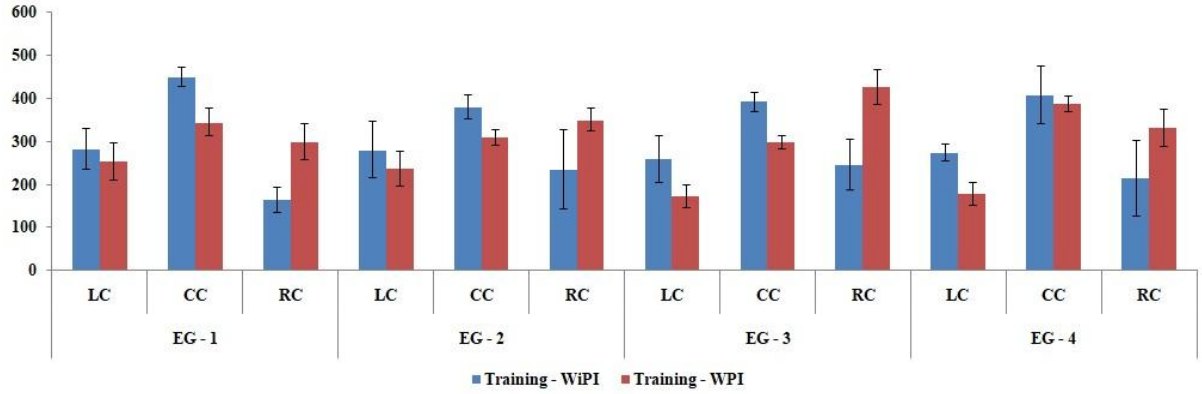

Figure 8 Comparative analysis of behavioral training (without probiotic infusions – WiPI Vs with probiotic infusions – WPI) showed that probiotic oral infusions played a major role in the development of synaptic plasticity (enhanced learning ability) compared to non-infusive training. (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

through the formation of neuronal/synaptic plasticity changes. Formed neuronal plasticity was further strengthened during the training phase after probiotic infusions. Thus, the obtained results showed that formed plasticity changes can be strengthened with the help of probiotic intake (Figure 8). Besides enhancing learning ability, probiotic oral infusions also enhance the efficient retrieval of learned information during testing after infusions. Thus, the comparative testing analysis proved that probiotic microorganisms play a pivotal role in strengthening formed neuronal connections with their intake in pure and mixed form (Figure 9).

3.3 Effect of probiotic oral microbial infusions on the development of stress-free cognitive functions

To prove the effect of probiotic oral microbial infusions on stress-free memory development, we used predator exposure test (PET) and open field test (OFT) in this study. All experimental animals performed PET and OFT to detect the absence of anxiety-like behavior, and fear memory formation in the infused groups. Scores of PET showed that probiotic oral microbial infusions were never involved in the formation of anxiety-like behavior in infused groups. The animals spent more time in the stress-free zone (inner compartments) than the stressed zone (outer compartments) in the experimental setup. It also proved that probiotic oral microbial infusions never induce anxiety-like behavior against pure and mixed doses of infusions (Figure 10). Followed by PET, OFT was performed to prove that probiotic oral infusions may prevent the formation of fear memory when experimental groups are exposed to their predators. Behavioral responses of the experimental groups showed that all animals spent more time near their predator, which showed that probiotic oral infusions might limit the production of cortisol through the hypothalamic-pituitary-adrenal (HPA) axis. Reduced amount of cortisol production may result in the prevention of stress formation (Figure 11). Statistical analysis showed no significant differences among the CBLP, PET, and OFT experimental groups. Nonsignificant differences were calculated by the observed p values with the help of mean average values and standard errors.

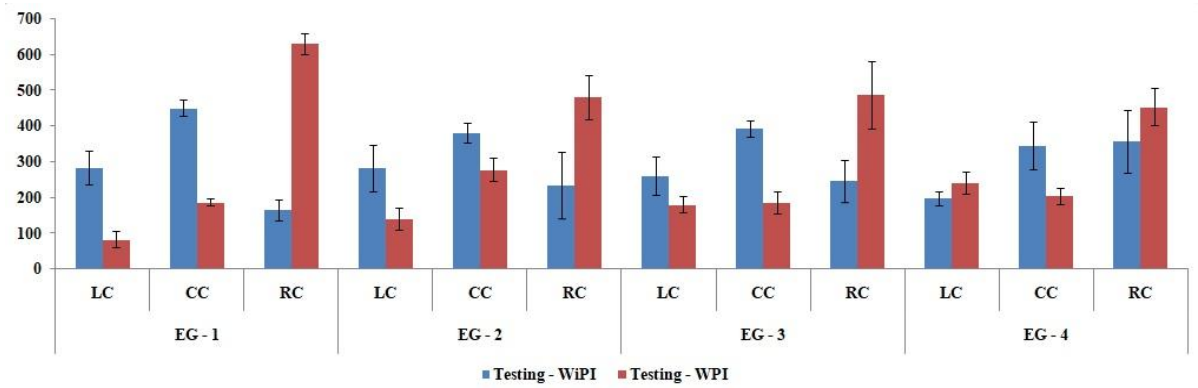

Figure 9 Comparative analysis of behavioral testing (without probiotic infusions – WiPI Vs with probiotic infusions – WPI) showed that probiotic oral infusions played an unavoidable role in the retrieval of learned information along with the development of synaptic plasticity during the testing phase. (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

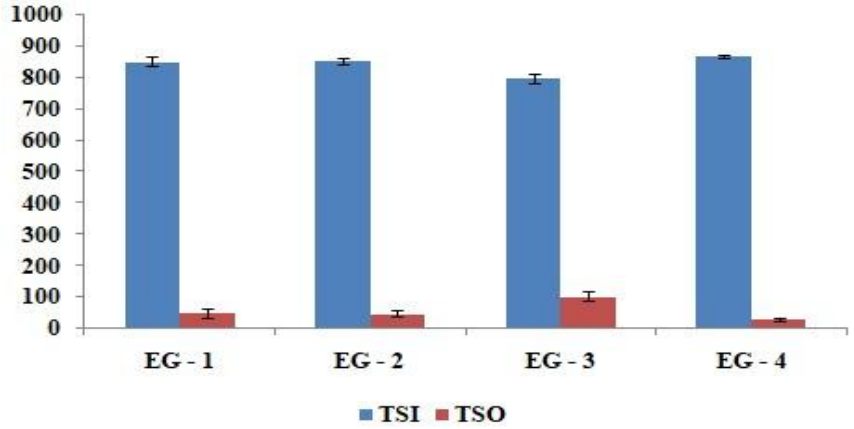

Figure 10 Behavioral responses of open field test (OFT) showed that infused experimental groups with probiotics in pure (EG – 1, 2, and 3) and mixed form (EG – 4) did not show any anxiety-like behavior development as animals spent more amount of time in the inner compartment (TSI) compared to the outer compartment (TSO). (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

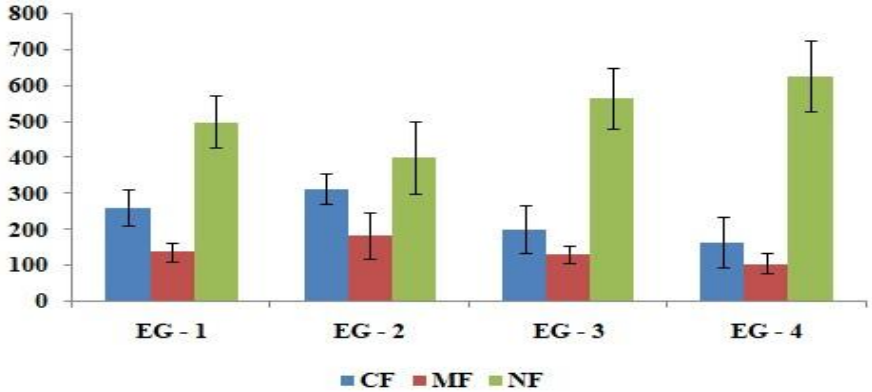

Figure 11 Behavioral scores of the predator exposure test (PET) proved that probiotic oral infusions do not show any fear memory development in the experimental groups. Responses showed that all experimental animals spend most of the time near the predator [No fear zone (NF)] other than mid fear zone (MF), and complete fear zone (CFZ). Thus observed result supports the role of probiotics in the alleviation of stress formation. (Y axis denotes time in seconds; EG – experimental group; LC- left chamber; RC – right chamber; CC – central chamber).

4 Discussion

In recent scenarios, oral and gut microflora research has become a prominent research area in the oral-gut-brain (OGB) axis. This OGB axis shows close interconnection between the oral cavity, brain, and gastrointestinal tract (GI) (Narengaowa et al. 2021; Paudel et al. 2022; Chaudhry et al. 2023; Mukilan et al. 2024b). Emerging clinical evidence shows that this oral and gut microbiota may contain many bacterial communities, including beneficial and harmful flora. In a healthy condition, these beneficial flora play a relevant role in the maintenance of the homeostasis mechanism in the GI, which results in the production and transmission of neurotransmitter precursor compounds (NPCs) from the gut to the CNS (Zheng et al. 2020; Chen et al. 2021; Varela-Trinidad et al. 2022; Ji et al. 2023). The BBB carried out transmission of NPCs through the vagus nerve in a direct/indirect way. Transmitted NPCs produce brain neurotransmitters and the subsequent activation of neuronal signaling molecules involved in cognitive memory formation (Alajangi et al. 2022; Sarubbo et al. 2022; Mukilan 2023). Dysbiosis of oral/gut microflora further plays a major role in forming immune dysfunction, lung diseases, and reduced cognitive functions within a host. Few reports showed that ageing, food habit alterations, pathogenic infections, and poor oral hygiene play an unavoidable role in the development of cognitive dysfunctions through oral-gut dysbiosis (Kandpal et al. 2022; Malik et al. 2023; Mitra et al. 2023). Formed oral-gut dysbiosis consequently results in the causation of gastritis and other systematic disorders. This formed dysbiosis state can be reversed by the intake of probiotic microorganisms along with diet for the maintenance of probiotic flora within the oral cavity and gut (Sandhu et al. 2017; Den et al. 2020; Zhu et al. 2021; Liu et al. 2023).

The present study strived to explore the role of probiotic microorganisms in developing and strengthening synaptic plasticity with the help of a cue-based learning paradigm. Recent reports have proved that pathogenic microbial colonization plays a significant role in developing cognitive dysfunctions during dysbiosis. In healthy conditions, this pathogenic microbial colonization may controlled by the action of native flora with its mutual relationship with the barrier immunity of the existing host immune system (Sarkar et al. 2021; Dash et al. 2022; Ahmed et al. 2024; Pitchaikani et al. 2024). To prevent the colonization of pathogens, probiotic intake has become an alternative way to safeguard the host from oral and gut microflora disturbances. Based on the information availed from the recent reports, the current study tried to traverse through the beneficial effect of probiotics in the strengthening of brain neuronal plasticity changes (Han et al. 2021; Wang et al. 2021; Dahiya and Nigam 2022; Gebrayel et al. 2022). Obtained results showed that probiotic oral infusions had a major role in regulating neurotransmitter production and its associated signaling molecules for the development of proper cognitive memory. Formed cognitive memory showed the proper production and transmission of NPCs from the gut to the brain via the BBB. Transported NPCs resulted in synthesizing neurotransmitters (serotonin) in the presynaptic neurons and their release into the SC. Once released into the SC, serotonin binds with 5-HT receptors, increasing calcium influx inside postsynaptic neurons. Increased calcium influx further activates neuronal signaling molecules needed for the maintenance of proper cognitive health (Chen et al. 2021; Dicks 2022; Miri et al. 2023; Margoob et al. 2024). This probiotic strain may also prevent stress formation by regulating the production of cortisol in the hypothalamic-pituitary-adrenal (HPA) axis (Freimer et al. 2022; Sabit et al. 2023). Thus, the present study revealed that probiotics are responsible for cognitive memory formation in the brain and alleviating stress formation in a host system.

Conclusion

The eventual upshots of the present study manifest the effect of probiotic infusions on the formation of cognitive memory. Recently, it was shown that pathogenic microbial colonization or conversion of normal flora into opportunistic pathogens may result in cognitive impairment through oral/gut dysbiosis. It was also reported that formed oral/gut dysbiosis can be reversed with the oral intake of probiotic microorganisms to reverse the formed cognitive impairment. In the current study, the ramifications of probiotics on the formation and strengthening of neuronal connections were studied in a controlled, habituated, serene environment. The studies showed that probiotic's impact on enhancing brain plasticity development was studied using a reward-based learning paradigm in a laboratory. Experimental behavioral responses showed that entering live probiotic microorganisms into the oral passage might prevent pathogenic colonization in the oral cavity and gut and positively impact cognitive health. Development of cognitive health results in the proper neurotransmitter production by transmitting the needed amount of neurotransmitter precursor compounds from the gut through the BBB. Formation of the needed quantity of neurotransmission results in the development of RNA/Protein mediated LT memory formation by regulating neuronal signaling molecules, microRNAs, and negative regulators of cognitive memory formation. It also proved that probiotics may control the synthesis and production of stress hormones like cortisol in the HPA axis other than enhanced neuronal plasticity changes. For the first time, the current study opened up the dual role of probiotic microorganisms in controlling stress formation and its effect on developing and strengthening existing neuronal connections to maintain proper cognitive health. The present study also revealed that probiotics can be stress relievers to control endocrine hormone production.

Authors Contributions

MM performed the conceptualization, research design, funding acquisition, original investigation, draft preparation, revision, and editing of this manuscript. RA and SP performed the experimentation and data collection.

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Conflict of Interest

Authors report no conflicts of interest in this work

Data Availability

Research data is available with the authors and shall be provided upon request.

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Hematite Nanoparticle Mediated Enhancement of *Chlorella minutissima* Lipid Productivity for Sustainable Biodiesel Production

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Biofuel Enhancement

ABSTRACT

This study aims to enhance lipid and biofuel productivity from *Chlorella minutissima* with hematite (α-Fe_2O_3) nanoparticles (IONPs) as a growth stimulant. The IONPs were synthesized using chemical method and characterized using X-ray diffraction (XRD), Scanning Electron Microscopy (SEM), and Energy Dispersive X-ray (EDX) analysis to confirm their structure and composition. The experimental setup involved inoculating various concentrations of IONPs (10, 20, and 30 $mg \cdot L^{-1}$) into the microalgal BG-11 growth medium to evaluate their impact on microalgal growth and biodiesel production. Results of this study showed that a concentration of 10 $mg \cdot L^{-1}$ of IONPs significantly increased the biomass concentration to 508.1 $mg \cdot L^{-1}$ over a 20-day cultivation period, achieving the highest biomass production rate of 31.7 $mg \cdot L^{-1} \cdot d^{-1}$ at this concentration. The lipid extracted from the microalgal biomass was subsequently transesterified into biodiesel. Key biodiesel properties, such as cetane number, calorific value, density, and viscosity, were measured to assess fuel quality. The findings demonstrate that incorporating hematite nanoparticles into the microalgal growth medium can significantly

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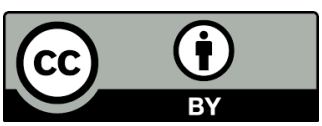

boost both lipid content and overall growth, thereby improving biodiesel production. This study suggests that the use of α-Fe_2O_3 nanoparticles presents a promising approach for scalable and sustainable biofuel production from microalgae.

1 Introduction

The development of microalgal biomass to produce carbon-neutral and renewable biofuel is essential for environmental sustainability, national economy, and energy security (Vasistha et al. 2021; Zhao et al. 2024a). Biodiesel, which is classified as an alkyl monoester of long-chain fatty acids, is a sustainable fuel that is extensively utilized in diesel engines across several nations (Yaşar 2020; Moschona et al. 2024). It has been proposed that biofuels such as biodiesel and bioethanol present captivating alternatives to renewable energy sources. Microalgae have been thoroughly investigated as a source for generating biofuels. The main components present in microalgal biomass are lipids, proteins, and polysaccharides. The lipid component is used as the primary material for biodiesel production. Some oleaginous species such as *Chlorella* (Saxena et al. 2020), *Nitzschia* (Huang et al. 2022), and *Scenedesmus obliquusand* (He et al. 2017) are the most widely researched species in biodiesel production (Ma et al. 2022). The intensive part of producing biofuel from microalgae is extracting biomass from the diluted culture broth (Muhammad et al. 2021). Consequently, one of the main obstacles to the commercialization and large-scale production of microalgae is the low cell concentration and lipid content. Thus, the methods to enhance the growth of microalgae with comparatively greater biomass concentration and lipid accumulations are significant requirements in biofuel production (Kaushik et al. 2009; Ganesh Saratale et al. 2022). Adding nanoparticles throughout the cultivation and harvesting phases is one of the many methods that may be employed to improve microalgae development (Vasistha et al. 2021). The application of NPs during the microalgal growth phase can increase the amount of CO_2 that is absorbed from the atmosphere and even improve the photobioreactor's ability to convert light efficiently, which accelerates the growth of the microalgae (Pahariya et al. 2023). While incorporating nano-additives into microalgae cultures, it is important to consider both the characteristics and concentration of the NPs carefully. However, the impact of NPs on the growth and biochemical composition of microalgae depends closely on their unique physical characteristics (Saxena et al. 2020). It is reported that the lipid content of *Chlorella fusca* LEB 111 increased by 10.9% and 16.7% when grown outdoors with 0.3 and 0.5 g L^{-1} of nanofibers, respectively. These nanofibers are composed of polymeric nanofibers (10% w/v) made from polyacrylonitrile (PAN) dissolved in dimethylformamide (DMF) with the addition of 4% (w/v) Fe_2O_3NPs. When exposed to uncoated nano-zero-valent iron (nZVI), *Tetraselmis suecica* showed a lipid augmentation of 41.90% in the study on the effects of nZVI on various microalgae species. Additionally, they noticed that adding inorganically coated nZVI powder to *Pavlova lutheri* resulted in a 46.34% increase in lipid levels (Kadar et al. 2012). The biodiesel yield in *Spirulina* was enhanced up to 81% by Fe_2O_3 nanoparticles, which were synthesized using a green procedure involving extracts from *Hibiscus rosa-sinensis* (Banerjee et al. 2019). Similarly, it was found that the addition of a 100 mg. L^{-1} dose of nZVI enhances the biomass concentration in *Isochrisis galbana* by 18.75%, accompanied by a 3.57% increase in lipid accumulation (Kadar et al. 2012). Similarly, the lipid content was increased by 16.7% in *Chlorella fusca* when nZVI was added at 0.5 g L^{-1}(Da Silva Vaz et al. 2020). The impact of nanoparticles on the growth and biochemical composition of microalgae is contingent upon their physical characteristics (Khan et al. 2018). *Candida rugosa* lipase was employed for the transesterification of algal lipids after being immobilized on graphene oxides magnetized with $NiFe_2O_4$ nanoparticles ($NiFe_2O_4$-GO). This nanocomposite proved to be an outstanding nano biocatalyst for efficient biodiesel generation, with biodiesel production efficiency three times better than that of free enzymes (Aghabeigi et al. 2023). The increase in lipid content is attributed to the use of NPs, which positively impact cellular activity. For example, iron (Fe) serves as a crucial micronutrient for microalgae, playing a vital role in essential cellular functions such as photosynthesis and respiration. The availability of iron regulates their productivity, community structure, and overall ecosystem functioning (Cheng et al. 2020). In contrast, Fe_2O_3 nanoparticles showed toxicity towards *C. sorokiniana* even at the low dose of 2 mg. L^{-1}, whereas the dose of 20 and 30 mg. L^{-1} in *C. pyrenoidosa* increased biomass productivity and lipid content (Rana et al. 2020). The effects of copper and selenium nano aqua chelate carboxylated with citric acid on biomass accumulation in *C. vulgaris* were also investigated. The inoculation of 0.67–4 mg. L^{-1} of Cu nano carboxylates resulted in an approximately 20% increase in *Chlorella* biomass. However, concentrations of 20–40 mg. L^{-1} significantly inhibited algal development after the 12th day of incubation. Se nanocarboxylates at concentrations of 0.4–4 mg. L^{-1} also fostered the growth of *C. vulgaris*, leading to a 40–45% rise in biomass (Mykhaylenko and Zolotareva 2017).

Previous research has shown that NPs concentration can significantly impact microalgae growth; the specific effects vary depending on several factors. Such factors include the type of NPs, the microalgae species itself, and environmental conditions like culture media and pH. Among microalgae, *C. minutissima* is a particularly promising strain for biofuel production. It is also known to be less sensitive to Fe NPs compared to other types, such as nZVI and Fe_3O_4. However, the impact of IONPs on *C. minutissima*'s growth and its potential for biofuel production remains unclear.

Therefore, the present research aims to investigate the effect of IONPs on both the growth and lipid accumulation of *C. minutissima*. It involved testing various concentrations of IONPs in BG11 media and measuring parameters like cell density and lipid accumulation within *C. minutissima*. By examining these factors, this research will provide valuable insights into the potential use of IONPs in *C. minutissima* cultivation for biofuel production. The findings will help us understand if specific IONPs concentrations can promote both microalgae growth and lipid accumulation, making them a viable tool for this purpose.

2 Materials and Methods

2.1 Microalgal species

The oleaginous microalga *C. minutissima* MCC 27 was obtained from the Centre for Blue Green Algae, Indian Agricultural Research Institute (IARI), New Delhi. India. A sterile culture BG 11 medium was prepared for the inoculation, and pH was maintained at 7.1.

2.2 Chemical synthesis of Fe_2O_3 nanoparticles

IONPs termed "Hematite" were synthesized using the sol-gel process as per the method described by Paulson and Jothibas (2021). In the experiment, 100 mL of distilled water was used to dissolve the 0.3 M of Ferric chloride hexahydrate, which was maintained by vigorous stirring at 300 rpm for 15 minutes. Following that, a reagent solution made up of 10 mL of NH_3-ammonia solution and 10 mL of distilled water was combined. The produced reagent solution was then carefully added to the ferric chloride anhydrous solution, and it was stirred vigorously (400 ± 20 rpm) for one hour at 80 °C. The gel was put into a petri dish once the initial cleaning was finished and kept in the oven at 80 ^{0}C for 24 hours. The resultant nano-powder sample was then stored for subsequent characterizations after being calcined at 400 °C for 3 hours in an open environment.

2.3 Materials Characterization

2.3.1 X-ray diffraction (XRD)

The X-ray diffraction was performed using a Philips XRD 3100 diffractometer (Philips Electronics Co., Eindhoven, Netherlands) with a medium scan rate of 0.3 degrees per second over a 2θ range of 20-70º.

2.3.2 Scanning Electron Microscopy (SEM)

The SEM and EDX study was performed using TESCAN Magna 200 eV-30 KV along with cross-sectional, morphology, and elemental analysis for Fe & O.

2.4 Evaluation of microalgal growth

Initial studies were carried out in 250 mL Erlenmeyer flasks with 100 mL of BG 11 medium to examine the biomass potential and growth profiles. 10% (v/v) of freshly grown *C. minutissima* was added to the medium containing flasks and incubated in a microalgae growth chamber at 25 ± 2 ^{0}C. All the experiments were carried out in triplicates. The flasks were kept under LED light (about 2500 Lux) for an 18:6 light and dark period (18-hour light-dark cycle 6 hours) for 20 days. Throughout 20 days, the optical density of the microalgae was measured at 680 nm utilizing an Agilent Technologies Cary 60 UV-Vis Spectrophotometer.

To measure the effect of iron nanoparticles, *C. minutissima* was cultured in 100 mL of BG11 media supplemented with different concentrations of nanoparticles (0, 10, 20, and 30 mg·L^{-1}). The cultures were maintained under the controlled conditions mentioned above. The growth of the microalgae was measured by sampling the culture every two days for 20 days. After 20 days, the microalgal biomass was harvested by centrifugation and washed with double distilled water to remove impurities. After centrifugation, the pellets were dried in an oven at 60 °C until they reached a consistent weight. After drying, the biomass was placed in desiccators to estimate the accumulation of lipids and biodiesel. Biomass concentration, biomass yield, and biomass productivity were calculated using corresponding equations (1), (2), and (3).

$$\text{Biomass concentration} = \text{Weight (mg)} / \text{volume of culture (L)} \quad (1)$$

$$B = X_f - X_0 \quad (2)$$

Where B= biomass yield (g.L^{-1})

X_f= final biomass concentration

X_0 = initial biomass concentration

$$P_b = (X_2 - X_1)/(t_1 - t_0) \quad (3)$$

Where P_0= biomass productivity

X_2 and X_1= biomass concentrations

2.5 Calculation of Chlorophyll a

Chlorophyll-a (chl-a) was estimated using the colorimetry method (Porra et al. 1989). In summary, a 1.5 mL Eppendorf tube containing an aliquot (1 mL) of microalgae culture was centrifuged at room temperature for 10 minutes at 6000 rpm. The sample was then rinsed three times with deionized water to remove the impurities. The pellets were again suspended in 1 millilitre of methanol after the supernatant was disposed off. The tubes were tightly sealed with parafilm and immersed in a water bath heated to

60 °C for 30 minutes to extract chlorophyll-a. Following cooling to room temperature, absorbance readings were taken at wavelengths of 652 nm, 665.2 nm, and 750 nm. Porra's equation was then used to estimate the concentration of chl-a in $\mu g \cdot mL^{-1}$.

$$Chl - a = 16.29\ (A_{665.2} - A_{750}) - 8.54\ (A_{652} - A_{750}) \quad (4)$$

2.6 Lipid extraction from harvested microalgae

The lipids from microalgae were extracted using the modified Bligh and Dyer (1959) method. Then, dried and powdered microalgal biomass was dispersed in distilled water. After microwaving at 540 W, the microalgal cells were disrupted, and the suspension was allowed to cool to room temperature. Subsequently, chloroform and methanol were introduced into the microalgal suspension. The mixture was vigorously shaken manually and left at room temperature for 4 hours. Following this, water was added to aid in the separation of phases. The mixture was subsequently left undisturbed until the organic layer settled and the upper phase became clear (Lee et al. 2010). The organic phase, containing chloroform and lipids, was carefully extracted, and its volume was recorded, and the lipid content was calculated using the following equation:

Lipid content (%) = Mass of lipid (in grams) X 100/ Mass of algae culture (in grams)

2.7 Fatty Acid Methyl Ester (FAME) formation

FAME formation was carried out by Transesterification (Mishra et al. 2014). Briefly, in this process, methanol was added to sodium hydroxide into the glass blender. The mixture of methanol and sodium hydroxide was appropriately mixed with the help of a magnetic stirrer after dissolving NaOH in methanol. Then, the extracted oil was transferred into a glass blender for 30 minutes. Following that, the mixture was put into a sterile container. The mixture is divided into two layers after two to three hours. The first layer was biodiesel, and the second was glycerine. The biodiesel was then washed multiple times with distilled water to remove traces of alcohol, catalyst and glycerol.

2.7.1 FT-IR Analysis

A Bruker Vertex 70 FTIR spectrophotometer with a Platinum ATR (attenuated total reflection) module was used to conduct the spectral study. FT-IR spectra were produced at 400–4000 cm^{-1} (Portaccio et al. 2023). The Origin software (version 5.0, 2007) was then used to evaluate these spectra.

2.8 Assessment of biodiesel fuel properties

The biodiesel obtained from *C. minutissima* grown in BG 11 was subjected to testing for its physical characteristics, including specific density (Prabakaran et al. 2021), viscosity (Al-Ansari et al. 2023), calorific value (Boopathi et al. 2023), cetane number (Tesfa et al. 2013), iodine value, and oxidative stability (Geng et al. 2023). These tests were conducted according to the protocols outlined by ASTM D6751 and EN 14214 fuel standards.

3 Results

3.1 Morphology of *C. minutissima*

Under the scanning microscope, *C. minutissima* has a unicellular structure, yellow-green color, and a spherical shape with a diameter ranging from 5 to 15 µm. The cells appear spherical or ellipsoidal under a scanning electron microscope, and their walls either have smooth surfaces or uneven coastal portions, as shown in Figure 1. The cells exist in either a unicellular state or in the palmella stage.

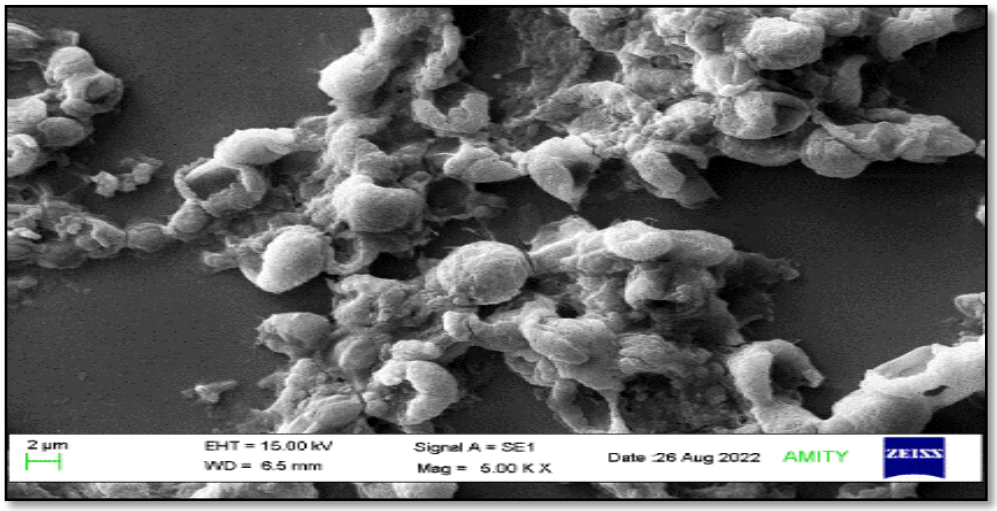

Figure 1 Scanning microscopic image of *C. minutissima*

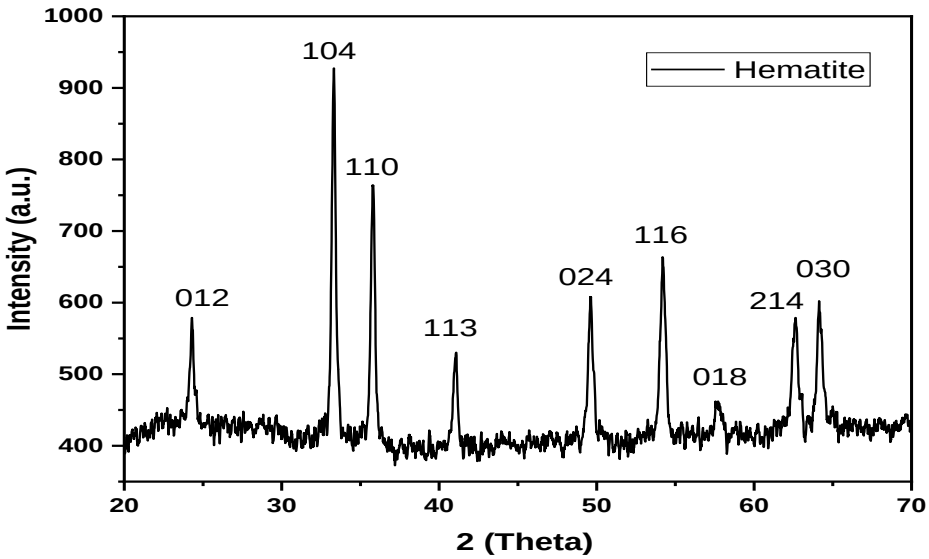

Figure 2 X-ray diffraction (XRD) pattern of synthesized nanoparticles of iron oxide (α-Fe_2O_3)

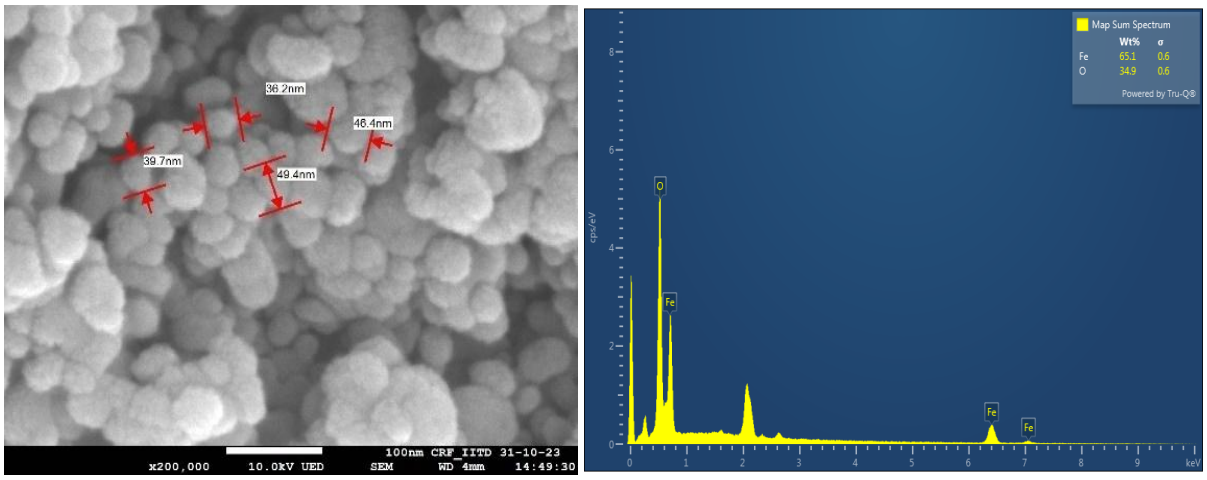

Figure 3 (a) SEM (b) EDX image of synthesized Fe_2O_3 (α-Fe_2O_3) nanoparticles

3.2 XRD analysis

Figure 2 shows the X-ray diffraction pattern for iron oxide (α-Fe_2O_3). The X-ray diffraction study for α-Fe_2O_3 shows the characteristic peaks for the 104 plane corresponding to the 2θ angle of 33.29, implying the formation of α-Fe_2O_3. The referred X-ray diffractions are from the JCPDS Card No. 86–0550. The crystallite size was determined using the Debye-Scherrer's equation: $D = K\lambda / \beta Cos\theta$

Where the wavelength of Cu-Kα radiation utilized is λ = 1.5418 Å, the form factor is k = 0.9, the full-width half maximum (FWHM) is expressed in radians (β), the crystallite's diameter is D, and Bragg's angle is θ (Sun et al. 2006). The crystallite size for the α-Fe_2O_3 was calculated to be 40 nm from FWHM of the most intense (104) diffraction peak using Debye-Scherrer's equation.

3.3 SEM analysis

Figure 3 (a, b) shows the FE-SEM micrograph for the α-Fe_2O_3 at a scale of 100nm, along with the elemental X-ray electron energy and their elemental mapping. The particle size calculated for sample α-Fe_2O_3 was ~46 nm using SEM, which is in good agreement with the size calculated using XRD data.

3.4 Assessment of growth parameters in *C. minutissima*

C. minutissima is the fastest-growing strain. After 20 days of incubation, microalgal cells were subjected to further analysis. To access the growth parameters, the strain was cultured in a BG 11 medium. Figure 4 shows that the OD of samples increased and reached the maximum in 14 days. The absorbance (A_{680}) of the culture microalgae strain was recorded using a spectrophotometer

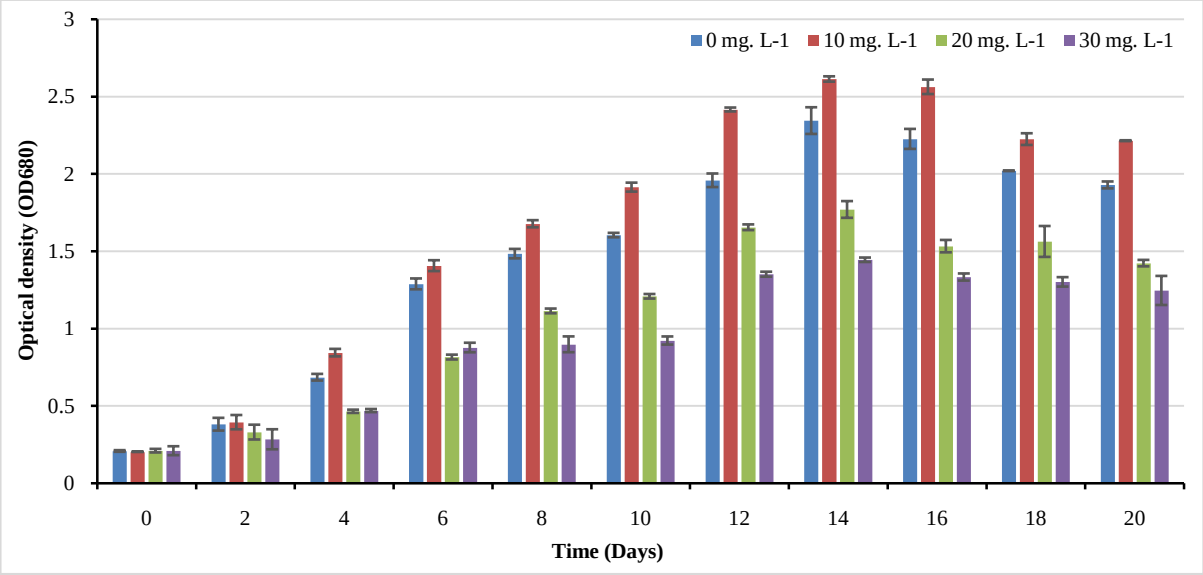

Figure 4 Graphical representation of optical density on various IONPs concentrations on *C. minutissima*

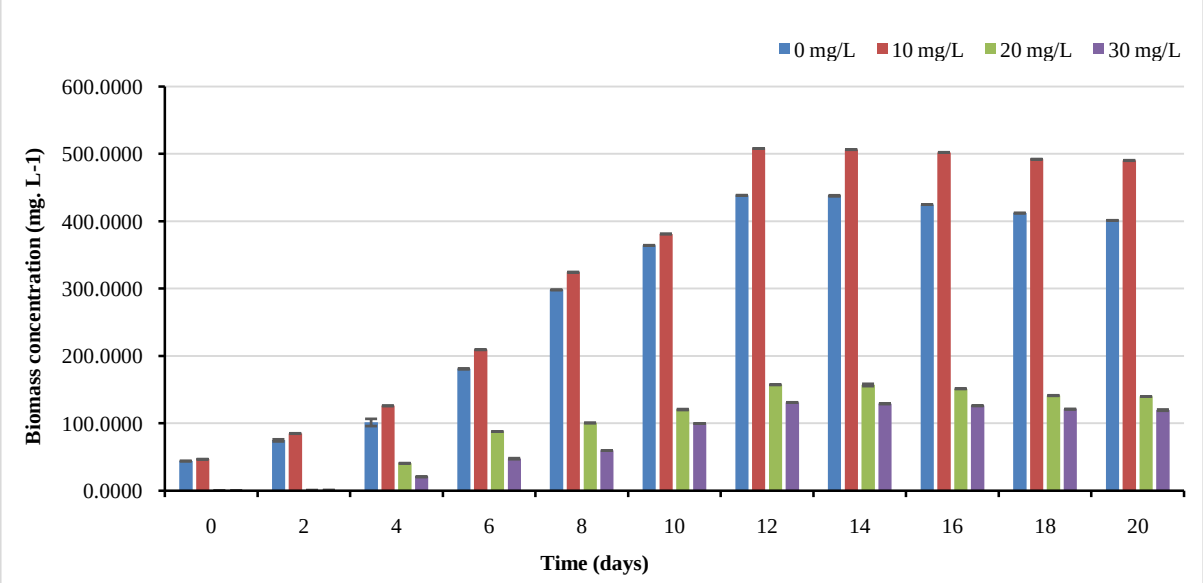

Figure 5 Graphical representation of biomass concentration on various IONPs concentration on *C. minutissima*

(Agilent Technologies Cary 60 UV-Vis). According to Figure 4, the optical density is at 0 mg. L^{-1} observed 2.34 for *C. minutissima*. Further, the highest *C. minutissima* optical density of 2.61 ± 0.08 was exhibited at a 10 mg·L^{-1} IONPs dose, while 20 and 30 mg·L^{-1} showed 1.76 ± 0.05 and 1.44 ± 0.01 on 14th day of inoculation respectively (Figure 4).

3.4.1 Estimation of biomass concentration on *C. minutissima*

After 20 days of the batch study, a gradual exponential growth phase extended until the 14th day (Figure 5). Biomass concentration was calculated based on the dry cell weight (DCW). The highest biomass concentration (438.533 ± 0.35 mg. L^{-1}) was reported for the control sample (0 mg. L^{-1}).

3.4.2 Estimation of biomass concentration on *C. minutissima* on different concentrations of synthesized Fe_2O_3

The growth pattern of biomass for *C. minutissima* is illustrated in Figure 5. Until the 8th day, there were no significant changes reported in various concentrations of IONPs in the culture medium, and the highest concentration of 508.1 mg.L^{-1} was reported in 10 mg·L^{-1}. Whereas 157.53 and 131.3 mg. L^{-1} recorded in 20 and 30 mg. L^{-1} dose of IONPs

3.4.3 Estimation of Chlorophyll-a in *C. minutissima*

During the growth, chlorophyll-a content also increased in correspondence to optical density. The results of the study revealed a concentration of 12.00 ± 0.5689 µg.mL^{-1} Chlorophyll-a in *C. minutissima* up to the 12th day. However, after the 12th day, chlorophyll-a concentration starts decreasing gradually (Figure 6).

3.4.4 Estimation of Chlorophyll-a in *C. minutissima* on different concentrations of synthesized Fe_2O_3

Exposure to various concentrations of IONPs ranging from 10 to 30 mg L^{-1} resulted in different levels of Chlorophyll-a in *C. minutissima* cultures (Figure 6). Further, Chlorophyll-a concentration was higher (17.11 ± 1.29 µg.mL^{-1}) at 10 mg L^{-1} IONPs dose until the 12th day of cultivation while at 30 and 20 mg. L^{-1} dose of IONPs chl-a concentrations were 6.3 and 9.073 µg·mL$^{-1,}$ respectively. However, impairment in chl-a concentration was noted at all IONPs doses starting on the 14th day.

3.4.5 Estimation of biomass productivity and yield on *C. minutissima*

Once inoculated into BG 11 media, *C. minutissima* exhibited exponential growth. The biomass productivity at 0 mg. L^{-1} was recorded 26.66 mg.L^{-1}d$^{-1,}$ and the final biomass yield was recorded 357.003 mg.L^{-1}

3.4.6 Estimation of biomass productivity and yield on *C. minutissima* on different concentrations of synthesized Fe_2O_3

As shown in Figure 7, biomass productivity was recorded 31.74 mg.L^{-1}d^{-1} at 10 mg. L^{-1} while 9.69 and 8.1 mg.L^{-1}d^{-1} was observed at 20 and 30 mg. L^{-1}, respectively. Similarly, At 10 mg.L^{-1} IONPs

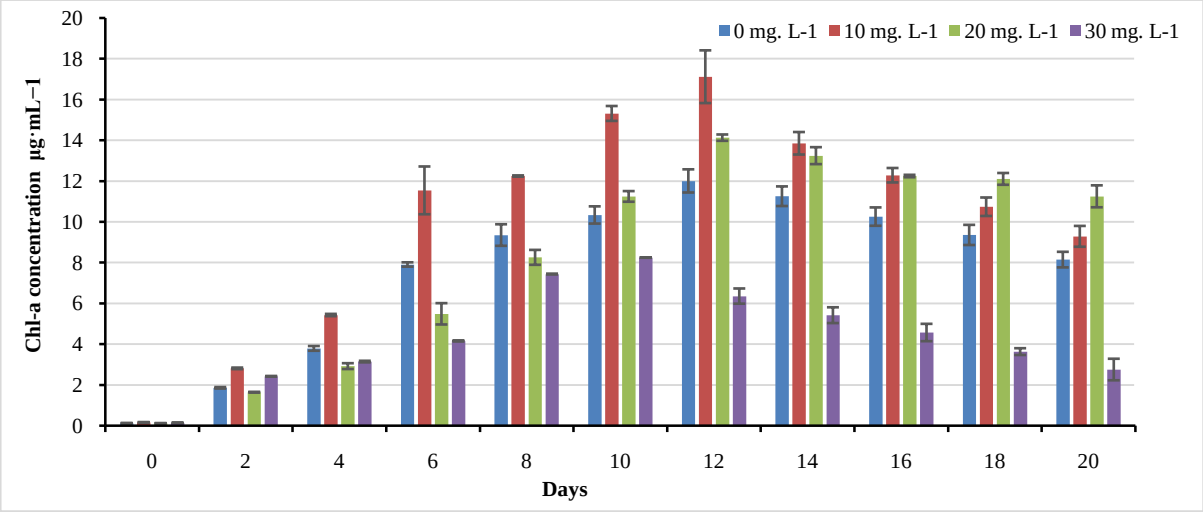

Figure 6 Graphical representation of Chl-a on various IONPs concentration on *C. minutissima*

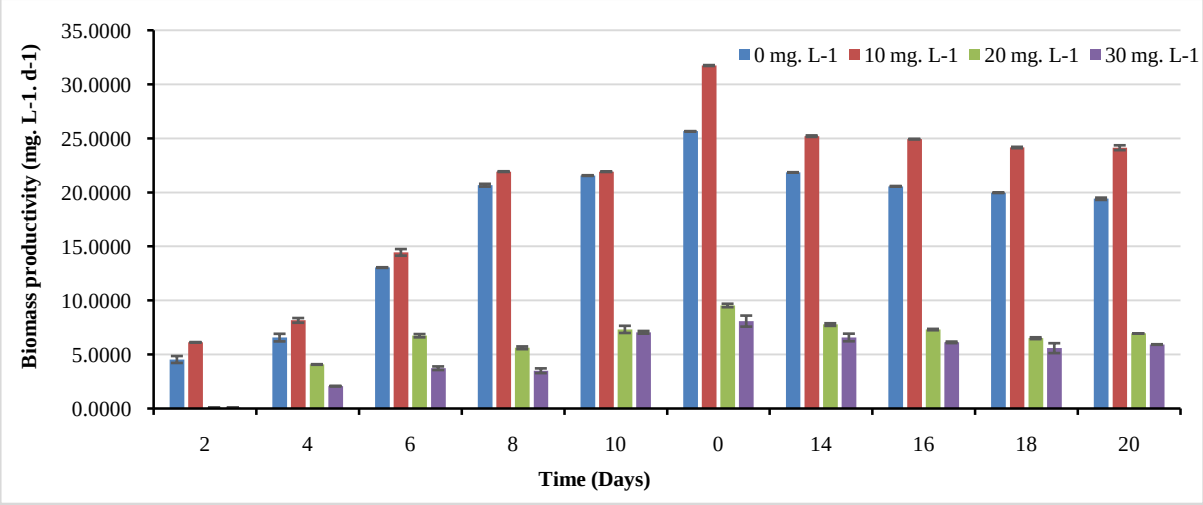

Figure 7 Graphical representation of biomass productivity on various IONPs concentrations on *C. minutissima*

dose, *C. minutissima* demonstrated the highest biomass yield of 443.167 mg.L^{-1}, whereas at 20 mg·L^{-1} and 30 mg.L^{-1}, the yields were 139.86 mg· L^{-1} and 119.75 mg.L^{-1}, respectively. At 10 mg.L^{-1}, biomass yield is 24% higher in comparison to other doses.

3.5 Lipid productivity

In this study, the assessment of the total lipid content or productivity of *C. minutissima* was conducted following the harvesting process. Among the different doses of IONPs, the highest lipid production (29.52%) was reported at 10 mg·L^{-1}, which was 28.52 % higher than control. The observed changes in growth and lipid production suggest the absorption of NPs and their potential effects. This phenomenon is intricately linked to the specific nanoparticles used and the choice of microalgal strain. The interaction between these factors plays a crucial role in shaping the outcomes, emphasizing the need for a detailed understanding of the interplay between IONPs, nanoparticles, and microalgal strains to optimize and tailor the effects for desired outcomes in applications such as biomass and lipid production.

3.5.1 FTIR

The FTIR spectra of the microalgal oil are displayed in Figure 8. The region indicates the presence of lipids in the sample in the spectra between 3100 and 2800 cm^{-1}, which arises due to the vibrations of both symmetric and asymmetric stretching of the $–CH_2–$ groups. These $–CH_2–$ groups serve as the foundational structure of lipids and demonstrate absorption, especially at 2923 and 2865 cm^{-1}. The algal oil's FTIR spectra revealed well-absorbed areas between 3500 and 3000 cm^{-1}, 1747 and 1172 cm^{-1}, and 800 and 700 cm^{-1}. The regular peaks at 2923 and 2860 cm^{-1} result from the $–CH_2–$ groups' symmetric and asymmetric stretching vibrations. The spectra of algal oil also showed peaks at 3005 cm^{-1} from double bond stretching and 1300–1100 cm^{-1} from the C–O bond (axial stretching). There were also observed absorption peaks at 722 cm^{-1} attributed to the $–CH_2–$ bending out of the plane and at 1365–1377 and 1465 cm^{-1} attributed to the $–CH_3$ bond. The existence of these peaks signifies the transformation of oil into biodiesel.

Table 1 compiles the different fuel qualities of *C. minutissima* biodiesel produced under ideal circumstances. The biodiesel showed a density of 0.86 g/cm^3, a viscosity of 3.24 mm^2 /s and an iodine value of 124 g I_2/100 g, which abided by the fuel standards. All the tested parameters were found to be closely aligned with the American Society for Testing and Materials (ASTM) D6751 and EN 14214 fuel standards, respectively.

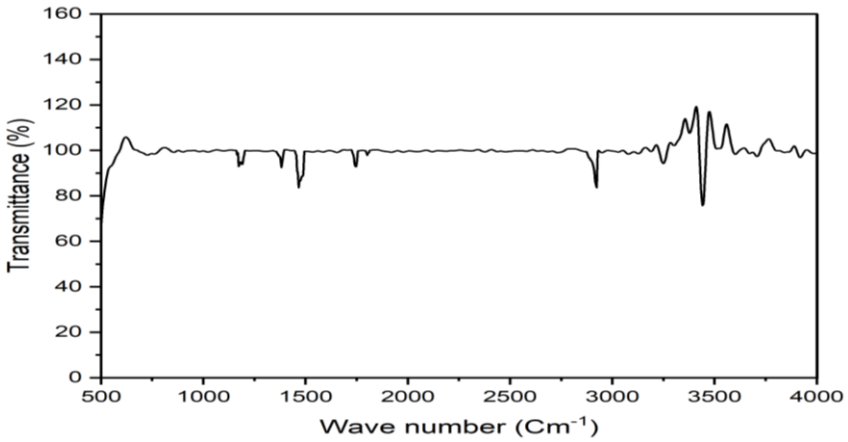

Figure 8 FTIR spectrum of *C. minutissima* algal oil

Table 1 Comparison of *C. minutissima* biodiesel with various biodiesel standards

Parameters	Present study	ASTM standards	EN 14214
Density (g cm^{-3})	0.86	-	0.86-0.90
Viscosity (mm^2/s)	3.5	1.9-6.0	3.5-5.0
Calorific value (MJ Kg^{-1})	35.58	-	-
Iodine value (g I_2/100 g)	124	-	<120
Cetane number	50.34	>47	>51
Degree of unsaturation (%)	70.15	-	-
Oxidative stability	6	-	>6

4 Discussion

Fe_2O_3 nanoparticles were chosen for the microalgae due to their demonstrated highest efficiency in harvesting in this research. Surface characterization was analyzed through XRD, as depicted in Figure 2. The pattern in the figure indicates the crystalline nature of Fe_2O_3 nanoparticles. The observed peaks are corresponding to pure Fe_2O_3 nanoparticles. The presence of narrow and sharp peaks suggests that the hematite products exhibit high crystallinity (Lassoued et al. 2017). The average grain size of the synthesized α-Fe_2O_3 was determined by SEM to be less than 100 nm. SEM analysis of the synthesized nanoparticles revealed the formation of spherical particles in shape and uniform distribution (Qayoom et al. 2020). Additionally, the elemental composition of Fe_2O_3 nanoparticles was examined through energy dispersive spectroscopy, as depicted in Figure 3(b). The analysis revealed the presence of peaks corresponding to Fe and O, indicating the absence of impurities.

The addition of IONPs demonstrated positive outcomes regarding the growth, biomass concentration, biomass productivity and biomass yield of *C. minutissima*. According to the current findings, C. *minutissima* and NPs doses of 10, 20, and 30 mg·L^{-1} were chosen for subsequent investigations and the growth of *C. minutissima* was analyzed at $OD_{680.}$ A similar result was reported by Attre et al. (2018), who cultured *C. minutissima* strain on glycine medium. Overall, the highest biomass concentration of 508.1 mg.L^{-1} was achieved at 10 mg·L^{-1}. In contrast, biomass concentrations for NPs doses of 20 and 30 mg·L^{-1} were recorded as 157.33 ± 0.32 and 131.3 ± 0.26 mg·$L^{-1,}$ respectively. It was noted that in the low-concentration range of nanoparticles, the biomass of algal cells initially increased but then declined with higher concentrations of Fe_3O_4 NPs (Wang et al. 2021). Results of Kaliamurthi et al. (2019) are contradictory to the findings of the present study, where no aggregation was reported at a lower concentration of 50 mgL^{-1} for ZnO nanoparticles, but higher microalgae accumulation was noted at 100 mgL^{-1} (Kaliamurthi et al. 2019). Further, carbon nanotubes inhibit cell growth in freshwater microalgae *Scenedesmus obliquus,* while nano Fe_2O_3 promotes growth in the same species (He et al. 2017). Up to the 14^{th} day, there was a notable distinction in biomass productivity among various doses of NPs. In the current study, biomass productivity is measured in the NP-treated samples, and higher biomass (31.74 mg.L^{-1}.d^{-1}) was observed at 10 mg. L^{-1} IONPs, while at higher concentrations, it decreased. The biomass productivity, in the context of using MIL-100(Fe), a type of metal-organic framework incorporating iron, was measured in *Arthrospira* sp., and it was determined to be 0.61 g.L^{-1} (Cheng et al. 2020). Biomass yield was 24.12% at 10 mg.$L^{-1,}$ which is higher than that of the control. The stimulation of algal proliferation may be linked to the dissociation of minute iron nanoparticles (Marsalek et al. 2012). Due to its association with the photosynthetic electron transport chain, iron may be required for both growth and metabolic alterations, as suggested by the enhanced growth of microalgal biomass; this supports the hypothesis. However, the rise in NPs concentrations is adversely correlated with the notable reduction in growth. The growth of marine phytoplankton *Skeletonema costatum* and *Nitzschia closterium* is inhibited by metal salt (Cu^{2+}), nano-metal (nano-Cu), and nano-metal oxide (nano-CuO). Cu^{2+} and nano-Cu EC50 values, which range from 0.356 to 0.991 mgL^{-1} and 0.663 to 2.455 mgL^{-1}, have been shown to have an impact on the extracellular polymeric compounds and amino acids secreted by *S. costatum* and *N. closterium* microalgae (Huang et al. 2022). Further, Chl-a concentrations also decreased at all IONPs doses starting on the 14th day. The highest chl-a was recorded (17.11 ± 0.09 µg·mL^{-1}) at 10 mg·L^{-1}. Moreover, the concentration of 30 mg·L^{-1} exhibited the lowest number of Chl-a. Studies have indicated that titanium oxide nanoparticles notably hindered the growth and biomass (including dry weight, chlorophyll A, and total chlorophyll) of *Chlorella vulgaris* due to their surface adsorption on the algal cell, which promotes growth inhibition (Roy et al. 2018). On the contrary, when Fe_2WO_6 NPs inoculated at different concentrations in the culture medium of *Dunaliella salina,* the cell density, chlorophyll a and b, increased at lower 20 ppm concentrations (Hassanpour et al. 2020).

As per a recent study, in *Scenedesmus obliquus,* lower concentrations of CNTs and Fe_2O_3 increased the chlorophyll content, while higher concentrations repressed photosynthesis (He et al. 2017).). Iron is essential for photosynthesis, cell respiration, and the creation of phytohormones and chlorophyll (Zhao et al. 2024b). From the current observations, it was hypothesized that the lower dose of NPs functions as a source of iron for the growth of microalgae. In general, NPs demonstrated favourable effects on *C. minutissima*, leading to increased biomass growth and chlorophyll-a concentration at 10 mg·L^{-1} NPs dose. In contrast, recent research signifies a significant advancement in the field. Cultivation of *C. minutissima* not only remained viable but also demonstrated an enhanced growth rate in NP-supplemented media. This opens up new possibilities for employing NPs in large-scale cultivation of targeted microalgae, leading to increased biomass production. The highest lipid production of 29.52% was observed at 10 mg·L^{-1} NPs dose, which was 28.52 % higher than that of control (15.98%). The change in growth and lipid production is a sign of metal oxide absorption and its consequences. Using Cabon Nano Tubes, Fe_2O_3, and MgO nanoparticles, increases in lipid production were reported at 8.9%, 39.6%, and 18.5%, respectively (He et al. 2017). The treatment with 50 mg/L of ZnO-NP increased the synthesis of neutral lipids (6.08-fold) and triacylglycerol (8.21-fold) without causing complete growth inhibition (Kaliamurthi et al. 2019). The rise in lipid synthesis indicates a change in cellular metabolism. Likewise, Chandra et al. (2019) observed 37.24 mg.$L^{-1}$$d^{-1}$ a lipid content in *C.*

minutissima at a concentration of 50 mg. L^{-1}. In contrast, a slight decrease in lipid (23.07 %) was also observed at NPs doses of 20 mg·L^{-1}. At this concentration, the relatively low lipid percentage partially suggests the absorption of NPs and the generation of reactive oxygen species (Shi et al. 2017). Hence, the gradual uptake of NPs at an optimal dose could help balance oxidative stress and enhance lipid productivity. Previous research indicates that in cultures containing NPs, microalgal biomass typically has only a few nanoparticles adhering to the cell surface (Taghizadeh et al. 2022). Nanomaterials can stimulate the generation of reactive oxygen species (ROS), initiating the cellular mechanism of oxidative stress (Wang et al. 2021). However, a significant decline in biomass growth represents a considerable constraint in conditions of nutrient scarcity. However, the NPs that were examined for this study did not exhibit any restrictions on the growth of microalgae. Overall, 10 mg. L^{-1} NPs dose was shown to be optimal for *C. minutissima* development and growth with improved lipid accumulation based on the acquired biomass potential and lipid yields. The region indicates the presence of lipids in the sample in the spectra between 3100 and 2800 cm^{-1}, which is caused by the symmetric and asymmetric stretching vibrations of the symmetric CH_2 and asymmetric CH_3 and CH_2 stretching (Wallach et al. 1979). These $–CH_2–$ groups serve as the lipids' structural backbone and exhibit absorption, especially at 2923 and 2865 cm^{-1} (Portaccio et al. 2023). The algal oil's FTIR spectra revealed well-absorbed areas between 3500 and 3000 cm^{-1}, 1747 and 1172 cm^{-1}, and 800 and 700 cm^{-1}. The normal peaks at 2923 and 2860 cm^{-1} result from the $–CH_2–$ groups' symmetric and asymmetric stretching vibrations (Arif et al. 2021). The spectra of algal oil also showed peaks at 3005 cm^{-1} from double bond stretching and 1300–1100 cm^{-1} from the C–O bond (axial stretching). There were also observed absorption peaks at 722 cm^{-1} attributed to the $–CH_2–$ bending out of the plane and at 1365–1377 and 1465 cm^{-1} attributed to the $–CH_3$ bond (Nematian et al. 2020). The existence of these peaks signifies the transformation of oil into biodiesel. The characteristics of biodiesel predominantly depend on its fatty acid esters (Mondal et al. 2021). In a study, Mallick et al. (2012) found that the calorific value of *C. minutissima* is comparable to *C. vulgaris*. Further, the Iodine value in *C. minutissima* shows a high oxidative value, which means the presence of a mixture of fatty acids; it is found within the range according to ASTM and EN 14214 (Thirugnanasambandham 2018). Density plays a significant role in airless combustion systems as it directly impacts the efficiency of fuel atomization. Density was recorded at 0.86 g cm^{-3} in *C. minutissima*, which is also in synchronization with ASTM and EN standards. The above results show that *C. minutissima* fuel properties are aligned well with commercial biodiesel.

Conclusion

The utilization of hematite (α-Fe_2O_3) nanoparticles demonstrates a promising approach to enhance lipid and biofuel productivity in microalgae cultivation. Characterization studies confirm the suitability of these nanoparticles for the effective conversion of microalgal lipids into biodiesel. Particularly, at 10 mg·L^{-1} concentration, iron oxide nanoparticles significantly boost biomass concentration and growth rates in *Chlorella minutissima* cultures. The findings highlight the feasibility of integrating hematite nanoparticles into microalgal media for sustainable biofuel production.

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Isolation and characterization of polygalacturonase producing thermophilic *Aspergillus niger* isolated from decayed tomato fruits

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Column chromatography

Polygalacturonase

Solid-state fermentation

ABSTRACT

This study aimed to isolate a fungal strain capable of producing acidophilic and thermostable polygalacturonase. In this study, the fungal isolate was isolated from decaying tomatoes. Based on the colony characteristics, microscopic and morphological observations, the isolated fungal pathogen has been identified as *Aspergillus niger*. The isolated fungus was used in solid-state fermentation to produce an acidic polygalacturonase enzyme. The enzyme was then purified using ammonium sulphate precipitation and column chromatography, and its activity was assayed by measuring the releasing sugar group from citrus pectin using a 3, 5-dinitrosalicylic acid (DNSA) reagent assay. The crude extract obtained from solid-state fermentation had an activity of 94.6 U/mL. Ammonium sulphate precipitation increased the enzyme's specific activity from 6.89 U/mg to 12.42 U/mg. Sephadex G-200 was used to purify the enzyme 3.58 times, and its specific activity was determined to be 24.66 U/mg. The Sephacryl S-100 column achieved a final fold purification of 9.93 times and a specific activity of 68.41 U/mg. The purified enzyme performed best when polygalacturonic acid was used as a substrate. The enzyme's optimum temperature and pH were 55°C and 5, respectively. $CaCl_2$ was found to be the best chelating ion for the enzyme. This enzyme is recommended for use in a variety of industrial applications as the enzyme was found to be stable at acidic pH and high temperature.

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1 Introduction

Enzymes are now the cornerstone of several industries worldwide, including pharmaceuticals, brewing, fabric, and most processed foods. As a result, the demand for enzymes has risen dramatically (Li et al. 2012; Cocok et al. 2017; Raveendran et al. 2018; Ramesh et al. 2020). Pectinases are a class of pectinolytic enzymes that catalyse the depolymerisation and degradation of pectinaceous materials using hydrolases, lyases (depolymerisation reaction), or esterases (de-esterification reaction) (Zhang et al. 2021). Pectinase can also hydrolyze the alpha-1, 4 glycosidic linkages between galacturonic acid residues and sugar (Rahman et al. 2019). Polygalacturonases, pectin lyases, and pectin methyl esterases are enzymes that hydrolyze the glycosidic bonds in pectic substances (Jayani et al. 2010; Khatri et al. 2015; Wang et al. 2015). Polygalacturonase is a depolymerizing enzyme that catalyses the α-1,4 glycosidic linkage in the pectin chain, resulting in galacturonic acid units (Ahmed et al. 2021). The biotechnological potential of polygalacturonase is expanding due to increasing applications in the food and feed industries. It belongs to the pectinase enzyme family, which accounts for 25% of all industrial enzymes worldwide (Munir et al. 2019). Polygalacturonases are the most exhaustively studied pectinolytic enzyme family (Jayani et al. 2005).

Microorganisms account for the majority of industrial demand for enzymes. Microorganisms are preferred in the industry for enzyme production due to their high growth capability, short life span, and ease of genetic manipulation (Haile and Ayele 2022). Microbial pectinolytic enzymes, produced mainly by fungi, are used in various large-scale industrial processes (Soares et al. 2012). Filamentous fungi are the primary sources of hydrolases because they produce multienzyme complexes composed of endo- and exo-enzymes that degrade polymers such as cellulose, hemicellulose, and pectin (Ramos-Ibarra et al. 2017). Commercial pectinases are primarily derived from *Aspergillus* (Ravi and Raghu 2017). *Aspergillus* sp. *pectinases* are widely used in industry because this strain has GRAS (Generally Recognized As Safe) status, which means that the metabolites produced by this strain can be used safely. This fungus produces pectinases such as polymethyl-galacturonase, polygalacturonase, and pectin esterase (Reddy and Sreeramulu 2012). Adding commercial pectinolytic enzyme preparations greatly improves the juice yield (Ribeiro et al. 2010). Thermophilic fungi are eukaryotes that have an exceptional ability to grow at high temperatures of 50°-60°C and can survive in a wide range of extreme environments (Majumdar et al. 2018). Furthermore, the maximum activation temperature of fungi-produced polygalacturonase enzymes is between 35 and 60°C (Thakur et al. 2010; Anand et al. 2016).

The most common methods for producing enzymes are submerged and solid-state fermentation, but solid-state fermentation is more productive than the others (Karimi et al. 2021). Solid-state fermentation has several advantages, such as high productivity, extended product stability, and low production costs (Yoon et al. 2014). Rice bran, sugar cane bagasse, orange bagasse, sugar beet pulp, wheat bran, and other food processing waste are all suitable substrates for pectinase production via solid-state fermentation (Alavi et al. 2020). The primary objective of this study was to identify an efficient fungal strain capable of producing polygalacturonase. To achieve this objective, comparatively less explored fruit sources such as tomatoes were investigated. Various endophytic and pathogenic fungi were reported to be present in tomato fruits (Paolo et al. 2018). Some endophytic fungi (Kaur et al. 2004) and pathogenic fungi (Samal et al. 2023) have been reported to produce a variety of industrially important enzymes. The diversity and interactions of endophytic and pathogenic fungi within tomato fruits present a fascinating area of research with the potential to uncover novel strategies for producing industrially important enzymes (Ikram et al. 2020). This study aimed to isolate and characterize the polygalacturonase-isolated enzyme from the thermophilic *Aspergillus niger* fungal pathogen found in decayed tomato fruits.

2 Materials And Methods

2.1 Sample collection

The ripened fruits of tomatoes were collected from a local fruit market in Ethiopia. These ripened fruits were transferred to the laboratory in sterilized polythene bags for further processing. These fruits were allowed to decay in the laboratory (Figure 1).

Figure 1 Decayed tomato fruits used in this study

2.2 Isolation of fungi and primary screening

The fruit samples were immersed in sterilised distilled water to prepare a stock solution. Each stock was serially diluted twice and aseptically poured into plates containing mineral salt agar media (0.2g $NaNO_3$, 0.05g KCl, 0.05g $MgSO_4$, 0.02g K_2HPO_4, 0.01g $FeSO_4$, 1g pectin, and 2g agar/100mL). Plates were incubated at 50°C for five to seven days. Following incubation, plates were poured with a potassium iodide-iodine solution (1.5g potassium

iodide and 0.3g iodine/100mL) to examine the pectin lysis zones on plates for primary screening of fungal isolates with minor modifications (Munir et al. 2019).

2.3 Secondary screening

Solid-state fermentation was used for secondary screening for polygalacturonase estimation. Bagasse from sugarcane was extracted and dried to make powder. This powdered extract was then used as a carbon source in a fermentation medium prepared according to Acuña-Argüelles et al. (1995). The fermentation medium (250 ml) was inoculated aseptically with spore inoculum (2 ml). The inoculated fermentation medium was incubated at 50°C for 4-5 days. Furthermore, the fermented media was extracted with 30 mL of distilled water. The flasks were vigorously shaken for 1 hour before being filtered through cheesecloth. The crude enzyme was extracted by adding 100 mL of citrate buffer to each flask (0.1 M, pH 5.0). The extract was centrifuged at 4°C for 15 min at 10,000 rpm, and the supernatant was sieved using Whatman No. 1 filter paper to remove all spores. The obtained supernatant (crude enzyme) was used to estimate polygalacturonase activity as per the method of Adedayo et al. (2021).

2.4 Identification of polygalacturonase-producing isolate

The fungus with the highest polygalacturonic acid hydrolysis value was identified by observing hyphal characteristics colony characteristics such as colour, texture, and spore structure following the methodology of Shamly et al. (2014). The conventional lactophenol cotton blue technique (LPCB) was used to study the fungal morphology.

2.5 Enzyme Purification

The culture filtrate was centrifuged at 10,000 rpm for 20 minutes at room temperature. The salting-out procedure was carried out following the method of Siddiqui et al. (2012). To achieve 20% saturation, solid ammonium sulphate was added slowly to the crude enzyme preparation in an ice bath with continuous stirring and then stored overnight at 4°C. The precipitated protein was removed by centrifugation at 4°C for 30 minutes at 10,000 rpm. After that, ammonium sulphate was added to the supernatant to reach 80% saturation. Again, centrifugation at 4°C for 30 min at 10,000 rpm separated the precipitated protein, which was then dissolved in sodium acetate buffer (0.1 M; pH 5.0). The crude enzyme was then loaded onto a Sephadex G-200 (150 cm) column pre-equilibrated with sodium acetate buffer (0.1 M; pH 5.0). At a flow rate of 24 ml/h, 3 mL volume fractions were collected. The eluted fractions were monitored using a spectrophotometer at 280 nm for protein and enzyme activity. The fractions with the highest polygalacturonase activity were loaded onto pre-equilibrated Sephacryl S-100 columns (1.6 cm X 60 cm) at a flow rate of 20 ml/h. Fractions (1.5 ml) were collected and examined regularly for protein and polygalacturonase activity.

2.6 Enzyme assay

Polygalacturonase activity was determined by measuring the releasing sugar group from citrus pectin using DNSA reagent assay, according to Adedayo et al. (2021). In a test tube, 2 ml of crude enzyme and 2 ml of citrus pectin were mixed in phosphate buffer and incubated at 50°C for 30 minutes. After incubation, the mixture was filtered, and 2 mL of DNSA reagent was added to 2 mL of the filtrate to stop the reaction and the mixture was kept in a boiling water bath at 100°C for 10 minutes until yellow colour developed. The tubes were then cooled with running water. A spectrophotometer was used to measure the optical density of the resulting coloured solution at 540 nm. The amount of enzyme that released 1 mol of galacturonic acid per minute was defined as one unit of pectinase activity (U).

2.7 Protein estimation

The protein concentration was determined as described by Lowry et al. (1951) using bovine serum albumin (BSA) as standard, and absorbance was read at 660 nm using a UV-Vis spectrophotometer.

2.8 Characterization of the enzyme

2.8.1 Substrate specificity

Purified polygalacturonase was evaluated for substrate specificity against polygalacturonic acid, pectin, xylan, galactose, and cellulose at 0.1% (w/v) (Thakur et al. 2010). The substrates were incubated with the purified enzyme for 4 hours in 50 mM citrate buffer (pH 4.4). Polygalacturonase activity was determined for each substrate, with pectin serving as control.

2.8.2 Effect of temperature

The enzyme activity was determined by incubating the reaction mixture (as described in the enzyme assay method) at different temperatures from 30 to 60°C. The optimum temperature for polygalacturonase activity was calculated by plotting enzyme activity against temperatures.

2.8.3 Effect of pH

The effect of reaction pH on polygalacturonase activity was assessed using citrate buffer (pH 2.0–4.0) and potassium-phosphate buffer (pH 5.0–8.0) following the method of Bentouhami et al. (2024). The reaction mixture was incubated for 4 hr at 50°C. Finally, the enzyme activity was determined to evaluate its stability in varying ionic strengths.

2.8.4 Effect of divalent cations on enzyme activity

The divalent cations tested include $CaCl_2$, $MgCl_2$, $MnSO_4$, and $FeCl_2$. To study the effect of various divalent cations on enzyme activity, 2 ml of crude enzyme and 2 ml of citrus pectin were mixed in phosphate buffer containing divalent cations to a final concentration of 10 mM and incubated at 50°C for 30 minutes. After incubation, the mixture was filtered, and 2 mL of DNSA reagent was added to 2 mL of the filtrate to stop the reaction. The remaining steps were performed as given in section 2.6.

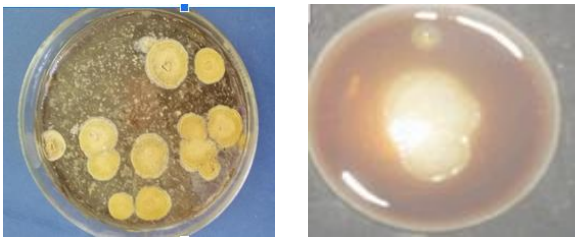
2.9 Statistical analysis

The experiments were conducted following a completely randomized block design. Each experiment was repeated three times to get triplicate data subjected to statistical analysis using the SPSS tool to compute mean and standard error (SE) values.

3 Results and Discussion

3.1 Isolation of fungi producing polygalacturonase

Fungi secrete various enzymes that can degrade complex plant-derived polysaccharides, such as cellulose and pectin (Badhan et al. 2018). Among the pectinolytic enzymes, polygalacturonase plays a crucial role in the degradation of polygalacturonic acid, the main component of pectin (Pedrolli et al. 2009). The activity of polygalacturonase can lead to the formation of clear zones in nutrient media, a widely used phenotypic characteristic for identifying and screening fungal isolates (Balabanova et al. 2018). The present study isolated twenty-five fungal strains from infected tomato fruits. Pure cultures of isolated fungal strains were sub-cultured onto pectin agar media and kept for enzymatic studies and identification. Among these twenty-five pure cultured fungal strains, ten strains that were able to grow on a medium containing polygalacturonic acid as the sole carbon source were isolated. At pH 5.6, these ten strains were tested for polygalacturonic acid hydrolysis using a plate assay. Polygalacturonase activity of a strain was indicated by a clear zone in the media (Figure 2). When a fungal strain presented at least 23 mm clear zones around colonies, the strains were classified as very good producers of pectin depolymerizing enzymes, while if the zones were at least of



(a) **(b)**

Figure 2 (a) Fungal isolate culture, (b) Secondary screening for potential fungal isolate (Zone of polygalacturonic acid hydrolysis).

Table 1 Polygalacturonase activity of different isolated fungal strains

S.N.	Polygalacturonase isolates	Zone of Inhibition in mm
1.	AT_1	20
2.	AT_3	23
3.	AT_5	19
4.	AT_7	17
5.	AT_{11}	21
6.	AT_{14}	25
7.	AT_{16}	30
8.	AT_{20}	20
9.	AT_{22}	15
10.	AT_{24}	18

18 mm and 15 mm these strains were considered as good producers and weak producers, respectively. Finally, the strain was considered poor producers when no polygalacturonase activity and clear zones were observed (Table 1). The strain with the most extensive zone (approximately 30 mm) was used for further parameter evaluation and to produce enzymes in liquid media.

3.2 Secondary screening

Solid-state fermentation has emerged as a promising approach for producing various enzymes, including polygalacturonase, a key enzyme involved in pectin degradation (Madamwar et al. 1989). Sugarcane bagasse, a readily available agricultural waste, has been extensively investigated as a carbon-rich substrate for solid-state fermentation due to its lignocellulosic composition (Garcia et al. 2018; Lamounier et al. 2018). The present study aims to explore the potential of utilizing sugarcane bagasse as a renewable and cost-effective carbon source for producing polygalacturonase by fungi in a solid-state fermentation system. The secondary screening was performed on the selected pectinolytic fungal strains AT3, 14, and 16 (Table 2). Among these, a fungal strain AT16 isolated from the tomato samples collected from Arba Minch had the highest polygalacturonase production (25.67 ± 0.10 U/ml/min) and protein content (13.74 mg/ml), with production carried out using sugarcane bagasse dried powder as substrate.

Table 2 Secondary screening of polygalacturonase fungal isolates for polygalacturonase production

S.N.	Polygalacturonase isolates	Polygalacturonase activity (U/ml/Min)
1	AT_3	19.83 ± 0.18
2	AT_{14}	16.57 ± 0.36
3	AT_{16}	25.67 ± 0.10

Values are mean of triplicates followed by ±SE

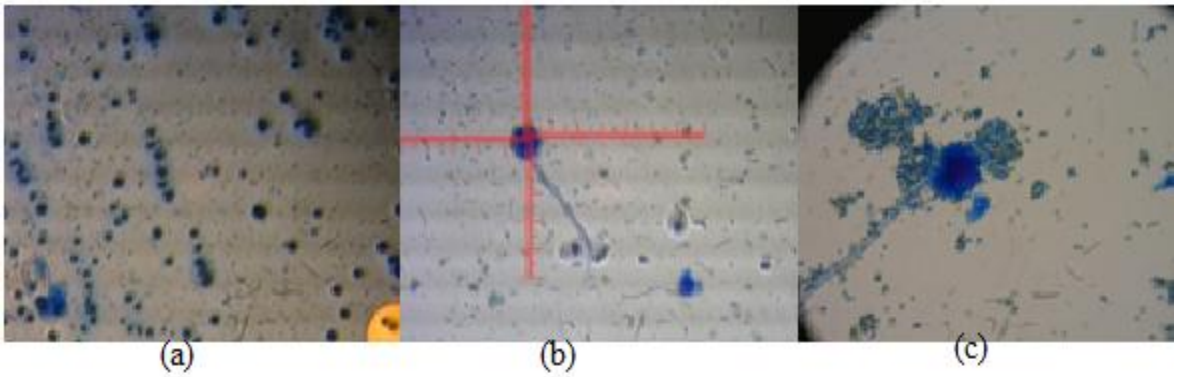


Figure 3 (a) Heavy sporulation of Isolate *Aspergillus niger* (b) Septate and hyaline hyphae of Isolate *Aspergillus niger* and (c) conidial head of Isolate *Aspergillus niger*

3.3 Identification of isolate

Isolated fungus strain was identified as *Aspergillus niger* based on morphological observation, cultural, hyphal characteristics and sporulation (Figure 3). This fungus was chosen for further investigation. Initially, the colonies were made up of a compact yellow felt, which later turned brown due to the conidiophores production, which is a characteristic of *A. niger* (Raper and Fennell 1965). The reverse side of the plate ranges from cream to yellow, which aligns with descriptions in the literature (Samson et al. 2010). The hyphal characteristics, including septate and hyaline hyphae, along with the conidial heads that radiate with heavy sporulation, are key identifying features of *A. niger* (Klich 2002). The absence of a teleomorph stage further supports the identification, as *A. niger* is typically known for its asexual reproduction (Gams et al. 1986). The conidia being globose to sub-globose is another hallmark trait corroborating the identification (Pitt and Hocking 2009).

3.4 Production of polygalacturonase

In the crude extract (150 ml) obtained using solid-state fermentation involving AT3, AT14, and AT16 strains, the protein content and polygalacturonase enzyme activity were found to be 13.74 mg/ml and 94.6 U/ml, respectively. Hence, the specific activity was 6.89 U/mg. The polygalacturonase activity recorded in the present study was higher than that reported by Alves et al. (2002) in the case of *Mucor genevensis* (5 U/ml) and Thakur et al. (2010) in *M. circinelloides* (9.15 U/ml), while the present study polygalacturonase activity was lower than the value reported by Gomes et al. (2009) in case of Penicillium viridicatum where the activity recorded, was 18 U/ml.

3.5 Enzyme purification

Polygalacturonase from the screened *A. niger* was purified from 150 mL of crude extract obtained from solid-state fermentation (Table 3). The polygalacturonase enzyme was initially purified by the salting out procedure, which involves the addition of up to 80% solid ammonium sulphate. Ammonium sulphate precipitation increased the enzyme's specific activity from 6.89 U/mg to 12.42 U/mg. Polygalacturonase can be precipitated with 0 - 90% ammonium sulphate, depending on the source of the enzyme (Buga et al. 2010; Chinedu et al. 2016). Sephadex G-200 column chromatography helped purify the enzyme 3.58 times, resulting in a specific activity of 24.66 U/mg. Finally, Sephacryl S-100 column chromatography increased the specific activity to 68.41 U/mg, which, compared to that of crude extract, was 9.93 times higher. The yield of the enzyme decreased as the purification fold increased.

Previous researchers have found significant variations in the purification fold and yield of polygalacturonase enzymes from various microbial sources. Cheng et al. (2016) isolated an acid-stable endo-polygalacturonase from *P. oxalicum* CZ1028. The purification process involved ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, and size exclusion chromatography. This resulted

Table 3 Purification of polygalacturonase from *A. niger*

Purification steps	Collected Volume (mL)	Total protein (mg/mL)	Total enzyme Activity (U/mL)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude extract	150	13.74	94.6	6.89	1	100
$(NH_4)_2 SO_4$ precipitation	80	2.93	36.4	12.42	1.8	38.48
SephadexG-200	12	1.16	28.6	24.66	3.58	30.23
Sephacryl S-100	1	0.29	19.84	68.41	9.93	20.97

in the enzyme purifying 29.9 times, achieving a specific activity of 2320 U/mg, with a final yield of 17.1%. Anand et al. (2016) purified polygalacturonase from *A. fumigatus* MTCC 2584 using acetone precipitation and Sephadex G-100 column, which resulted in 18.43 fold increase in enzyme purity, with a specific activity of 38.9 U/mg. Satapathy et al. (2021) extracted pectinase from *A. parvisclerotigenus* KX928754 using apple pomace as the substrate. The crude filtrate underwent ammonium sulfate precipitation, dialysis, and elution on a Sephadex G-100 column. As a result, the enzyme was purified 2.10-fold, with a yield rate of 2.91% and a specific activity of 1081.66 U/mg. Almowallad et al. (2022) partially purified exo-polygalacturonase from *P. oxalicum* AUMC 4153 using sugar beet manufacturing waste as the sole carbon source. The enzyme purification process included ammonium sulfate precipitation, acetone precipitation, and gel filtration chromatography. This resulted in a 28-fold increase in enzyme purity, with a final yield of 57%.

3.6 Characterization of the enzyme

3.6.1 Effect of temperature on the activity of polygalacturonase

Temperature is a critical factor in both microbial growth and product formation. The incubation temperature significantly impacts microbial growth rate, enzyme secretion, enzyme inhibition, and protein denaturation (Adeyefa and Ebuehi 2020). The effect of reaction temperature on polygalacturonase activity is depicted in Figure 4. Enzyme activity was detected at temperatures ranging from 30 to 60°C, with 55°C being the optimal temperature, followed by 50°C and 45°C. This finding demonstrated that polygalacturonase activity increased with increasing temperature until the optimal temperature was reached. However, polygalacturonase activity dropped dramatically above 55°C. The present study results align with those that Kaur et al. (2004) revealed, who found that exo-polygalacturonase produced from thermophilic mould *Sporotrichum thermophile* was optimally active at 55 °C. The decrease in enzyme activity at very high temperatures is attributed to the denaturation of the enzymes (Almowallad et al. 2022).

3.6.2 Effect of various substrates on the activity of polygalacturonase

The purified enzyme's affinity for different substrates was determined (Figure 5). The best substrates were polygalacturonic acid, pectin, cellulose, xylan, and galactose. Siddiqui et al. (2012) observed a maximal enzyme activity of 8.34 U/ml when polygalacturonic acid was used as a substrate. In the present study, the substrate polygalacturonic acid led to a maximum enzyme activity of 10.1 U/ml.

3.6.3 Effect of pH on the activity of polygalacturonase

The initial pH of the fermentation medium is critical in determining metabolite synthesis levels. The stability of the microbial metabolite is also affected by the medium's hydrogen ion concentration (Adeyefa and Ebuehi 2020). pH is important in polygalacturonase because it promotes and regulates extracellular enzyme synthesis by microorganisms, particularly fungi (Siddiqui et al. 2012). Fungi, particularly *Aspergillus* species, have been shown to thrive in acidic or slightly alkaline environments (Ahmed Olaitan 2019). The present

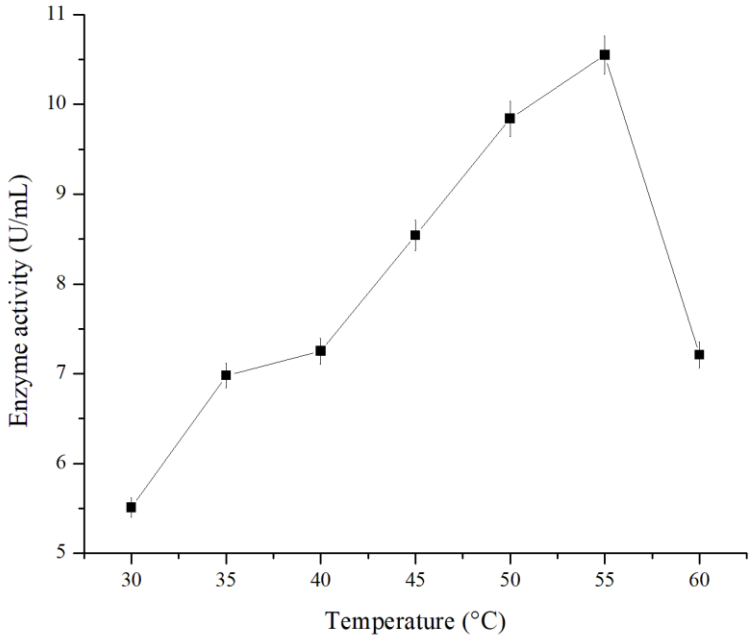


Figure 4 Effect of temperature on polygalacturonase activity from *Aspergillus niger*; values are mean of triplicates; error bar indicates ±SE.

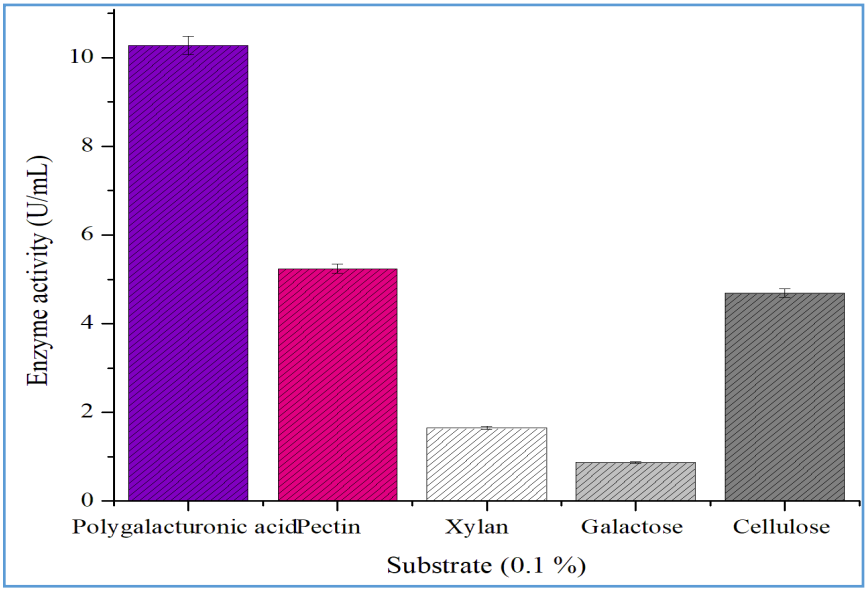


Figure 5 Substrate specificity of polygalacturonase from *Aspergillus niger*; values are mean of triplicates; error bar indicates ±SE.

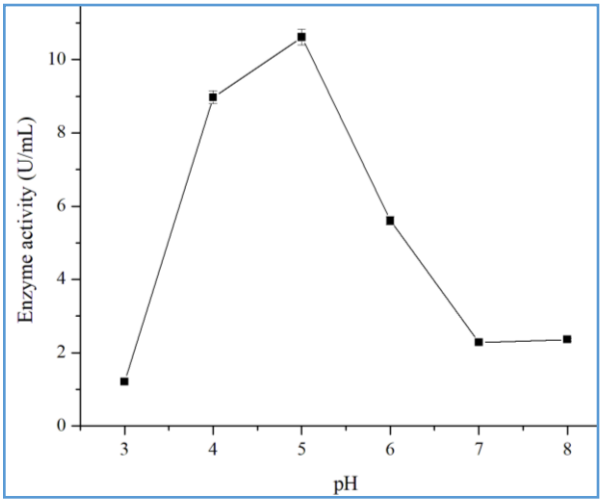


Figure 6 Effect of pH on polygalacturonase activity from *Aspergillus niger*; values are mean of triplicates; error bar indicates ±SE.

study found that pH 5 was the optimum for polygalacturonase activity (100% relative activity) produced by *A. niger* (Figure 6). The results agreed with Aminzadeh et al. (2007), who found that polygalacturonase from *Tetracoccosporium* sp. was more active at an acidic pH of 5. Similar observations were reported about the optimum pH for polygalacturonase from *A. fumigatus* (Wang et al. 2015), *P. oxalicum* CZ102 (Cheng et al. 2016), *Thermoascus aurantiacus* (Martins et al. 2012), *P. oxalicum* AUMC 4153 (Almowallad et al. 2022) and *A. tubingensis* (Tai et al. 2013).

3.6.4 Effect of divalent cations on enzyme activity

The effects of different metal ions on enzymatic activity were studied using a concentration of 10 mM of each metal ion in the reaction solution (Figure 7). Among all the divalent cations, Ca^{2+} was the optimum for the maximal polygalacturonase activity. On the contrary, Zn^{2+} inhibited the enzyme activity. The mechanism behind the increased activity of fungal polygalacturonases in the presence of certain divalent cations is not fully understood, but it is believed to involve several factors. First, divalent cations may help stabilize the enzyme structure, increasing catalytic efficiency (Pedrolli et al. 2009). Additionally, these cations may facilitate the interaction between the enzyme and the pectin substrate, a complex polysaccharide composed of galacturonic acid residues (Balabanova et al. 2018). Furthermore, divalent cations can influence the charge and conformation of the pectin substrate, making it more accessible to the polygalacturonase enzyme (Oumer 2017).

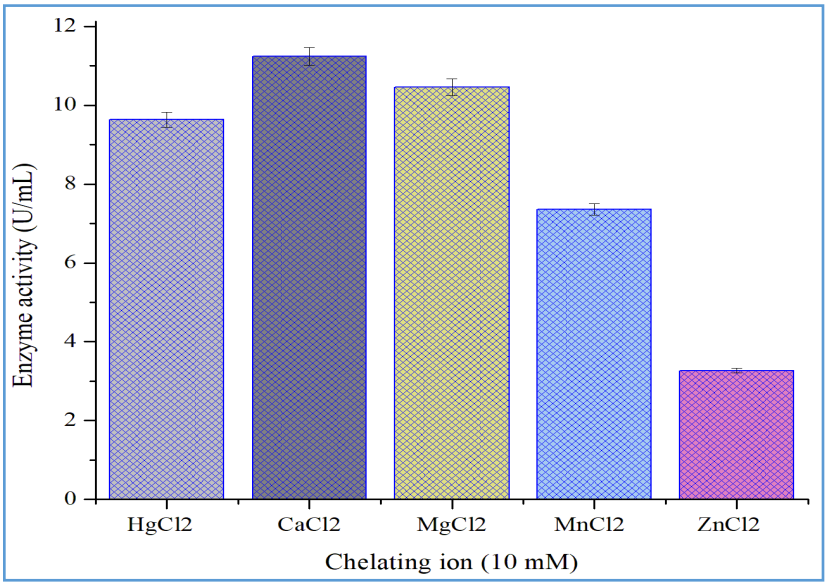


Figure 7 Effect of divalent cations on enzyme activity; values are mean of triplicates; error bar indicates ±SE.

Conclusion

Polygalacturonases are important members of the pectinase enzyme family and have significant biotechnological and commercial potential. In the current study, polygalacturonase was purified from *A. niger* 9.93 times by ammonium sulphate precipitation and column chromatography, resulting in specific activity of 68.41 U/mg protein. The purified polygalacturonase was naturally acidic, with an optimum pH of 5.0. The optimal temperature for maximum enzyme activity was 55°C, indicating that the enzyme is resistant to heat. $CaCl_2$ was revealed to be the most effective chelating ion for the enzyme. The homogeneity of the enzyme will be investigated using SDS PAGE shortly. Fungi typically produce polygalacturonase, which is essential for producing organic vegetable oil and fruit juice and being used to manufacture easily digestible animal feed.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Data statement

Data will be made available on reasonable request.

Authors' contribution

Gebiru S conceived the idea and performed the isolation of fungi, solid state fermentation, and enzyme purification. Jeyaramraja P R supervised the entire work. Sasikumar J M contributed in manuscript drafting. Abate Ayele performed enzyme assays. All authors have read and approved the final manuscript for publication.

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Germination of *Senegalia mellifera* seeds in response to presowing treatments

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KEYWORDS

Germination

Seed dormancy

Seed dimensions

Pre-sowing treatments

ABSTRACT

This study aimed to evaluate the size of *Senegalia mellifera* seeds and determine the most effective scarification techniques to improve germination. The experiment, conducted at Botswana University of Agriculture and Natural Resources, involved ten presowing treatments, including control, nicking, soaking in sulphuric acid for different durations, and boiling water for varying periods. A completely randomized design (CRD) was used for the experiment. Germination data was transformed using arcsine to meet normal distribution requirements and then analyzed using analysis of variance (ANOVA). The results showed that the presowing treatments had a statistically significant effect on germination ($P<0.00001$). Seeds treated with sulphuric acid, nicking, and those left untreated exhibited the highest germination rates, while seeds treated with boiling water showed the lowest germination percentages. These findings indicate that the seed coats of *S. mellifera* are permeable to water and air, and presowing treatments do not show any significant effect on the successful germination of *S. mellifera* seed.

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1 Introduction

Forests play a crucial role in the environment by providing a wide range of goods and services to both the environment and humans (Opoku et al. 2018; Turner-Skoff and Cavender 2019). Besides offering various environmental services, forests also supply economically important goods such as fruits, traditional medicine, animal feed, charcoal, firewood, and timber for building and household purposes (Cavendish 2000). They also serve as habitats for wildlife, protect water and soil, improve air quality, and store carbon. Anthropogenic activities, particularly deforestation, pose significant conservation issues in tropical regions, leading to a decrease in biodiversity and contributing to poverty in rural areas (Blakesley et al. 2002). This situation has been worsened by forest fires, occasional droughts, and climate change, as noted by Rampart et al. (2021). Deforestation not only endangers the flora and fauna that rely on forests and their resources but also threatens the livelihoods of indigenous people. Moreover, in arid and semi-arid regions, native woody species are extensively harvested without being replaced, despite the essential goods and services they provide.

The lack of attention given to incorporating native woody plants into planting projects and the excessive harvesting of these resources are putting their ongoing existence at risk. Planting initiatives struggle to include these indigenous woody plants due to a lack of knowledge on how to effectively propagate them in tropical areas. Additionally, the use of poor planting materials propagated in tree nurseries also contributes to their exclusion from planting programs (Elliot et al. 2002; McNamara et al. 2006). In recent years, there has been a growing trend towards replanting indigenous woody plants to restore native woodlands (Shono et al. 2007; Raman et al. 2009; Rasebeka et al. 2014; Meli et al. 2014).

Before acacias were renamed, *Senegalia mellifera* (Benth.) Seigler & Ebinger, was known as *Acacia mellifera* (Vahl) Benth. (Kyalangalilwa et al. 2013). *S. mellifera* belongs to the family Fabaceae (Timberlake 1980; Venter and Venter 2012). The tree is also known by several common or vernacular names, such as hook thorn, blackthorn, wait-a-bit, hook thorn, mongana (Timberlake 1980; Smit 2008), and many other names varying from place to place. The species is well-known for its tolerance and is common in arid and semi-arid regions of Africa and the Arabian peninsula (Hagos and Smit 2005; Heuze and Tran 2015). The species thrives in dry conditions and on a variety of soil types, including clayey and sandy Kalahari soils (Timberlake 1980; Tietema et al. 1992; Smit 2008). *S. mellifera* is a thorny shrub that grows up to 4 meters in height with multiple stems (Timberlake 1980) and rarely reaches 6 to 9 meters high (Venter and Venter 2012; Heuze and Tran 2015). The distinguishing feature of *S. mellifera* is a flat, round, or spreading crown with the potential to touch the ground (Venter and Venter 2012). Furthermore, the *S. mellifera* tree has a robust set of thorns with a sturdy hook at the nodes (Timberlake 1980; Smit 2008; Venter and Venter 2012).

S. mellifera is a valuable multipurpose tree species. Different parts of the tree, such as leaves, twigs, pods, shoots, and flowers, are eaten by domestic animals as feed (Palmer and Pitman 1972; Venter and Venter 2012; Abdalkreem et al. 2017). It provides nectar used by honeybees to produce high-quality honey (Bein et al. 1996; Smit 1999; Nonyane 2013). The termite-resistant wood of the tree is also used for making axe and pick handles (Venter and Venter 2012), constructing fences, and producing firewood and charcoal (Orwa et al. 2009; Nonyane 2013; Heuzé and Tran 2015). Local communities utilize the bark, leaves, and roots to treat a variety of illnesses (Hines and Eckman 1993; Mutai et al. 2004; Koch et al. 2005; Fatima and Mamoun 2013). According to Orwa et al. (2009), the tree has been used in agroforestry systems to mark farm boundaries and create live fences.

The purpose of a plant's seed is to establish new plants. However, the process of germination can only occur once because it is essentially a permanent one (Smýkal et al. 2014). Plant seeds have a very long dormancy period, and various mechanisms have evolved to minimize the period of germination and to overcome this dormancy (Foley 2001). Many woody plants, especially legumes, have tough outer seed coats that prevent the entry of water and oxygen to stimulate or initiate germination. Seeds with impermeable or physical dormancy are not able to germinate when conditions are conducive for non-dormant seeds to germinate (Vleeshouwers et al. 1995; Bewley 1997). Seed dormancy influences germination ecology and plant seed ecology (Forbis et al. 2002). Several researchers have used different pre-sowing techniques to crack impermeable seed coats to improve the germination of several plants (Ren and Tao 2004; Anand et al. 2012; Gilani et al. 2019; Botumile et al. 2020; Latiwa et al. 2023; Dasari et al. 2024; Longjam and Kotiya 2024).

The survival of plants depends not only on dormancy but also on seed qualities such as size and weight (Moles et al. 2005). Seed weight and size are critical factors for growth in the early stages of the plant life cycle, particularly in resource-limited areas (Saeed and Shaukat 2000; Moles et al. 2005). Seed size is also crucial for seed germination, dispersal, and the survival rates of plants (Tripathi and Khan 1990; Bonfil 1998; Moles and Westoby 2006). Limited information is available about the seed characteristics, dormancy, and germination requirements of various woody species, including *S. mellifera* in Botswana. This information is crucial for foresters to effectively propagate seedlings of native woody plants for afforestation and replanting initiatives. Therefore, the present study aimed to assess seed characteristics and determine the most effective scarification method for achieving speedy, successful, and consistent

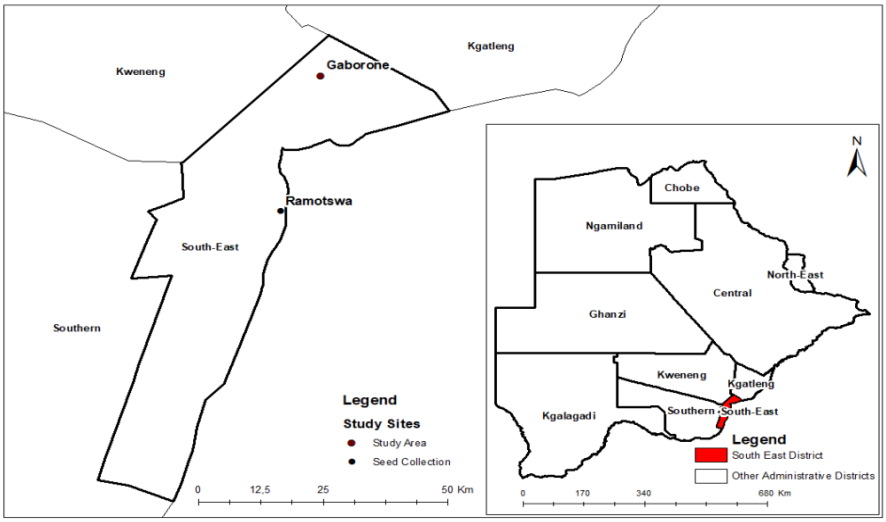

Figure 1 Map of Botswana showing Gaborone, where BUAN is situated, and Ramotswa, in the Southeast, where seeds were gathered (Cartographer T.I. Makoi)

Figure 2 *S. mellifera* pods and seeds (Photographer: F.O. Madisa)

germination in *S. mellifera*. The study's goals included measuring the size and weight of seeds, as well as testing various scarification methods on germination.

2 Materials and Methods

2.1 The study area description

This study was conducted in 2021 at the Botswana University of Agriculture and Natural Resources (BUAN) in Botswana. The experimental site at BUAN is located at an elevation of 994 meters above sea level, with an altitude of 24° 33'S and a longitude of 25° 54'E. Gaborone has a subtropical, semi-arid climate characterized by warm and wet periods from November to March and dry periods from April to October. The coolest month is July (13.5°C), and the hottest month is January. Gaborone receives approximately 485mm of rainfall each year.

2.2 Collection of plant material

Mature fruits were collected from various *S. mellifera* trees in the Disaneng grazing land of Ramotswa, located in the Southeast District of Botswana (Figure 1). Disaneng has semi-arid conditions with consistently sunny summers. The savanna vegetation at Disaneng is primarily composed of shrubs. Dry fruits were manually harvested from randomly chosen trees. Paper bags were used to gather the fruits, which were then transported to BUAN. The seeds were extracted by splitting and opening the fruit, followed by removing the outer covering (Figure 2).

2.3 Seed properties

A digital vernier calliper was used to measure the length, width, and thickness of each seed from five sets of ten seeds. Five replicates of ten seeds were weighed on a digital precision scale to determine the

weight of individual seeds. Similarly, the weight of 1000 seeds was determined by weighing five batches of 100 seeds each.

2.4 Effect of various seed treatments on germination of *S. mellifera*

In this study, ten treatments were conducted, including control or untreated seeds. The treatments included nicking, soaking in boiling water for one, three, and five minutes, using hot water (cooled for 24 hours), treatment with concentrated sulphuric acid (H_2SO_4) for 15, 30, 45, and 60 minutes, as well as untreated seeds. The study used a completely randomized block design (CRB), and each treatment, including the control, was repeated four times. Before starting the experiment, the viability of the seeds was tested by soaking them in water. Viable seeds settled at the bottom of the jar, while those that floated were considered not viable and were discarded. Each replicate contained 25 seeds sown inside 90 mm petri dishes lined with cotton wool, kept moist by adding distilled water as needed. Seeds were considered germinated when the radical had penetrated the seed coat. All germinated seeds were counted and then removed from the petri dish to avoid counting them the next day. The germination study was conducted for 30 days.

2.4.1 Nicking treatment

In this treatment, 100 seeds were divided into four groups of 25 seeds for each replication. Each seed underwent a 1-2mm seed coat incision on the curved edge opposite the embryo location to prevent damage or displacement of the embryo.

2.4.2 Sulphuric acid treatments

Seeds were treated with concentrated H_2SO_4 for 15, 30, 45, and 60 minutes using the method described by Teketay (1996b). Each soaking time and treatment involved twenty-five seeds placed in separate heat-resistant beakers. These beakers were filled with enough H_2SO_4 to soak the seeds and were stirred every five minutes to ensure uniform acid distribution. After each soaking time, an acid-resistant sieve was used to separate the seeds from the acid. The acid was then transferred to a different beaker. The seeds were cleaned and washed with running tap water to eliminate any acidic residues.

2.4.3 Boiling water treatment

In this treatment, seeds were subjected to three different boiling water periods: one, three, and five minutes. Each soaking period comprised of four replicates of 25 seeds enclosed inside coffee filter papers. The seeds were placed in a pot of boiling water on a stove and then removed after one, three, and five minutes. Finally, they were cooled in a small bowl of cold water.

2.4.4 Hot water treatment

For this treatment, four coffee filters were filled with 25 seeds each and placed in a 250 ml beaker. After that, boiling water was taken off the heat and poured into the beaker, and the seeds were left for 24 hours to cool down.

2.4.5 Control

In this study, each experiment used a control group consisting of four replicates of 25 untreated seeds.

2.5 Statistical analyses

The percent germination of the seeds was calculated by directly counting the germinated seeds and using the following formula.

$$\text{Seed germination (\%)} = \frac{\text{Number of seedlings emerged}}{\text{Total number of seeds sown}} \times 100$$

The data was analyzed using Statistix Software, Version 10 (Statistix 10, 1984-2003) for one-way ANOVA and descriptive statistical analysis. The percentage germination data was transformed with arcsine to meet normality requirements before being subjected to analysis of variance (ANOVA) (Zar 1996). The Tukey's Honestly Significant Difference (HSD) Test was utilized to identify significant differences in means at a significance level of $P < 0.05$.

3 Results

3.1 Characteristics of seeds

Each pod contained between 2 and 3 seeds, with 1 to 2 of these being sound seeds. No insect-damaged or aborted seeds were found in the collected samples. The observed measurements for the length, width, and thickness were 10.32 ± 0.27 mm, 8.52 ± 0.06 mm, and 1.76 ± 0.08 mm, respectively (Table 1). The results in Table 1 show that the weight of individual seeds and one thousand seeds were 0.14 ± 0.01 g and 111.66 ± 1.11 g, respectively.

3.2 Effect of seed treatment on germination of *S. mellifera*

The influence of pre-sowing treatments on the germination of *S. melifera* was found to be extremely significant ($P < 0.00001$).

Table 1 Different characteristics of the *Senegalia mellifera* seed samples

Seed length (mm)	Seed width (mm)	Seed breast (mm)	Single seed weight (g)	Thousand seed Wight (g)
10.32 ± 0.27	8.52 ± 0.06	1.76 ± 0.08	0.14±0.01	111.66±1.11

The "±" standard error of mean

Table 2 Effect of various pre-sowing treatments on the cumulative germination of *S. mellifera* seeds

Treatment	Germination percentage	
	Mean germination	Range
Control	79.2± 4.2[a]	69.7 – 90.0
Nicking	80.8 ± 5.4[a]	69.7 – 90.0
Sulphuric acid (15 min)	78.5 ± 0.0[a]	78.5 – 78.5
Sulphuric acid (30 min)	80.1 ± 3.5[a]	73.6 – 90.0
Sulphuric acid (45 min)	87.1 ± 2.9[a]	78.5 –90.0
Sulphuric acid (60 min)	80.1± 3.5[a]	73.6 – 90.0
Boiling Water (1 min)	27.7 ± 3.0[c]	20.3 –34.5
Boiling Water (3 min)	8.0 ± 4.9[d]	0 – 20
Boiling Water (5 min)	2.9 ± 2.9[d]	0 – 11
Hot water (allowed to cool for 24 hrs)	54.3 ± 0.70[b]	53.1–55.6

Values are means ± standard error; Means were separated using Tukey Honestly Significant Differences (HSD) Test at $p \leq 0.05$; Means within columns followed by the same letters are not significantly different.

When compared to untreated seeds, the germination percentage for seeds that were boiled for one, three, and five minutes, as well as those soaked in hot water (and then allowed to cool for 24 hours), was significantly lower (Table 2). Moreover, there were no noteworthy variances in germination percentage between seeds treated with sulphuric acid (for 15, 30, 45, and 60 minutes), nicking, and the control. The seeds that exhibited the highest germination percentage were those treated with sulphuric acid for 45 minutes (87.1%), followed by nicking (80.8%), sulfuric acid treatment for 30 and 60 minutes (80.1%), and the control (79.2%). Conversely, the seeds subjected to the five-minute boiling treatment displayed the lowest germination percentage (2.9%) (Table 2).

4 Discussion

4.1 Seed Properties

The size of a plant's seeds can influence its ability to regenerate and maintain health (Chacon et al. 1998). Larger seeds have been found to have a positive impact on germination and plant survival (Zimmermann and Weis 1983; Chacon et al. 1998; Moles and Westoby 2006). The current study did not observe any damaged or aborted seeds, indicating that these seeds are unlikely to be affected by living or non-living factors. These findings are consistent with a study by Kahaka et al. (2018), which reported no damage or seed abortion in other legume plant species native to Botswana. Table 1 presents the findings on the seed dimensions (length, width, and breadth) of *S. mellifera*. These dimensions are similar to those of other species native to Botswana, particularly *Senegalia erubescens* (Kahaka et al. 2018) and *Senegalia galpini* (Botumile et al. 2020). The length and width of *S. mellifera* seeds are comparable to those of *Vachellia robusta* (Botumile et al. 2020). The weight of a single seed and 1,000 seeds is similar to that of *S. erubescens* (Kahaka et al. 2018) and smaller than that of *Senegalia galpinii* (Botumile et al. 2020). The mass of 1,000 seeds is higher compared to *Vachelia rehmanniana* (Mojeremane et al. 2017), *Dichrostachys cineria* (Kahaka et al. 2018), and *Vachelia erioloba* (Odirile et al. 2019). Furthermore, in comparison to small seeds, various studies have indicated that large seeds produce seedlings that are more resistant to drought and have increased survival and/or growth in nutrient-deficient environments (Stock et al. 1990; Milberg et al. 1998; Lloret et al. 1999; Seiwa et al. 2002).

4.2 Effect of seed treatment on *S. mellifera* germination

Leguminous woody plants have impermeable seed coats, which prevent the absorption of water and oxygen, leading to seed dormancy (Considine and Considine 2016). These plants are naturally found in dry environments and have impermeable seed coats to survive tough conditions such as intense heat, animal damage, extreme drought, and physical harm. The low germination rate caused by these impermeable seed coats hinders the use of local species in planting initiatives in dry environments (Smith et al. 2003; Mojeremane et al. 2021).

Based on the data presented in Table 2, *S. mellifera* does not have impermeable seed coats that would prevent water absorption or gas exchange. The results indicate no significant difference in seed germination between seeds treated with sulphuric acid, nicking, and untreated seeds. Untreated seeds showed a germination rate of 79.2%, which is not significantly different from the germination rates of seeds subjected to sulphuric acid and nicking treatments. This suggests that the species lacks the typical hard, impermeable seed coat found in other leguminous woody plants. These findings align with those of Tietema et al. (1992), who reported a 65%

germination rate for untreated *S. mellifera* seeds five days after sowing. Previous reports have indicated that untreated *S. mellifera* seeds can achieve higher germination rates without any prior treatment (Roodt 2008; Venter and Venter 2012; Nonyane 2013) and that soaking the seeds in warm water overnight can expedite their germination process.

Boiling seeds for one, three, or five minutes resulted in the lowest germination rates, ranging from 2.9% to 27.7%. The seeds' sensitivity to high temperatures may explain the low germination rates observed in these treatments (Kahaka et al. 2018). These recent study results are consistent with previous research on various types of trees in Botswana (Kahaka et al. 2018; Odirile et al. 2019; Botumile et al. 2020; Mojeremane et al. 2021; Latiwa et al. 2023). The germination rate of seeds soaked in hot water (allowed to cool for 24 hours) was also found to be lower compared to that of untreated seeds. This finding is supported by studies conducted in Botswana (Mojeremane et al. 2017; Mojeremane et al. 2020) and elsewhere (Diallo et al. 1996; Teketay 1996a, 1996b; Teketay 1998; Fredrick et al. 2017).

Conclusions

The laboratory germination tests revealed that *S. mellifera* seeds are not impacted by the impermeability of the seed coat, which typically hinders water intake and gaseous exchange necessary for germination. Untreated seeds displayed comparable germination rates to those treated with nicking and sulphuric acid. Consequently, these seeds do not necessitate any prior treatment before planting.

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Declarations

The authors have no conflicting interests.

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Effect of Preparation and Drying Techniques on the Physicochemical, Functional and Nutritional Properties of products from Beetroot (*Beta Vulgaris L.*) varieties

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ABSTRACT

Beetroot (*Beta vulgaris* L.) is rich in biologically active compounds. This study aimed to assess how different methods of preparation and drying affect the physical, chemical, functional, and nutritional properties of iron-rich beetroot powder. Two beetroot varieties, Detroit Dark Red (DetR) and Crimson Globe (CrimG), were processed using three drying techniques: sun drying (SD), oven drying (OD), and freeze drying (FD), with both boiled and fresh beetroots. The properties evaluated in the study included water activity, color, total phenolics and flavonoids, oxalate content, and mineral content. The results showed significant ($p<0.05$) differences in these properties between the dried and fresh samples. Notably, drying increased calcium, zinc, and phosphorus levels while decreasing the iron content. Boiling followed by sun drying was the best method for retaining iron, particularly for the CrimG variety. The study suggests that drying can help preserve or even enhance the physicochemical properties and micronutrient content, especially iron while reducing phytochemical levels affecting iron absorption. These findings are important for developing iron-rich beetroot products to improve dietary iron intake, especially for adolescent children.

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1 Introduction

Beetroot (*Beta vulgaris* L.), known for its high fibre and sugar content, is also recognized for its moderate caloric value (Kovarovič et al. 2017). This vegetable, particularly the red beetroot variety (*Beta vulgaris* L. var. *vulgaris*), is appreciated worldwide for its rich composition and numerous health benefits (Székely and Máté 2023). The intense red coloration of beetroot is due to betalains, specifically betacyanins and betaxanthins, which offer significant antioxidant and anti-inflammatory properties (Șeremet and Oana-Viorela 2020). These compounds are noted for their health-promoting effects, such as inhibiting lipid peroxidation and enhancing low-density lipoprotein oxidation resistance (Șeremet and Oana-Viorela 2020).

Furthermore, consuming beetroot is linked to various health benefits, including improved blood circulation, respiratory health, skincare, and immune system support (Șeremet and Oana-Viorela 2020). Additionally, beetroot has shown potential in treating anemia by promoting red blood cell production and boosting hemoglobin levels (Ali and Bilal 2023). In today's market, which favors convenient and nutritious food options, beetroot powder has emerged as a promising product, especially for developing iron-rich supplements to enhance dietary iron intake among school-aged children (Șeremet and Oana-Viorela 2020). The drying techniques used in processing beetroot are crucial for preserving its nutrients, transforming it into powder form, and enhancing its functionality in food preparation or as a beverage ingredient (Șeremet and Oana-Viorela 2020).

However, the impact of these processing methods, including drying and thermal techniques, on the physicochemical, functional, and nutritional properties of beetroot powder needs careful consideration (Hamid and Mohamed Nour 2018). Previous research indicates that various drying techniques substantially influence the chemical composition, mineral content, bioactive compounds, and color characteristics of beetroot slices (Hamid and Mohamed Nour 2018). Various studies have assessed the effects of different drying conditions, such as traditional drying, swell drying, and blanching combined with drying, on the levels and activity of antioxidants in red beetroots (Alonzo-Macías et al. 2020). Moreover, the choice of drying method, including freeze-drying, microwave-assisted drying, or conductive hydro drying, affects the retention of bioactive compounds, phenolics, and antioxidant activity in beetroot products (Preethi et al. 2020; Liu et al. 2022). Drying is a common preservation method, but the effects of these methods on the phytochemicals and antioxidant properties of food products are necessary (Sarkar et al. 2021). Previous studies highlight the importance of evaluating different pre-treatments and temperatures to optimize the drying characteristics of beetroot slices (Mudgal 2023). Additionally, techniques like osmotic dehydration and ultrasound pre-treatments have been explored to enhance dried beetroot chips' nutritional quality and sensory attributes (Peters et al. 2021). Choosing the right preparation and drying techniques is vital for maintaining the quality, functionality, and nutritional value of beetroot products. Understanding how different processing methods impact the physicochemical properties of beetroot powder can facilitate the development of innovative and nutritious food products. This research aims to produce beetroot powder using various preparation and drying techniques and assess their effects on the physicochemical, functional, and nutritional attributes of dried beetroot products.

2 Materials and Methods

2.1 Raw materials and sample preparation

This study used two beetroot varieties, Crimson Globe (CrimG) and Detroit Dark Red (DetR). The mature, fresh beetroots were obtained directly from farmers in Kabale, Western Uganda, then transported to the laboratory in Kampala. Upon arrival, the beetroots were sorted, washed under running potable water, peeled using a ceramic knife, and placed in clean water to prevent discoloration.

2.2 Processing of samples into powder

The beetroots were peeled, cleaned, and sliced into 1-2 mm thick pieces. The slices were divided into two portions. One portion was boiled at 90°C for 30 minutes in a stainless-steel pan with distilled water, while the other was left unboiled. These portions were further divided into four sub-portions for different drying treatments: (i) Fresh Sample: grated into mash and used as a control, (ii) Oven Drying (OD): dried at 65°C for 48 hours, (iii) Sun Drying (SD): spread on raised plastic mesh using wooden stands, and (iv) Freeze Drying (FD): dried at -80°C for 48 hours. The dried samples were pulverized using a pestle and mortar, passed through a 60 mm plastic mesh sieve, and kept in dark, airtight plastic containers for further analysis.

2.3 Estimation of physicochemical and functional characteristics

2.3.1 Water activity

Measurements were conducted using an Aqua Lab water activity meter (Aqualab Series 3, Decagon Devices, Inc., Pullman, WA, USA) with temperature compensation. The measurements were performed in triplicate.

2.3.2 Color measurement

The color was determined using a Minolta CR-10 color reader (Minolta Co. Ltd, Japan), following the method of Youssef and Mokhtar (2014) with some modifications. The chroma meter was

calibrated using a white plate and light trap provided by the manufacturer. Color values were expressed using the CIE Lab* system.

2.3.3 Bulk density

Bulk density was determined using a graduated cylinder following the method described by Roongruangsri and Bronlund (2016).

2.3.4 Water solubility and water absorption

The parameters were determined using the methods outlined by Roongruangsri and Bronlund (2016) with necessary adjustments. One gram (1.0 g) of beetroot powder was mixed with 10 milliliters of distilled water and then placed in an incubator at 60°C for 20 minutes. Afterwards, the mixture was centrifuged at 3000 rpm for 15 minutes. The water solubility and absorption were then calculated based on the weights of the remaining solids and precipitates. The following formula was used for the calculation:

$$\text{Water solubility} = \frac{\text{Weight of residue}}{\text{Weight of beetroot powder}} \text{ x } 100$$

$$\text{Water absoprtion} = \frac{\text{Weight of centrifuged precipitate}}{\text{Weight of beetroot powder}}$$

2.4 Phytochemical analysis

2.4.1 Extraction of phenolic compounds

To extract phenolic compounds, the method described by Wang et al. (2020) and Serna-Vázquez et al. (2021) was followed with some minor adjustments. In summary, 100 mg beetroot sample was blended with a solution of 80% methanol and 2% formic acid (10 mL). This blend was homogenized at room temperature using a Poltroon PT 1200E handheld homogenizer. Next, we sonicated the homogenate for 30 minutes at room temperature using a Bandelin Electronic (Germany) sonicator with DT1028. After sonication, the mixture was centrifuged at 3000xg for 25 minutes using an MSE MSB080.CR2.K centrifuge (UK). The supernatant was carefully transferred to a separate container and stored at -18°C. We re-extracted the remaining pellet under the same conditions to ensure maximum extraction efficiency. The supernatants from both extractions were combined and stored in an airtight container in a Sanyo Biomedical Freezer MDF-U333 (Sanyo Electric Biomedical Co. Ltd, Japan) at -18°C. These extracts were subsequently used to determine the total phenolic content (TPC) and total flavonoid content (TFC).

2.4.2 Total phenolic content (TPC)

The total phenolic content of the samples was measured using the Folin-Ciocalteu reagent, following the method outlined by Özderin (2024). Briefly, 50.0 µL of beetroot powder extract was added to a 50.0 mL Falcon tube and diluted to 3.0 mL with distilled water. After this, 250 µL of Folin-Ciocalteu reagent (diluted 2-fold before use) was added to the mixture and allowed to react for 5 minutes. Subsequently, 250 µL of 7% (w/v) sodium carbonate solution was added, and the mixture was topped up to 5 mL with distilled water. This mixture was then incubated at ambient temperature for 90 minutes to develop the color. Absorbance was then measured at 765 nm using a UV/Vis spectrophotometer (Jenway 6405 UV/Vis, UK). A calibration curve with gallic acid standards (0, 20, 40, 60, 80, and 100 mg/mL) was used to quantify the total phenolic content. Results were expressed as milligrams of gallic acid equivalent (GAE) per 100 mL on a dry weight basis (DW).

2.4.3 Total flavonoid measurement

The total flavonoid content was measured using a method adapted from Sinurat et al. (2022). To summarize, 125 µL of either catechin standard solution or beetroot powder extract was placed in a 5 mL disposable test tube and mixed with 0.625 mL of deionized water and 37.5 µL of 5% (w/v) sodium nitrite solution. The reaction proceeded at room temperature for 6 minutes, after which 75 µL of 10% (w/v) aluminium chloride was added. The mixture was left to react for another 6 minutes before adding 0.25 mL of 4.0M sodium hydroxide. Finally, 0.4 mL of distilled water was added, and the absorbance was recorded at 510 nm using a UV/Vis spectrophotometer (Jenway 6405 UV/Vis, UK). A standard calibration curve was created using varying concentrations of catechin (ranging from 0.2 to 1.00 mg/mL). The total flavonoid content was expressed as milligrams of catechin equivalents per 100 mL of beetroot extract (mg CE/100 mL) on a dry weight basis (DW).

2.4.4 Measurement of Oxalates

The oxalate content of the samples was determined using a method similar to the classical titrimetric method described by Karamad et al. (2019). For one hour, 2.0 g of beetroot sample was digested with 10 mL of 6M hydrochloric acid. After cooling, the mixture was brought to a final volume of 250 mL with distilled water and filtered. Two portions of 125 mL were taken from this filtrate and placed in beakers. 3-4 drops of methyl red indicator were added to each portion. Concentrated ammonium hydroxide was added drop by drop until the solution changed from salmon pink to pale yellow, and the pH was recorded. Each portion was then heated to 90°C, cooled, and filtered to remove any precipitate. The filtrate was reheated to 90°C, and 10 mL of 5% calcium chloride solution was added while stirring continuously. The resulting solution was decanted, and the precipitate was dissolved entirely in 10 mL of 20% (v/v) sulfuric acid solution. This solution was then topped up to 300 mL with distilled water. An aliquot of 125 mL of this filtrate was heated until near boiling and titrated against 0.05 M standardized potassium permanganate until a pink color persisted

for 30 seconds at the endpoint. The oxalate content was calculated using a specific formula.

$$\text{Oxalate content} = \frac{\text{T X Vme X Df X 105}}{\text{ME X MF}}$$

Where: T = Titer value of Potassium permanganate, Vme= volume- mass equivalent (that is, 1 ml of 0.05 m Potassium permanganate, = 0.00228 g of anhydrous oxalic acid), Df= dilute factor (Vt/A that is, total volume of titrate/ Aliquot used), Mf= mass of sample used, ME = molar equivalence of Potassium permanganate in oxalate concentration in g/dm^3.

2.5 Nutritional analysis

2.5.1 Digestion and Mineral Content Analysis

The dried, ground beetroot sample underwent complete digestion using a solution composed of 50% nitric acid (HNO_3) and 30% hydrogen peroxide (H_2O_2), following a modified version of the method described by He et al. (2013). Briefly, 1.0 g of the ground sample was placed in a 50 mL grainer falcon tube. Approximately 0.5 mL of 50% HNO_3 and 0.2 mL of 30% H_2O_2 were added to the sample. The mixture was left overnight to allow for cold digestion. The next day, the samples were subjected to warm digestion on a heating block, initially at 80°C for 30 minutes, then increasing to 125°C for 2 hours. Once the solution became clear with a slightly yellowish tint, digestion was halted, and the volume was brought up to 25 mL with deionized water. The samples were then stored at room temperature in preparation for further analysis.

The digested samples were transferred into vials and placed in an autosampler (SPS 4 Autosampler, Agilent Technologies). Mineral content and control samples were analyzed using microwave plasma atomic emission spectroscopy (4200 MP-AES, Agilent Technologies). The concentration of each analyte was calculated in parts per million (PPM). The final concentration in mg/kg was determined using the formula:

$$\text{Final Concentration (mg/kg)} = \text{Sample Concentration (PPM) X } \frac{\text{Total Dilution}}{\text{Weight of Sample}}$$

2.6 Data analysis

Experimental data were presented as mean ± standard deviation of triplicate measurements and analyzed using IBM SPSS Statistics version 16 (IBM Corporation, New York, USA). Statistical significance was determined at $p \leq 0.05$ using Student's t-test and one-way ANOVA, followed by Fisher's Least Significant Difference test (LSD) for post hoc analysis.

3 Results and Discussion

3.1 Functional and physicochemical properties

Table 1 illustrates the significant influence of preparation and drying methods on beetroot powder's functional and physicochemical properties.

3.1.1 Water Activity

The water activity of beetroot samples decreased significantly ($p < 0.05$) with boiling and drying methods. The highest values were observed in boiled freeze-dried samples (0.54 ± 0.00 for Detroit Dark Red and 0.52 ± 0.00 for Crimson Globe). The lowest water activity values were reported in boiled sun-dried (0.25 ± 0.01) and sun-dried (0.23 ± 0.01) samples for the Crimson Globe variety. This reduction in water activity improves shelf-life stability by inhibiting microbial growth, which aligns with the findings of Stavropoulou and Bezirtzoglou (2019), who emphasized the importance of low water activity in food preservation.

3.1.2 Bulk Density

The bulk density varied significantly ($p < 0.05$) depending on the preparation and drying methods. The boiled oven-dried samples exhibited the highest bulk density (0.86 ± 0.01 for Detroit Dark

Table 1 Functional and physicochemical properties of crimson globe and detroit dark red beetroot powders

Treatment	Bulk Density(g/mL)		WaterAbsorptivity index		Water Solubility Index (%)		Water activity (a_w)	
	DetR	CrimG	DetR	CrimG	DetR	CrimG	DetR	CrimG
FDR	0.18 ± 0.03^{c}	0.22 ± 0.02^{d}	4.59 ± 0.08^{c}	5.09 ± 0.36^{c}	39.00 ± 8.00^{f}	41.00 ± 4.00^{d}	0.52 ± 0.02^{b}	0.52 ± 0.01^{b}
FDB	0.27 ± 0.01^{c}	0.26 ± 0.04^{d}	8.84 ± 0.50^{a}	8.40 ± 0.48^{a}	64.00 ± 10.00^{d}	44.00 ± 6.00^{d}	0.54 ± 0.03^{b}	0.52 ± 0.02^{b}
ODR	0.66 ± 0.02^{b}	0.69 ± 0.01^{c}	3.35 ± 0.07^{d}	6.49 ± 0.57^{b}	77.00 ± 6.00^{b}	78.00 ± 5.00^{a}	0.29 ± 0.01^{cd}	0.31 ± 0.03^{c}
ODB	0.86 ± 0.02^{a}	0.79 ± 0.02^{b}	4.73 ± 0.17^{c}	4.74 ± 0.08^{d}	67.00 ± 11.00^{d}	72.00 ± 4.00^{b}	0.33 ± 0.02^{c}	0.37 ± 0.02^{c}
SDR	0.68 ± 0.01^{b}	0.74 ± 0.01^{b}	5.34 ± 0.12^{b}	5.44 ± 0.10^{c}	78.00 ± 5.00^{a}	75.00 ± 4.00^{b}	0.26 ± 0.01^{d}	0.23 ± 0.01^{d}
SDB	0.82 ± 0.02^{a}	0.90 ± 0.02^{a}	5.26 ± 0.19^{b}	4.03 ± 0.03^{f}	71,00 ± 8.00^{c}	60.00 ± 6.00^{c}	0.25 ± 0.01^{d}	0.26 ± 0.02^{f}

Values represent means ± standard deviation from three separate experiments, different superscript letters in the same column indicate significant differences ($P < 0.05$); here DetR (Detroit Dark Red), CrimG (Crimson Globe), FDR (Freeze-Dried Raw), FDB (Freeze-Dried Boiled), ODR (Oven-Dried Raw), ODB (Oven-Dried Boiled), SDB (Sun-Dried Boiled), SDR (Sun-Dried Raw)

Red and 0.90 ± 0.02 for Crimson Globe), while the boiled freeze-dried samples had lower bulk densities. The higher bulk densities in the boiled samples indicate a more compact structure, which is in line with the findings of Hamid and Mohamed Nour (2018), who observed that drying methods significantly impact the physical properties of beetroot slices.

3.1.3 Water Absorption and Solubility

The highest water absorption index was found in the boiled freeze-dried samples for both varieties, while the lowest was observed in the oven-dried Detroit Dark Red and boiled sun-dried Crimson Globe samples. On the other hand, the water solubility index was highest in the sun-dried Detroit Dark Red and oven-dried Crimson Globe samples, with the lowest values in freeze-dried samples. The porous structure of freeze-dried products allows for higher water uptake, which is beneficial for rehydration (Razzak 2024). This is crucial due to the impact of the speed at which a powder dissolves in water on its rehydration properties and the quality of the finished product (Grabowski et al. 2006; Kim et al. 2012).

3.2 Color

Table 2 shows the color characteristics of beetroot powders based on different processing methods. The lightness (L) values increased when the beetroot was boiled and dried, with the highest values reported in oven-dried and freeze-dried boiled samples. The redness (a) values were highest in the freeze-dried samples, while sun-dried and oven-dried samples showed a significant reduction. The yellowness (b) values increased significantly with sun and oven drying, indicating a relationship with drying temperature. The pigment analysis revealed that the betacyanin content in freeze-dried samples was similar to that of fresh samples but decreased as the temperature increased. Conversely, the betaxanthin content increased with higher drying temperatures. This suggests that betacyanin, the red pigment, is sensitive to heat and degrades with increased temperature, possibly converting into betaxanthin (Gokhale and Lele 2011). The increase in yellow betaxanthin could be due to the chemical transformation of betacyanin or enhanced extractability at higher temperatures. These findings are consistent with the observation that freeze-drying preserves texture and minimizes shrinkage, unlike sun and oven drying, which cause considerable shrinkage (Hamid and Mohamed Nour 2018).

3.3 Nutritional Characteristics

The assessed nutrients included mineral levels, oxalates, and the total contents of phenolics and flavonoids in various dried samples of beetroots. The results of these analyses are presented in Tables 3, 4, and 5.

3.3.1 Mineral Content

Table 3 shows the mineral content of fresh and dried beetroot samples (calcium, iron, magnesium, zinc, and phosphorus). The study found a significant increase ($p < 0.05$) in the calcium content after drying, with the highest level of calcium reported in oven-dried boiled Crimson Globe and sun-dried boiled Detroit Dark Red samples. These results are supported by Asante et al. (2024), who found that boiling before drying enhanced calcium content. Iron also significantly increased ($p < 0.05$) in boiled-dried samples, with the highest levels in boiled and sun-dried samples, consistent with the findings of Joshi and Mehta (2010). Additionally, there was a significant increase in the levels of magnesium and zinc in boiled dried samples, particularly in oven-dried boiled samples, which aligns with the results of Alassane et al. (2022). Furthermore, fresh samples had higher phosphorus content than dried samples ($p < 0.05$), contrary to the findings of Asante et al. (2024).

Table 2 Colour characteristics of the crimson globe and detroit dark red beetroot varieties

Treatment	L* (Lightness)		a* (Redness)		b* (Yellowness)	
	DetR	CrimG	DetR	CrimG	DetR	CrimG
FB	34.55±0.07 [b]	35.65±0.35[b]	7.0±0.42 [c]	8.55±0.42[b]	8.2±0.14[c]	9.25±0.70[b]
FDR	35.40±0.40 [a]	38.10±0.20[a]	11.50±0.40[b]	12.40±0.00[a]	7.80±0.10[f]	8.85±0.10 [c]
FDB	34.40±0.20 [b]	38.70±0.20 [a]	15.10±0.40 [a]	14.80±0.10[a]	7.65±0.10[f]	9.15±0.10 [b]
ODR	36.00±0.11 [a]	38.60±0.20 [a]	6.50±0.00 [c]	6.20±0.30[c]	10.30±0.00[a]	11.65±0.20[a]
ODB	34.60±0.20 [b]	37.40±0.10 [a]	2.70±0.10 [f]	2.70±0.20[g]	8.60±0.00[c]	11.40±0.00[a]
SDR	35.60±0.10 [a]	35.50±0.10 [b]	7.20±0.40 [c]	6.90±0.30[c]	9.70±0.00[b]	10.30±0.10[a]
SDB	32.50±0.20 [c]	32.80±0.08[c]	4.70±0.20 [d]	4.40±0.00[f]	8.05±0.10[c]	8.30±0.00[c]

Values are presented as means ± standard deviation from three independent experiments, different superscript letters in the same column denote significant differences ($p < 0.05$), here DetR (Detroit Dark Red), CrimG (Crimson Globe), FDR (Freeze-Dried Raw), FDB (Freeze-Dried Boiled), ODR (Oven-Dried Raw), ODB (Oven-Dried Boiled), SDB (Sun-Dried Boiled), SDR (Sun-Dried Raw), FB (Fresh Beetroot)

Table 3 Mineral content of the processed crimson globe and Detroit dark red beetroot products

Treatment	Ca mg/100gDW		Fe mg/100gDW		Mg mg/100gDW		Zn mg/100gDW		P mg/100gDW	
	DetR	CrimG	DetR	CrimG	DetR	CrimG	DetR	CrimG	DetR	CrimG
FDR	72.65±2.55^{f}	103.89±6.5^{d}	2.94±0.9^{d}	5.71±0.5^{d}	144.36±2.8^{c}	130.23±10.8^{d}	4.62±0.92^{c}	6.63±0.4^{b}	146.02±1.9^{f}	105.56±4.5^{f}
FDB	117.97±1.3^{c}	105.31±5.9^{d}	5.72±0.6^{b}	5.10±1.0^{f}	171.35±27.4^{b}	147.96±5.28^{c}	6.21±0.2^{b}	5.90±0.1^{c}	167.68±4.8^{c}	149.92±1.6^{d}
F	100.74±3.3^{d}	124.24±1.4^{a}	4.19±0.2^{c}	7.23±0.8^{b}	183.03±17.8^{a}	183.73±11.9^{a}	3.83±0.7^{d}	4.58±0.2^{d}	213.24±5.1^{a}	240.69±13.9^{a}
FB	118.74±9.9^{c}	110.52±5.1^{c}	6.36±2.0^{b}	5.47±0.3^{d}	154.12±1.4^{c}	143.72±2.7^{c}	6.26±0.4^{b}	4.35±0.6^{d}	217.09±1.7^{a}	184.92±6.4^{b}
ODR	117.35±2.4^{c}	98.81±3.6^{f}	4.55±0.3^{c}	5.72±0.4^{d}	166.31±21.4^{b}	154.43±23.2^{b}	5.23±0.1^{b}	5.77±0.1^{c}	211.19±4.4^{a}	148.37±1.2^{a}
ODB	143.89±4.1^{a}	227.25±8.5^{a}	6.10±0.1^{b}	6.68±0.7^{c}	195.50±18.9^{a}	190.10±29.3^{a}	6.83±0.3^{a}	8.72±0.3^{a}	192.55±4.58^{b}	197.72±9.46^{b}
SDR	126.10±4.8^{b}	118.64±7.5^{b}	8.49±0.3^{a}	7.54±0.3^{b}	131.58±22.8^{d}	113.10±6.9^{f}	6.14±0.3^{b}	5.35±0.2^{c}	168.97±7.85^{c}	153.01±0.4^{c}
SDB	165.19±3.8^{a}	128.10±8.4^{a}	9.25±0.7^{a}	9.51±0.7^{a}	153.17±18.1^{c}	129.63±20.1^{d}	5.95±0.1^{b}	4.61±0.1^{d}	166.80±5.6^{c}	158.86±2.7^{c}

Values are presented as means ± standard deviation from three independent experiments, different superscript letters in the same column denote significant differences ($p < 0.05$), DetR (Detroit Dark Red), CrimG (Crimson Globe), FDR (Freeze-Dried Raw), FDB (Freeze-Dried Boiled), ODR (Oven-Dried Raw), ODB (Oven-Dried Boiled), SDB (Sun-Dried Boiled), SDR (Sun-Dried Raw), F (Fresh Non-Boiled Beetroot), FB (Fresh Boiled Beetroot)

Table 4 Total phenolic and flavonoid content of the crimson globe and detroit dark red beetroot powder

Treatment	Total phenolic content mg GAE 100mg^{-1}) DW		Flavonoid content (mg CE 100 mg^{-1}) DW	
	DetR	CrimG	DetR	CrimG
FDR	0.53±0.00^{b}	0.41± 0.01^{d}	0.46±0.01^{g}	0.42±0.01^{f}
FDB	0.74±0.08^{a}	0.68±0.02^{b}	1.84±0.02^{a}	1.00±0.02^{a}
ODR	0.78±0.01^{a}	0.56±0.02^{c}	0.98±0.02^{c}	0.75±0.02^{d}
ODB	0.55±0.02^{b}	0.48±0.01^{d}	0.56±0.01^{d}	0.88±0.01^{c}
SDR	0.50±0.03^{b}	0.35±0.03^{f}	0.57±0.04^{d}	0.45±0.03^{f}
SDB	0.51±0.02^{b}	0.80±0.04^{a}	0.63±0.03^{f}	1.01±0.02^{a}

Values are presented as means ± standard deviation from three independent experiments, different superscript letters in the same column denote significant differences ($p < 0.05$), here DetR (Detroit Dark Red), CrimG (Crimson Globe), FDR (Freeze-Dried Raw), FDB (Freeze-Dried Boiled), ODR (Oven-Dried Raw), ODB (Oven-Dried Boiled), SDB (Sun-Dried Boiled), SDR (Sun-Dried Raw), F (Fresh Non-Boiled Beetroot)

Table 5 Oxalate content of the crimson globe and detroit dark red beetroot powder

Treatment	Oxalate content (calcium oxalate) mg/100g					
	Insoluble		Soluble		Total	
	DetR	CrimG	DetR	CrimG	DetR	CrimG
FDR	13333.30±721.70[a]	9583.30±721.70[a]	5833.30±721.70[a]	5416.70±721,70[a]	19166.70±721.70[a]	15000.00±1250.00[a]
FDB	10833.30±721.70[b]	5416.70±721.70[g]	4166.70±721.70[b]	5416.70±721.70[a]	15000.00±1250.00[b]	10833.30±721.70[b]
F	7083.33±721.70[c]	7500±721.00[b]	2500±0.7210[c]	1666.67±721.70[c]	9583.33±721.69[c]	9166.67±721.69[c]
FB	5000±721.70[g]	4166.67±721.67[d]	1666.67±721.70[f]	1250.00±721.00[b]	6666.67±721.67[f]	5416.67±721.69[f]
ODR	6666.70±721.70[d]	4583.30±721.70[f]	1666.70±721.70[f]	1666.70±721.70[c]	8333.30±1443.40[d]	6250.00±721.70[d]
ODB	7083.30±721.70[c]	6666.70±721.70[c]	2500.00±0.00[c]	2500.00±721.70[b]	9583.30±721.70[c]	9166.70±721.70[c]
SDR	7083.30±721.70[c]	7500.00±721700[b]	2083.30±721.70[d]	1666.70±721.70[c]	9166.70±721.70[c]	9166.70±721.70[c]
SDB	5416.70±721.70[f]	5000.00 ±721.70[h]	2500.00±721.60[c]	1666.7±721.70[c]	7916.70±721.70[h]	6666.70±721.70[g]

Values are presented as means ± standard deviation from three independent experiments, different superscript letters in the same column denote significant differences ($p < 0.05$), here DetR (Detroit Dark Red), CrimG (Crimson Globe), FDR (Freeze-Dried Raw), FDB (Freeze-Dried Boiled), ODR (Oven-Dried Raw), ODB (Oven-Dried Boiled), SDB (Sun-Dried Boiled), SDR (Sun-Dried Raw), F (Fresh Non-Boiled Beetroot), FB (Fresh Boiled Beetroot)

3.3.2 Total Phenolic Content (TPC)

The processing methods significantly impacted the total phenolic content (TPC), with the highest levels of TPC reported in raw oven-dried beetroot powder for dark red and boiled sun-dried samples for light red beetroot powder (Table 4). These findings are consistent with those of Guldiken et al. (2016), who observed a 36% increase in TPC in dried red beetroot compared to fresh samples. However, it differs from the results of Youssef and Mokhtar (2014), who noted a reduction in TPC during drying.

3.3.3 Total Flavonoid Content (TFC)

The treatment methods significantly affected TF content ($p < 0.05$). The highest TF content was found in boiled freeze-dried dark red samples and boiled sun-dried crimson globe samples (Table 4). The influence of drying methods on flavonoid content has been extensively researched. Mandale et al. (2023) underscored the importance of drying methods in preserving nutritional quality; these findings are also consistent with those of Liu et al. (2021).

3.3.4 Oxalates

Table 5 displays the oxalate content of beetroot powders. The total oxalate content varied significantly among treatments and varieties, with the highest levels found in raw freeze-dried samples. Oxalic acid, known to chelate metal cations, can contribute to the formation of kidney stones (Holmes and Assimos 2004; Weaver et al. 2006). The observed oxalate levels are higher than those reported by Wruss et al. (2015), which aligns with the typically high oxalic acid content found in beetroots (Duke 2001).

Conclusion

The study showed that beetroot is a rich source of essential minerals such as iron, zinc, phosphorus, magnesium, calcium, phenolic compounds, and flavonoids. These nutrients are important for physiological functions and overall health. The research also found that how beetroot powder is prepared and dried significantly affects these properties. Specifically, the treatment methods can protect or enhance the physical and micronutrient properties, especially iron, while potentially reducing phytochemicals affecting iron bioavailability. This understanding is crucial for developing iron-rich beetroot products that can effectively supplement dietary iron intake, especially for adolescent school children.

Conflict of interest

The authors declare that there is no conflict of interest concerning the publication of this research.

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Clonal propagated 'Ek Pothi Lehsun' as a potential antifungal agent against *Candida* sp.

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ABSTRACT

'Ek Pothi Lehsun', also known as snow mountain garlic, is a type of garlic grown in the high mountainous region of Jammu and Kashmir state of India. The present study aimed to develop a protocol for propagating snow mountain garlic *in-vitro* using corm seed as an explant. The study also assessed the antifungal potential of in vitro-grown bulbils against different *Candida* species. Four different concentrations of NAA and 2,4-D were tested for their effectiveness in promoting root formation, and eighteen different combinations of BAP (µM), KN (µM) and TDZ (µM) were investigated for effective proliferation of shoots with varied lengths. Shoot with maximum length (5.03±1.40) was obtained in MS medium containing 1.0 µM TDZ after 24 days of inoculation, whereas MS basal media was found effective for rooting plantlets. Rooted micro shoots were acclimatized successfully in hardening treys with a percent survival of nearly 80%. Seven different concentrations of Sucrose, i.e. 5%, 7%, 10%, 15%, 17%, 20%, and 25% were investigated for effective bulbil formation. Bulbil with a maximum diameter of 0.86 cm was obtained in 20% sucrose-containing MS media in 5 days. Further, the antifungal potential of aqueous extract (TC-SMG) of *in vitro* grown bulbils was investigated against three *Candida* sp. A zone of inhibition of 22.30±0.33 mm, 17.3±0.33 mm and 19.3±0.33 mm was observed against *C. albicans*, *C. tropicalis* and *C. glabrata* respectively, by using 200 mg/mL extract after 24 hrs depicting the remarkable potential of TC-SMG as an antifungal agent. *In vitro* culture of snow mountain garlic has demonstrated promising antifungal properties against *Candida* species.

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1 Introduction

For millennia, plants have served humanity as sources of beneficial drugs, food, flavouring agents, colourants, binders and lubricants. They have been used to develop and maintain various communities and cultures' physical, psychological and spiritual health (Davis and Choicy 2024). *Allium* is one such plant genera with many valuable medicinal properties. Several health benefits of this plant species have already been documented in Charaka Samhita, one of the oldest Indian medicinal treatizes (Devi et al. 2014). *Allium* has been recognized as one of the initial instances of the plant to be utilized for medicinal purposes. This genus includes many economically important crops like garlic, onion, and other ornamental species (Devi et al. 2014). *Allium sativum* (garlic) is a valuable bulbous crop of this genus, widely used as a spice/ condiment throughout India. It is a natural antibiotic and a remedy for various physical ailments (Parekh and Chanda 2007; Papu et al. 2014). Earlier, various civilizations, viz., Egyptian, Phoenicians, Greek, Indian, Roman, Babylonian, and Chinese, have demonstrated this plant species to be used for curing many ailments such as heart disorders, arthritis, pulmonary disorders, uterine growths, skin disease, symptoms of ageing, diarrhoea, headache, worms and tumours. Egyptians have been demonstrated to provide garlic to the labour force involved in heavy pyramid construction work (Woodward 1996; Sasi et al. 2021).

'Ek pothi lehsun', commonly known as snow mountain garlic or 'Kashmiri garlic', is a type of garlic found to grow in mountainous regions of the Himalayas at an altitude of 1800 meters from MSL. Solid cloves of snow mountain garlic are the cold, hardy corm seeds developed from the elephant garlic, planted in September or October and harvested in the summers. If left in the field, each clove miraculously transforms into complete elephant garlic in the following year. Earlier, mountaineers were found to use it to enhance their energy levels and remove toxins in extreme environmental conditions (https://specialtyproduce.com/produce/Kashmiri_Garlic_13356.php).

Candida sp. is an opportunistic fungal pathogen of the human oral-gastrointestinal tract. These pathogenic species have been recognized as one of the most prevalent reasons for nosocomial infections in patients (Lemar et al. 2002). Therefore, it is also known as "the disease of the diseased" (Al-Dorzi et al. 2020). Antifungal agents such as flucytosine and amphotericin B (AmB) are conventionally used to treat such infections (Kim et al. 2012). However, with the increase in fungal resistance to conventional medicines and the side effects of using these medicines, especially in immune-compromised patients, there is an urgent necessity to hunt for new sources of alternative medicines. Various studies have indicated the hidden power of garlic (*A. sativum*) as an alternative source of antimicrobial properties (Bayan et al. 2020). It has been found to contain a compound named 'allicin' containing diallylsulphide and thiosulfinate, which has been responsible for its antimicrobial potential (Rounds et al. 2012; Heshmati et al. 2010). Elephant garlic extracts, like other *Allium* species, have also been found to contain eight different thiosulfinates responsible for their antimicrobial activity (Huang and Ren 2013). Snow mountain garlic is a variant species of *Allium*. It has been considered a survival strategy for elephant garlic in various abiotic and biotic stress conditions. Therefore, it might be reasonable to systematically study the effectiveness of snow mountain garlic against various *Candida* sp.

However, the long life cycle and low productivity have made this plant species less acceptable among the farmers. Further, the necessity of environmental stress conditions has restricted this crop plant to the farmers of particular habitats. Therefore, in the present study, an attempt has been made to establish an *in vitro* propagation protocol of this plant species to reduce its life cycle so that increased commercial demand can be satisfied without the requirement of environmental stress conditions followed by an assessment of the effectiveness of water extract of *in vitro* grown bulb (TC-SMG) against different *Candida* species.

2 Materials and Methods

2.1 Collection of plant material and sterilization

Certified snow mountain garlic corm seeds were obtained from Srinagar, Jammu & Kashmir, India. Collected seeds were kept in running tap water for three hours followed by surface sterilization in three steps for establishment of *in vitro* tissue culture of snow mountain garlic which involved sterilization of corm seeds with ethanol (70% (v/v)) for 120 seconds, mercuric chloride (0.04% (w/v, 60 seconds) followed by extensive washing for 4–5 times, with autoclaved distilled water in order to remove the remaining traces of sterilizing agents and then transferred to MS medium having 3% sucrose as carbon source (Morales et al. 2006).

2.2 Establishment of aseptic culture of snow mountain garlic using corm bud as explant

In-vitro culture of snow mountain garlic was established using corm seeds containing axillary bud as a source of explants (Figure 1). For this, the thick coat of each of these seeds was removed using a scalpel blade to expose the bud. After this, seeds were shifted to MS medium augmented with 3.00% (w/v) sucrose, 0.78% agar, and numeral concentrations of plant growth regulators, i.e., 6-benzylaminopurine (BAP), thidiazuron (TDZ) and kinetin (KN) for effective shoot multiplication. Agar-agar was added to the culture medium after setting the pH (5.80). The medium thus prepared was transferred to various culture vessels for effective sterilization in an autoclave at a pressure of 15 psi for 15 min. A Laminar air chamber was used to inoculate explants on a sterilized medium under aseptic conditions. All the inoculated cultures were then kept at a

Figure 1 A) Cloves of Snow Mountain Garlic B) Peeled cloves exposing the axillary bud

temperature of 22 ±2°C, humidity of 70–80%, and in photoperiod of 16/8 h light/dark in the culture room containing optimal light intensity (40 µmolm^{-2}s^{-1}) using white fluorescent lamps (cool). Data was recorded 25 days after inoculation.

2.3 Effect of different auxins on root induction from explant

Four different concentrations of NAA (1, 1.5, 3.0, 4.0 µM µM) and 2,4 D (1, 1.5, 3.0, 4.0 µM) were investigated for effective root formation. For this, shoots in the early stages of development were transferred to MS medium containing different concentrations of NAA and 2,4 D.

2.4 Effect of different concentrations of Sucrose on bulb formation

Seven different Sucrose concentrations, 5%, 7%, 10%, 15%, 17%, 20%, and 25%, were studied for effective bulb formation, keeping the standard 3% sucrose concentration as control.

2.5 Hardening and acclimatization of *in vitro* grown plants

Hardening of *in vitro* grown plants of snow Mountain Garlic was carried out in hardening trays. For this, *in vitro* grown plants of 80 days were taken out from tubes and inoculated into hardening media consisting of a mixture of cocopeat and vermicompost in the ratio of 1:1. Before transferring, the plants were given treatment of a fungicide bavistin (0.04%), followed by thorough washing to remove traces of bavistin. Plants were kept in a hardening chamber. Frequent water sprays were given to maintain humidity.

2.6 Preparation of aqueous extract of in vitro grown garlic bulbils

Aqueous extract of in vitro developed garlic bulbils was prepared using the method given by Suleria et al. (2012). Seed bulbs from *in vitro* grown plants were collected and weighed (5.0 g), followed by their thorough homogenization with double distilled water to obtain fine garlic juice. The homogenized mixture was filtered through 2-3 layers of muslin cloth. The resultant aqueous garlic extract was passed through Whatman^{TM} grade 1 filter paper. The recovered filtrate was freeze-dried and stored at 4°C until further use. Different concentrations of garlic extract were prepared by diluting it with sterile water (Suleria et al. 2012). Prior to each antifungal assay, aqueous extract was filtered through a Whatman^{TM} 0.22µm PVDF membrane filter.

2.7 Analysis of the antifungal activity of aqueous extract of Snow Mountain Garlic against different *Candida* species

Antifungal potential of aqueous extract of in vitro grown snow mountain garlic was assessed through agar well diffusion assay. Three species of *Candida* viz., *Candida glabrata*, *C. tropicalis*, and *C. albicans* were obtained from MTCC (Microbial type culture collection), CSIR- IMTECH, Chandigarh, India. Procured fungal cells were grown on Yeast Malt Agar (YMA) at 30 °C and sub-cultured 2-3 times to confirm the purity and viability of cells. Single colony of each species was activated in YM broth for 4-5 hrs (OD was 0.4- 0.5). Each activated pathogen inoculum was spread on YM agar containing petridishes using a sterile L-shaped spreader and allowed to dry at room temperature for 2-3 minutes. Wells of 6mm diameter were made by using a cork borer. Six different concentrations (200, 100, 80, 60, 40, 20 mg/mL) of garlic extract were dispensed in the holes, followed by incubation at 30 °C for 24 and 48 hrs, respectively. The negative control well was poured with sterile MQ water. The zone of inhibition diameter was measured with an antibiotic zone scale after 24 and 48 hrs for each selected pathogenic strain, respectively.

2.8 Minimum Inhibitory Concentration (MIC)

The MIC value of TC-SMG (aqueous extract) was analyzed using the Tholen et al. (2004) protocol with slight modifications. This study used six different concentrations viz., 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 mg/mL of in vitro grown tissue culture (TC-SMG extract were prepared by serial dilution). Twenty microliters of each concentration was dispersed in 80 µL of YM broth, followed by

100 µL of cell suspension inoculums with 0.003-0.004 OD at 600 nm, which was added to the 96-well plate. Two wells of 100 µL of YM broth and fungal cell suspension were kept as a positive control. Plates were incubated at 30 °C, and OD was measured at 600 nm at 24 and 48 hours using an ELISA microplate reader (Thermofischer, USA).

2.9 Statistical analysis

All the experimental results were presented as the mean ± standard error (SE), and all experiments were performed in triplicate. Independent t-test was used in SPSS 17.0 (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL) to determine the significance of the results. A probability (p) value of ≤ 0.05 was treated significantly for ANOVA and the marked correlations between the numeral assays.

3 Results

Sterilized cloves of snow mountain garlic were transferred to MS medium supplemented with different combinations of auxins and cytokinins. Germination started within 10-12 days in explants of snow mountain garlic.

3.1 Effect of different cytokinins on shoot induction from explant

Hard coat of sterilized cloves of Kashmiri garlic was removed carefully and transferred to MS medium containing distinct concentrations of BAP (µM), KN (µM), and TDZ (µM). A total of 18 various compositions of three selected cytokinins were studied to assess their effect on shoot proliferation of varied lengths. MS basal without the addition of any growth hormones was used as a control. Single shoot was found to develop in all the investigated hormones. In the case of BAP and KN, the highest length of shoot, i.e., 1.36±0.23 cm and 1.43±0.133 cm, was witnessed in a medium containing 5.0 µM concentration of each respective hormone. However, the maximum length of the shoot (5.03±1.40) was observed in MS medium supplemented with 1.0 µM TDZ by direct organogenesis after 24 days of inoculation, whereas no shoot formation was observed in control. A sudden increase in shoot length was observed in shoots after 15 days of inoculation, which showed further enhancement in length with time. Thus, basal medium (MS) containing 1.0 µM TDZ was selected as the optimum medium for effective shoot proliferation compared to BAP and KN supplemented medium (Table 1).

Table 1 Effect of different concentrations of cytokinins on shoot development

S. N.	Medium Code	Medium composition	No of Shoots	Length of shoots
1.	SMGK0A	MSBM	0.00^{a}	0.00^{a}
2.	SMGK1	MSBM + 1.0 µM KN	0.67 ± 0.33^{c}	0.50 ± 0.05^{ab}
3.	SMGK2	MSBM + 2.0 µM KN	1.00 ± 0.00^{d}	1.43 ± 0.14^{bc}
4.	SMGK3	MSBM + 3.0 µM KN	1.00 ± 0.00^{d}	1.00 ± 0.15^{b}
5.	SMGK5	MSBM + 5.0 µM KN	1.00 ± 0.00^{d}	1.43 ± 0.13^{bc}
6.	SMGK7	MSBM + 7.0 µM KN	0.67 ± 0.33^{c}	0.80 ± 0.44^{ab}
7.	SMGB1	MSBM + 1.0 µM BAP	1.00 ± 0.00^{d}	1.00 ± 0.19^{b}
8.	SMGB3	MSBM + 3.0 µM BAP	1.00 ± 0.00^{d}	1.03 ± 0.08^{b}
9.	SMGB5	MSBM + 5.0 µM BAP	0.67 ± 0.33^{c}	1.36 ± 0.23^{bc}
10.	SMGB7	MSBM + 7.0 µM BAP	0.33 ± 0.33^{b}	0.32 ± 0.33^{a}
11.	SMGT0.5	MSBM + 0.5 µM TDZ	1.00 ± 0.0^{d}	3.77 ± 0.89^{d}
12.	SMGT1	MSBM + 1.0 µM TDZ	1.00 ± 0.0^{d}	5.03 ± 1.40^{e}
13.	SMGT3	MSBM + 3.0 µM TDZ	1.00 ± 0.0^{d}	4.87 ± 0.73^{e}
14.	SMGT5	MSBM + 5.0 µM TDZ	1.00 ± 0.0^{d}	2.30 ± 0.05^{c}
15.	SMGG0.5	MSBM + 5.0 µM TDZ+ 0.5 µM GA3	1.00 ± 0.0^{d}	2.06 ± 0.08^{c}
16.	SMGG1	MSBM + 1.0 µM TDZ+ 1.0 µM GA3	1.00 ± 0.0^{d}	3.17 ± 0.07^{d}
17.	SMGG3	MSBM + 3.0 µM TDZ+ 1.0 µM GA3	1.00 ± 0.0^{d}	3.20 ± 0.52^{d}
18.	SMGG5	MSBM + 5.0 µM TDZ+ 1.0 µM GA3	1.00 ± 0.0^{d}	2.63 ± 0.06^{cd}

MSBM - MS basal medium; Values are in means ± SEM of three determinations; Values with distinctive superscript (little letter set) letters inside a column were altogether distinctive ($p\leq0.05$)

Table 2 Effect of different concentrations of auxins on root development

S. N.	Medium Code	Concentration of NAA (µM)	No of roots (cm)	Length of roots (cm)
1.	SMG0	0.0 µM NAA	4.33±0.33[a]	1.43±0.14[a]
2.	SMG1	1.0 µM NAA	4.00±0.57[a]	1.50±0.11[a]
3.	SMG2	1.5 µM NAA	2.33±0.33[b]	1.27±0.03[ab]
4.	SMG3	3.0 µM NAA	1.33±0.33[c]	0.91±0.02[b]
5.	SMG4	4.0 µM NAA	0.67±0.33[d]	0.58±0.11[c]

Values are means ± SEM of three determinations. The values having distinctive superscript (little letter set) letters inside a column were altogether distinctive ($p \leq 0.05$).

3.2 Effect of different auxins on root and callus induction from explants

Four different concentrations of NAA **(**µM) were studied for effective root formation. Initiation of root development was observed after 10 days of inoculation. A significant variation in the number of roots was observed in a medium supplemented with different concentrations of NAA **(**µM). Among all the studied concentrations of NAA, the highest no of roots (4.0±0.578) with the highest length (1.5±0.11 cm) were found to develop in 1.0 µM concentration of NAA. However, the maximum no of roots (4.33±0.33) was found to develop in the MS basal medium (control) (Table 2).

In the case of 2,4 D, callus formation was observed in all the studied concentrations of 2,4D viz. 1 µM, 1.5 µM, 3 µM and 4 µM (Figure 2).

Based on the above results, MS medium supplemented with 1.0 µM TDZ was selected as the optimum medium for effective shoot and root development and selected further for effective multiplication of plants (Figure 3).

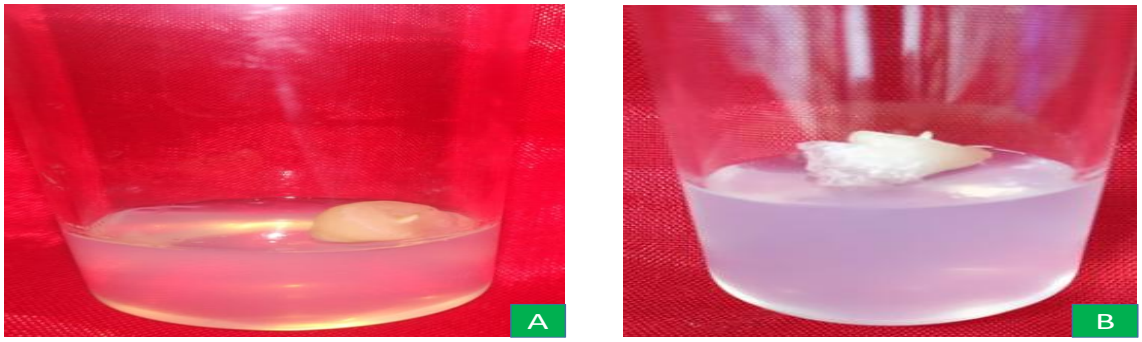

Figure 2 A) Inoculation of explant on MS medium containing 2, 4 D B) Formation of callus (15 days)

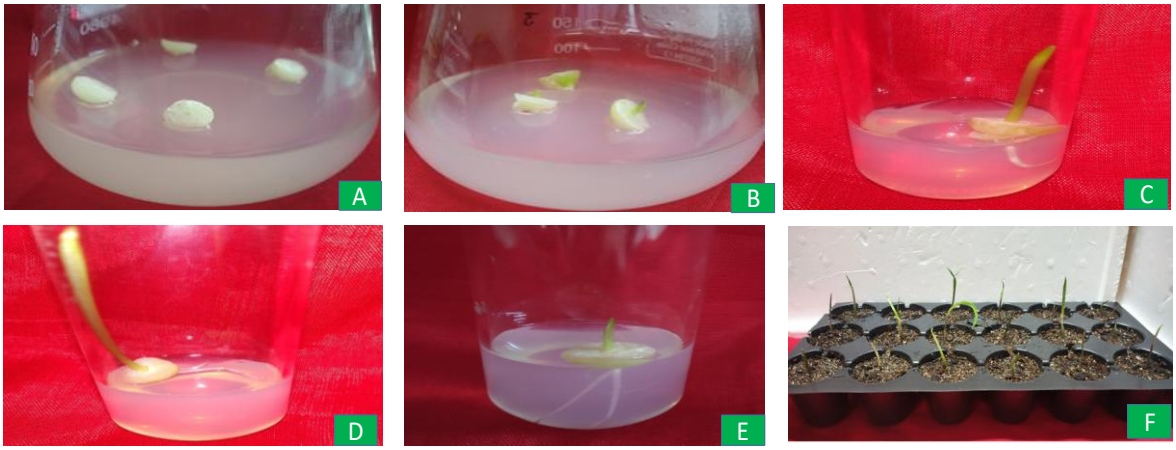

Figure 3 A) Explant transferred to MS medium supplemented with different concentrations of growth hormones B) Initiation of germination (10-12 days) C) Initiation of shoot formation on MS medium supplemented with 1.0 µM TDZ D) Elongation of shoot (15 days) E) Development of roots in MS basal medium F) Hardening of plants in controlled conditions

Table 3 Effect of various sucrose concentrations on bulb formation

S. N.			
1.	3.0	25.33±0.88[d]	0.27±0.008[a]
2.	5.0	14.67±0.67[c]	0.28±0.011[a]
3.	7.0	14.00±0.58[c]	0.49±0.052[b]
4.	10.0	12.33±0.33[b]	0.55±0.014[b]
5.	15.0	5.00±0.58[a]	0.74±0.015[c]
6.	17.0	4.67±0.33[a]	0.84±0.012[d]
7.	20.0	5.00±0.00[a]	0.89±0.012[d]
8.	25.0	5.00±0.00[a]	0.88±0.008[d]

Values are as means ± SEM of three determinations. Values with distinctive superscript (little letter set) letters inside a column were altogether distinctive ($p \leq 0.05$).

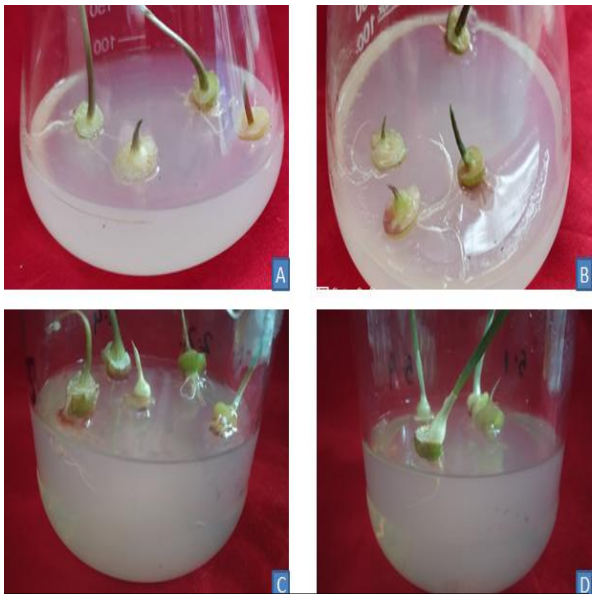

Figure 4A: A) Explant in 3% sucrose B) Explant in 5% sucrose C) Explant in 7% sucrose D) Explant in 9% sucrose

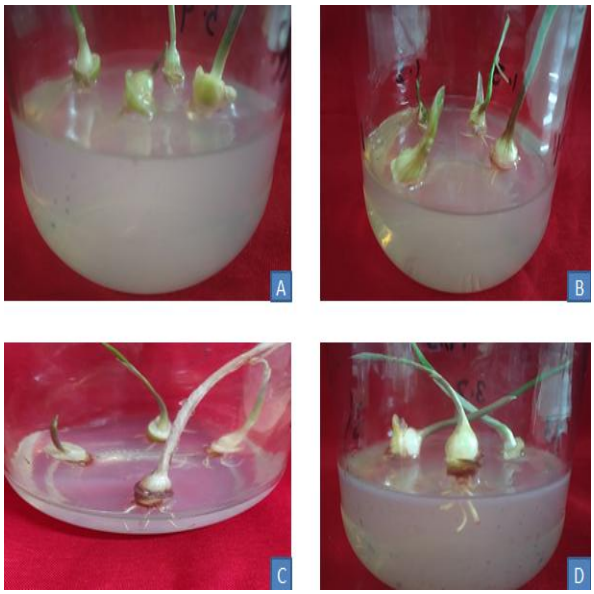

Figure 4B: A) Explant in 15% sucrose B) Explant in 17% sucrose C) Explant in 19% sucrose D) Explant in 20% sucrose

3.3 Effect of different concentrations of Sucrose on bulb formation

Development of corms from shoots can play an important role in their successful acclimatization and *in vitro* mass cultivation. Corms formed under invitro conditions are better adapted to storage conditions. Thus, in this study, six different concentrations of Sucrose in MS medium, i.e., 5, 7, 10, 15, 17, 20, and 25%, were investigated for *in vitro* corm development while 3% sucrose concentration was used as control. The average diameter of bulb was noticed to enlarge with the increment in concentration of Sucrose, whereas the duration of bulb development was found to decrease with the increase of sucrose concentration. A maximum diameter of 0.86 cm was obtained in MS medium supplemented with 20% sucrose in 5 days, while further increase in the concentration of Sucrose has left no further impact on the increase of diameter as well as on-time duration. Further, a diameter of 0.25 cm was observed in MS medium supplemented with 3.0% sucrose (control) after an average duration of 25.33 days (Table 3, Figure 4A-B). Investigation of liquid medium containing optimized concentration of Sucrose, i.e., 20% for effective bulbil formation, has resulted in a further decrease in time duration (3 days) of bulb formation (Figure 5).

3.4 Hardening and acclimatization of *in vitro* grown plants

Rooted plants were hardened and acclimatized in plastic trays in a hardening chamber. A frequent sprinkling of water was carried out to ensure humidity maintenance. Approximately 80% survival of plantlets was observed after 3 weeks of transplantation (Figure 3).

Figure 5 Development of bulbil in liquid MS media supplemented with 20% sucrose (3 days)

3.5 Analysis of the antifungal activity of aqueous extract of Snow Mountain Garlic against different *Candida* species

The agar well diffusion method evaluated the effectiveness of TC-SMG aqueous extract against 3 selected *Candida* species. Six different concentrations of TC-SMG viz., 20, 40, 60, 80, 100, and 200 mg/mL were tested against all the selected pathogens at 24 and 48 hrs, respectively. In the case of *C. albicans*, the maximum zone of inhibition (22.30±0.33 mm) was observed using 200 mg/ml extract after 24 hrs. Further, a non-significant inhibition zone reduction was observed after 48 hrs (Table 4). In the case of *C. tropicalis*, no inhibition was observed by using 20 mg/mL concentration at both time intervals (Table 4). However, the zone of inhibition was found to increase by further increasing the concentration, whereas the maximum zone of inhibition was achieved by using 200 mg/mL of TC-SMG after 24 (17.3±0.33 mm) and 48 hrs (16.3±0.88 mm). Similarly, in the case of *C. glabrata*, no zone of inhibition was observed using 20 mg/mL TC-SMG aqueous extract, and the highest zone of inhibition (19.30±0.33 mm) was reported after 24 hrs (Table 4). In all three

Table 4 Antifungal activity of various concentrations of TC-SMG extracts against selected *Candida sps*

S. N.	TC-SMG Concentration (mg/mL)	Zone of Inhibition (mm) at 24 hours			Zone of Inhibition (mm) at 48 hours			Positive control
		C. albicans	*C. tropicalis*	*C. glabrata*	*C. albicans*	*C. tropicalis*	*C. glabrata*	
1.	20	13.70±0.33^{a}	0.00±0.00^{a}	0.00±0.00^{a}	7.00±3.51^{a}	0.00±0.00^{a}	0.00±0.00^{a}	Growth
2.	40	16.70±0.33^{b}	12.00±0.00^{b}	4.00±4.00^{b}	14.70±0.33^{b}	6.70±3.33^{b}	4.00±4.00^{a}	Growth
3.	60	18.70±0.33^{c}	14.30±0.33^{c}	8.30±4.26^{c}	16.70±0.33^{bc}	12.30±0.33^{c}	7.70±3.93^{b}	Growth
4.	80	19.70±0.33^{c}	15.70±0.33^{d}	15.00±0.58^{d}	17.70±0.33^{bc}	14.30±0.33^{d}	13.30±1.33^{c}	Growth
5.	100	20.70±0.33^{d}	16.30±0.33^{d}	16.70±0.67^{de}	18.70±0.33^{c}	14.70±0.33^{d}	15.00±1.00^{d}	Growth
6.	200	22.30±0.33^{e}	17.30±0.33^{e}	19.30±0.33^{e}	21.00±0.58^{d}	16.30±0.88^{e}	18.70±0.67^{e}	Growth

Values are as means ± SEM of three determinations. Values with distinctive superscript (little letter set) letters inside a column were altogether distinctive ($p \leq 0.05$).

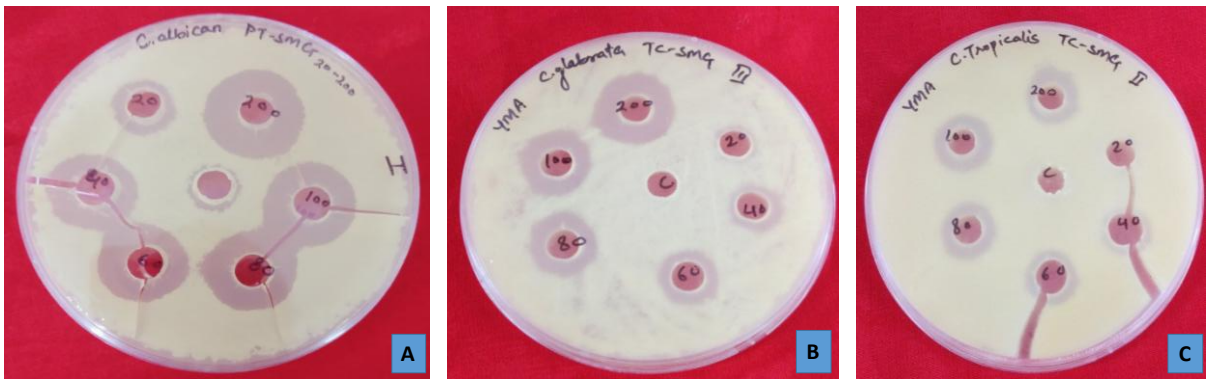

Figure 6 Antifungal effect of different concentrations of TC-SMG after 24 hrs on A) *Candida albicans*, B) *C. glaborata*, C) *C. tropicalis*

cases, depicting its remarkable potential to be used as an antifungal agent, a non-significant reduction in the size inhibition zone was observed after 48 hrs (Figure 6).

3.6 Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) against all the test pathogens was also evaluated in the range of 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 mg/ml. A MIC_{99} value of 3.2 mg/mL was observed for *C. albicans* at 24 and 48 hrs. For *C. tropicalis, a* MIC_{99} value of 1.6mg/mL was observed at 24 hrs, whereas at 48 hrs, it was observed to be 3.2 mg/mL. Further, in the case of *C. glabrata*, MIC_{99} of 6.4 mg/mL was obtained at both 24 and 48 hrs. From these results, it is visible that *C. tropicalis* has more susceptibility towards TC- SMG aqueous extract than the other two pathogens. On the other hand, *C. glabrata* was found more resistant to TC-SMG extract as it has shown the highest MIC amongst all three *Candida* spp.

4 Discussion

For thousands of years, garlic has remained an important herb for human consumption. When antibiotics and other pharmaceutical products did not exist, a bulb of garlic was used to represent the pharmaceutical industry due to its broad spectrum effects (Petrovska and Cekovska 2010). Various clinical investigations have revealed many beneficial effects of garlic, such as (i) reduced risk factors towards cardiovascular disorders, (ii) cancer risk, (iii) enhanced antioxidant and antimicrobial activity, and (v) detoxification of foreign components and liver protection (Aviello et al. 2009; Colín-González et al. 2012). These therapeutical properties of garlic and other members of the genera *Allium* have been attributed to various organosulfur compounds, and the most important sulfurous compound present in intact bulbs is allicin (S-allylcysteine sulfoxide). In addition to this, whole bulbs have also been found to contain several other sulphur compounds such as g-glutamyl-S-allylcysteine (GSAC), S-methylcysteine sulfoxide (methiin), S-trans-1-propenylcysteine sulfoxide, S-2-carboxypropylglutathione and S-allylcysteine (SAC), though some of these are available in much smaller amounts (Amagase 2006). These sulphur-rich compounds have been suggested to be responsible for the various medicinal properties of garlic (Iciek et al. 2009).

Snow mountain garlic is one of the variants found to grow in the mountainous region of Jammu & Kashmir state of India. In ancient times, snow mountain garlic has been observed to be used by various mountain climbers to regain energy and by Greeks to increase the efficiency of athletes participating in Olympics (Sasi et al. 2021). In literature, very little information is available regarding snow mountain garlic. However, for the last few years, an increment in commercial demand for snow mountain garlic has been observed. This increased demand in the market can be attributed to its reduced pungent flavor and beneficial medicinal properties, such as anti-inflammatory, antioxidant, and arthritic properties (Kaur et al. 2024). This garlic is a good source of various minerals, viz., manganese, copper, selenium, phosphorus, and vitamins B6 and C (Kaur et al. 2022).

However, the long life cycle, low productivity, and requirement of long, cold winters have restricted this species to specific areas of the country. A single seed bulb can grow into one plant only, so the availability of a large stock of seed corms is another limitation of its large-scale propagation. Thus, *in vitro* propagation and the development of seed bulbs are important approaches to quickly producing many propagules. In the present study, corm seeds containing axillary bud were used as a source of explant and inoculated on MS medium containing eighteen different concentrations of three selected cytokinins viz., BAP (µM), KN, and TDZ (µM) for efficient shoot proliferation, whereas, basal MS medium was used as control. Maximum shoot length was observed in MS medium supplemented with 1.0 µM TDZ, whereas BAP and KN were less efficient for shoot proliferation. Similarly, Mahajan et al. (2013) reported the lower efficiency of the combinations of BAP and KN for in vitro shoot proliferation compared to individual concentration. However, for the first time, the effect of TDZ has been studied in our investigation, and it has been found more promising than BAP and KN for *in vitro* shoot development.

The long life cycle and development of fewer seed propagules are the key limitations of large-scale cultivation of this species. Therefore, an attempt has been made to develop *in vitro* corm seeds that can be distributed to farmers and can be utilized to satisfy increased commercial demands. For this, different concentrations of Sucrose were applied in MS media. An increase in the bulb's diameter was found with the increase in sucrose concentration. This increase in bulb size with the increase in sucrose concentration can be attributed to the enhancement in the starch and total carbohydrate content (Khokhar 2022). Further, a liquid medium containing an optimized concentration of Sucrose was found to develop corm seed using less medium quantity, depicting the cost-effective *in vitro* development of bulb. It can be attributed to the ease of nutrient uptake resulting in bulbs' development. *Candida* sp has been observed to be able to switch between yeast and hyphae forms, ultimately leading to the formation of biofilms and developing resistance to various antifungal agents (Brand 2012). Since ancient times, plants have been renowned as natural sources of medicine. Several plant species viz., *Osmium sanctum* (Khan et al. 2010); *Glycyrrhiza glabra* (Martins et al., 2016); *Euphorbia hirta* (Rajeh et al., 2010); *Terminalia bellerica* (Valsaraj et al. 1997) have been tested against various *Candida* sp. However, none of these plant products has reached the marketing stage due to insufficient information and efficacy. Therefore, hunting for new plant species with antifungal properties is an alternative to solve these problems.

In the present investigation, aqueous extract of *in vitro* grown Snow Mountain garlic bulbs (TC-SMG) were studied for their efficacy against three *Candida* sp. viz., *C. albicans*, *C. glabrata*, and *C. tropicalis* after 24 and 48 hrs by agar well diffusion method. Six different concentrations were studied against all the selected pathogens. The diameter of the zone of inhibition was found to increase with the increase in extract concentration in all three cases at both time durations. Further, a non-significant reduction in the diameter of the zone of inhibition was observed after 48 hrs, depicting the remarkable potential of this extract to be used as an antifungal agent against different *Candida* sp, specifically in *C. albicans* where the zone of inhibition was found in all concentrations. *Candida* cidal activity of snow mountain garlic has also been investigated by Kaur et al. (2022). It has been suggested that allicin, the major compound found in garlic, can inhibit the thiol-containing amino acids and proteins, thus resulting in the interference in cell metabolism and ultimately leading to the death of the organism (Ankri and Mirelman 1999; Soliman et al. 2017, Kaur et al. 2023).

Conclusion

Food commodities with medicinal properties have remained popular for curing many health problems since immemorial and are being continued until today. Snow mountain garlic is one such plant with several medicinal properties but with a long life cycle and limited productivity. In the present investigation, an effective micropropagation protocol for multiplication of this plant followed by the development of bulbils under in vitro conditions for a reduction in its life cycle in order to their usage as seeds have been investigated and found that 1.0 µM TDZ and 20gm Sucrose can be a best suitable combination for the in-vitro micropropagation of this garlic. Further, water extract at the 200 mg/ml concentration was found fully effective in suppressing *Candida* species growth completely. It will be interesting to determine the effect of this extract on the molecular level of *Candida* proteins targeted by the extract. Further, this protocol could be used to commercialize the cultivation of this snow mountain garlic. To our knowledge, this is the first investigation of antifungal properties of aqueous extract of *in vitro* grown bulbs of snow mountain garlic against different *Candida* sp., depicting its strong rationale for industrial applications.

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Author Contributions

AS wrote draft preparation and carried out experiments, SS performed the tissue culture experiments, MKP contributed to the collection of samples, OPC supervised project administration and funding acquisition, and SSa supervised the work and edited the manuscript. All authors have seen the draft copy and approved the final version.

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Competing of Interest

There is no competing interest among the authors.

Ethics approval

There is no need for ethics approval as this investigation was unrelated to any animal or human subject.

Consent for publication

All authors have approved the manuscript and agree with its submission to JEBAS.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Principal component analysis of morpho-floral traits in *Oryza sativa* × *Oryza longistaminata* advanced backcross lines of rice

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ABSTRACT

Hybrid rice technology substantially improves the food security of South Asian countries where rice (*Oryza sativa* L.) is a staple food. Several traits contribute to hybrid seed production efficiency, among which stigma exsertion is crucial for enhancing production by facilitating out-crossing pollination. This study evaluated the variation patterns and relative impact of 12 morpho-floral traits on overall variability in advanced backcross lines derived from crosses CRMS 32B cv. *Oryza sativa* and *Oryza longistaminata*. For this study, 290 BC_4F_2 lines were grown during Kharif 2019 in 3 replications using a randomized complete block design (RCBD). Principle component analysis (PCA) was performed on all traits, and the findings revealed 11 principal components (PCs). Out of 11 PCs, the first five displayed eigenvalues exceeding 1, collectively explaining 78.78% of the total variability. PC1, PC2, PC3, PC4, and PC5 contributed 26.36%, 19.94%, 14.22%, 9.81%, and 8.44% of the variation, with eigenvalues of 3.16, 2.39, 1.71, 1.18 and 1.01, respectively. PC1 was predominantly associated with yield-related traits such as panicle length, plant height, grain yield per plant, grains per panicle, and effective tillers per plant. On the other hand, PC2 was mainly associated with outcrossing-related floral traits such as total stigma exsertion percentage, dual stigma exsertion percentage, and single stigma exsertion percentage. However, PC3 and PC4 were associated with both floral and yield-related traits, *i.e.*, days to 50% flowering (DF), days to maturity (DM), plant height (PH), effective tillers per plant (ETPP), spikelet fertility percentage (SFP), grain yield per plant (GYPP) and grains per panicle (GPP). Therefore, PC1, PC2, PC3, and PC4 were major contributors to rice hybrid seed production.

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1 Introduction

Rice (*Oryza sativa* L.) is a major staple crop, serving as the primary food source for over half of the global population and fulfilling more than 21% of the world's caloric requirements (Sathe et al. 2021). The world's population is increasing and is estimated to reach 9.7 billion people by 2050; thus, increasing the productivity of major cereal crops such as rice is urgently needed to keep pace with the population surge (Kumar et al. 2022). Although increased rice yields were successfully achieved by integrating semidwarf genes, the limited genetic diversity among breeding lines has decreased the yield of released rice varieties. It is now crucial to expand the genetic diversity of rice varieties to surpass the yield limit and satisfy the rising demand for rice. Hybrid rice technology has emerged as the most feasible and adaptable option to address this situation, providing a yield advantage of 15-20% over conventional varieties (Qian et al. 2016). However, the major challenge hindering the widespread adoption of hybrid rice is the low seed yield ($\leq$2.5 ton ha^{-1}), which consequently leads to high seed costs, and farmers often purchase hybrid seeds at a relatively high price for every cropping season (Xie 2009). Rice is an autogamous plant that prevents it from being naturally out-crossing; therefore, the most efficient strategy for enhancing hybrid seed production is to create male sterile lines with a high out-crossing rate. The out-crossing rate is affected by multiple traits, including both parents' flowering behavior and morphological traits (Marathi and Jena 2015). Among these traits, the stigma exsertion rate is critical for enhancing out-crossing and ensuring efficient hybrid rice seed production (Zhang et al. 2018). It is a genetic trait that varies among male sterile lines and can be improved through targeted breeding efforts with appropriate donor varieties. Wild rice species serve as valuable repositories for revealing novel variations in flowering and morphological traits and could be utilized to increase the genetic background of elite cultivars (Ramos et al. 2016). Furthermore, these wild rice species exhibit a higher out-crossing rate than cultivated rice, ranging from 3.2% to 70.0%, while some wild rice species, such as *Oryza longistaminata* and *O. rufipogon* exhibit out-crossing rates of up to 100% (Prahalada et al. 2021). Incorporating favorable traits from wild species into elite breeding materials through wide hybridization has been a longstanding strategy (Zeliang and Pattanayak 2013). Although backcross introgression is also a feasible method for incorporating favorable traits from wild species, this method primarily involves the genome of recurrent parents with minimal donor segments and offers advantages for accurately estimating novel genes or QTLs and diversifying existing germplasms (Todorovska et al. 2013; Balakrishnan et al. 2016).

The effectiveness of any breeding program relies on understanding genetic variability to determine appropriate selection strategies for improving targeted traits. Multivariate analysis aids plant breeders in devising these selection approaches (Ravindra et al. 2012). Principal component analysis (PCA) is a widely utilized multivariate statistical tool for compressing, reducing, and transforming data. This approach aims to simplify complex datasets by reducing their dimensionality to a minimum number of components while retaining most of the variance (Rahangdale et al. 2021). Through orthogonal transformation, PCA transforms a set of possibly related variables into a fresh set of linearly unrelated variables, facilitating data simplification and interpretation. PCA is extensively used to assess morphological and physiological traits by analyzing multiple parameters of each individual simultaneously. The eigenvalue of a given principal component signifies the extent of variance in attributes explained by that principal component, making it crucial for trait selection (Singh et al. 2020). Hence, the present research aimed to explore the genetic variations and to identify key factors contributing to the overall variance among selected morpho-floral traits of rice advanced backcross lines using PCA.

2 Materials and Methods

The plant material utilized in this study consisted of 290 BC_4F_2 lines with recurrent parents. These advanced backcross lines (BC_4F_2) were developed through interspecific crossing between the recurrent parent CRMS 32B (maintainer of male sterile line with low stigma exsertion) cv. *O. sativa* and the donor parent *O. longistaminata* (wild rice with high stigma exsertion). The BC_4F_2 lines were evaluated during *Kharif* 2019 at the Research Farm of the Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh. This study site is located in northern India's northern Gangetic Alluvial Plain zone, at latitude 25.18° N and longitude 83.03° E. The material was planted with three replications using a randomized complete block design (RCBD), with each row 3.0 meters long and a 20 × 15 cm spacing. Standard packages and practices were implemented to ensure optimal crop growth and quality. Observations were taken for 12 quantitative traits, which included days to 50% flowering (DF), days to maturity (DM), plant height (PH), effective tillers per plant (ETPP), panicle length (PL), grains per panicle (GPP), spikelet fertility percentage (SFP), grain yield per plant (GYPP), test weight (TW), single stigma exsertion percentage (SSE%), dual stigma exsertion percentage (DSE%) and total stigma exsertion percentage (TSE%). These traits were measured on five randomly selected plants from each line in every replicate. Morphological characterization followed the standard evaluation system (SES) for rice, as the IRRI (2013) outlined. After the data were collected, the mean values for each trait were calculated and subjected to further statistical analysis. PCA was performed to identify key traits that significantly impacted the overall variability, and biplots were generated to visually represent the data effectively. The analysis, including PCA and biplot, was carried out using R software version 4.3.2.

3 Results and Discussion

Principal component analysis is pivotal for identifying the primary contributors to overall variability. It reduces the dimensionality of large datasets by extracting a small set of major independent variables that encapsulate the original variability without sacrificing its integrity (Sharma et al. 2022). PCA was performed on a set of 12 morpho-floral traits observed in 290 advanced backcross lines of rice, and the findings revealed 11 principal components (PCs). The eigenvalues, percentage of variability, and cumulative percentage are provided in Table 1. Eigenvalues represent the variance explained by each principal component, with higher values indicating more significant contributions to variability. Generally, components with eigenvalues >1 are considered essential because they capture more variance than does a single original variable (Brejda et al. 2000). Out of 11 PCs, five PCs displayed eigenvalues exceeding 1, and these PCs collectively explained 78.78% of the total variability. The first principal component (PC1) explained 26.36% of the overall variation with an eigenvalue of 3.16, whereas PC2, PC3, PC4, and PC5 individually accounted for 19.94%, 14.22%, 9.81%, and 8.44% of the variation with eigenvalues of 2.39, 1.71, 1.18 and 1.01, respectively. The results demonstrated that the first five principal components captured a substantial portion of the total variability (78.78%), effectively summarizing the information in the original set of traits. This reduction in dimensionality while retaining most of the variance proved valuable for identifying key traits that influenced the genetic variation among the rice lines. Similarly, Sheela et al. (2020) reported four principal components that explained 72.24% of the total observed variability in rice germplasm.

The analysis of factor loadings revealed that the phenotypic traits having the most significant impact on variation displayed high positive loadings across various principal components (Table 1). In PC1, traits such as PL, PH, GYPP, GPP, and ETPP exhibited positive loadings, indicating that these traits were positively correlated and contributed significantly to the variation explained by PC1. Conversely, the remaining traits displayed negative loadings in PC1, suggesting an inverse relationship with this principal component. Thus, lines with high values of PC1 tended to have longer panicles, taller plants, greater grain yield, more grains per panicle, and more effective tillers. In PC2, the TSE%, DSE%, SSE%, GYPP, and PL traits had more positive loadings than did the other traits, suggesting that these traits, particularly those related to stigma exsertion, were significant contributors to the

Table 1 Eigenvalues, variability percentages, cumulative percentages, and factor loadings of different morph-floral traits across principal components

Principal Components	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11
Eigenvalues	3.163	2.393	1.707	1.177	1.013	0.889	0.494	0.456	0.262	0.234	0.211
Variability Percentage	26.362	19.940	14.224	9.808	8.445	7.405	4.121	3.799	2.187	1.953	1.758
Cumulative Percentage	26.362	46.302	60.526	70.333	78.778	86.183	90.304	94.102	96.289	98.242	100.000
Component matrix	Factor loadings										
DF	-0.556	-0.070	0.666	-0.088	-0.086	-0.320	-0.032	0.006	-0.201	-0.280	0.066
DM	-0.562	-0.104	0.624	-0.023	0.001	-0.400	0.083	-0.020	0.222	0.256	-0.031
PH	0.669	0.203	0.072	-0.415	-0.032	-0.249	-0.129	0.491	0.005	0.003	-0.111
ETPP	0.469	0.245	0.327	0.723	-0.048	-0.026	-0.013	-0.021	0.091	-0.124	-0.250
PL	0.755	0.301	0.107	-0.342	0.023	-0.133	-0.003	-0.261	0.280	-0.172	0.131
GPP	0.564	0.256	0.499	-0.383	-0.064	0.207	0.137	-0.260	-0.222	0.134	-0.145
SFP	-0.255	-0.113	0.587	-0.104	0.098	0.696	0.024	0.210	0.150	-0.063	0.041
GYPP	0.641	0.343	0.356	0.435	0.156	-0.030	-0.076	0.095	-0.116	0.139	0.289
TW	0.005	-0.163	0.008	-0.059	0.976	-0.088	-0.009	-0.038	-0.031	-0.032	-0.073
SSE%	-0.483	0.756	-0.009	-0.068	0.037	0.078	-0.416	-0.084	0.009	0.035	-0.036
DSE%	-0.256	0.785	-0.158	0.020	0.075	-0.044	0.516	0.128	-0.002	-0.046	0.022
TSE%	-0.455	0.881	-0.076	-0.040	0.059	0.036	-0.070	-0.004	0.005	0.005	-0.016

PC- principal component; DF-days to 50% flowering (days); DM-days to maturity (days); PH-plant height (cm); ETPP-effective tillers per plant (no.); PL-panicle length (cm); GPP-grains per panicle (no.); SFP-spikelet fertility%; GYPP-grain yield per plant (g); TW-test weight (g); SSE%- single stigma exsertion%; DSE%- dual stigma exsertion%; TSE%- total stigma exsertion%

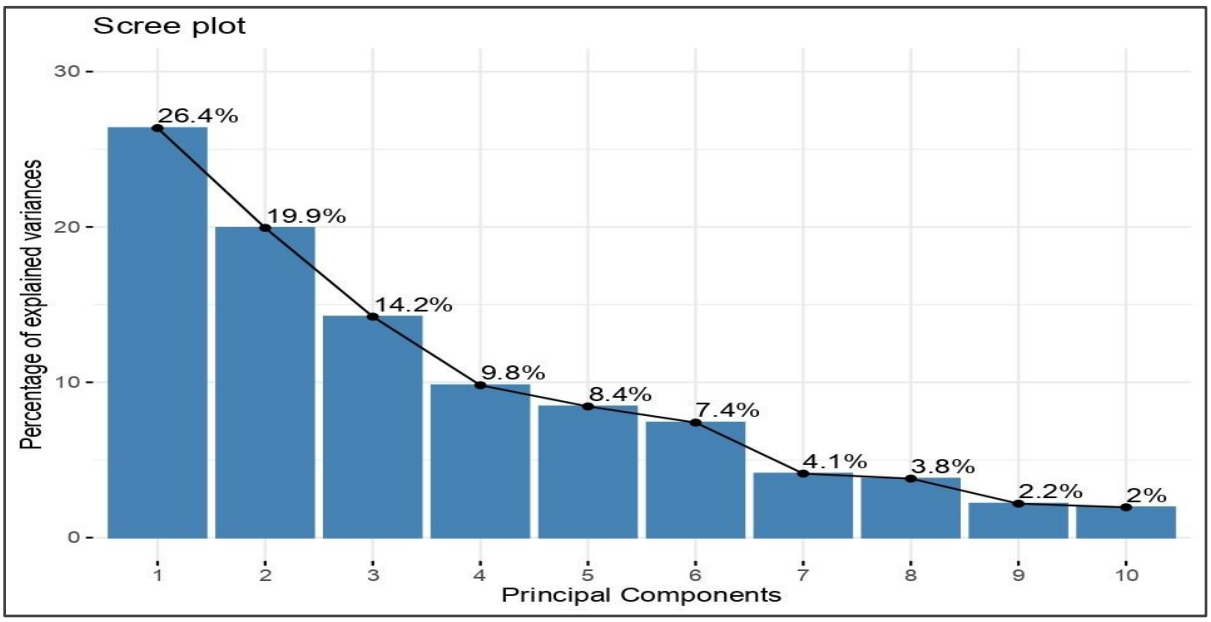

Figure 1 Scree plot visually representing the distribution of variance among principal components.

variability explained by PC2. The DF, DM, SFP, and GPP traits showed high positive loadings in PC3, indicating that these traits were important for the variability captured by PC3. On the other hand, the ETPP, GYPP, and TW traits showed substantial positive loadings in PC4 and PC5, respectively. This suggests that the ETPP and GYPP traits were key contributors to the variability explained by PC4, whereas TW was the primary contributor to the variability explained by PC5. These results demonstrated the complex interplay of various morpho-floral traits contributing to the overall genetic variability among the rice advanced backcross lines. The scree plot in Figure 1 illustrates the variance distribution across principal components, with PC1 displaying the highest variability (26.36%), followed by a decreasing trend in variability for the subsequent PCs. This indicates that PC1 captured the most significant variation in the dataset, followed by diminishing contributions from the subsequent principal components.

The interpretation of the rotated component matrix demonstrated that every principal component is distinctly loaded with various morphological and floral characteristics. The contributions of 12 traits to the first five PCs are detailed in Table 2. PC1 was primarily influenced by yield-related morphological traits, notably PL (17.998%), PH (14.163%), and GYPP (12.970%). On the other hand, PC2 was primarily contributed by outcrossing-related floral traits, particularly the stigma exsertion traits TSE% (32.402%), DSE% (25.739%), and SSE% (23.884%). PC3 contributed to both floral and yield-related traits, *viz.*, DF (26.019%), DM (22.791%), SFP (20.164%), and GPP (14.562%).

In PC4, the traits ETPP (44.402%), GYPP (16.082%), PH (14.637%), and GPP (12.455%) were prominent contributors. Therefore, PC1, PC2, PC3, and PC4 were key contributors to rice hybrid seed production. PC5 was mainly driven by the TW (94.083%) trait, and similar findings were reported by Bhargava et al. (2023). Overall, the results suggested that yield-related morphological traits played a significant role in PC1, highlighting their potential for increased productivity. Conversely, outcrossing-related floral traits were the major contributors to PC2, reflecting their role in reproductive success and potential for genetic diversity. PC3 and PC4 were influenced by a combination of flowering time-related and panicle characteristics, indicating their importance in determining the developmental and yield-related aspects of the plants. PC5 was influenced mainly by TW, indicating its role in determining grain size and weight. These results are consistent with Riaz et al. (2018), who reported that PC1 and PC2 were predominantly associated with yield-contributing traits, while flowering time-related traits, such as DF and DM, were clustered within the PC3 component.

The biplot is a powerful graphical tool that integrates information from both variables and observations into a single plot, offering a visual representation of the relationships between variables (traits) and observations (advanced backcross lines) in the reduced-dimensional space defined by the principal components. In the biplot, the length and direction of the vectors representing variables indicate the magnitude and direction of their contributions to the principal components, respectively. The clustering of variables in the biplot revealed the patterns of correlation or covariance among traits, while the positioning of observations relative to variable vectors provides insights into the performance of individual lines across various traits. A biplot between PC1 and PC2 was created using different morpho-floral

Table 2 Contribution of morpho-floral traits to the variation observed in the first five principal components

Traits	PC1	PC2	PC3	PC4	PC5
DF	9.784	0.203	26.019	0.651	0.726
DM	10.000	0.450	22.791	0.047	0.000
PH	14.163	1.730	0.304	14.637	0.104
ETPP	6.965	2.511	6.276	44.402	0.229
PL	17.998	3.790	0.666	9.949	0.051
GPP	10.057	2.737	14.562	12.455	0.403
SFP	2.050	0.535	20.164	0.925	0.953
GYPP	12.970	4.908	7.415	16.082	2.415
TW	0.001	1.111	0.003	0.292	94.083
SSE%	7.377	23.884	0.005	0.394	0.134
DSE%	2.079	25.739	1.459	0.034	0.557
TSE%	6.556	32.402	0.336	0.133	0.344

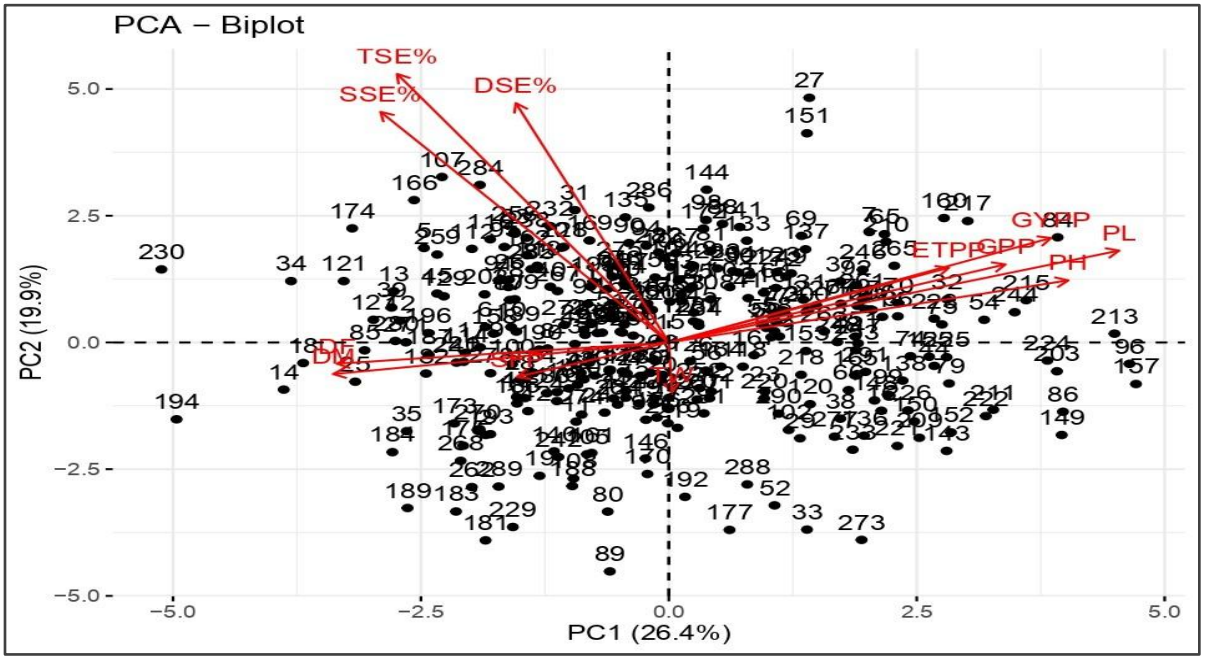

Figure 2 Biplot between PC1 and PC2 using different morpho-floral traits of rice advanced lines

traits, as depicted in Figure 2. The results indicated that traits showing positive correlations were clustered together, while those with negative correlations were positioned on opposite sides of the plot origin. The PL, GYPP, and PH traits had greater vector lengths (distance from the origin) in PC1, while the TSE%, DSE%, and SSE% traits had greater vector lengths in PC2, indicating a good representation of these traits compared to other traits. Figure 2 also illustrates the distribution and diversity of both variables in the advanced backcross lines. Overall, PC1 and PC2 accounted for 46.30% of the total variability. Among the traits, TSE% exhibited the greatest vector length, suggesting its substantial contribution to the overall divergence, followed by SSE%, DSE%, PL, GYPP, PH, and GPP. Advanced backcross lines adjacent to the trait vector within the same quadrant were more likely to exhibit favorable performance for the corresponding traits. Lines 27, 151, 107, 284, 213, 84, 230, 157, 96, 194, and 89 significantly contributed to the overall diversity, owing to their high PC scores and considerable distance from the origin of axes. Therefore, rigorous selection methods can be devised to expedite the enhancement of the aforementioned morpho-floral traits in advanced backcross

populations of rice. Similarly, Rahangdale et al. (2021) identified superior genotypes for yield and quality traits by focusing on high PC scores in their PCA.

Conclusion

PCA was employed to assess the contribution of different morpho-floral traits to overall variability. The analysis revealed that the first five PCs collectively accounted for an average of 78.78% of the total variability. The important traits (PL, PH, GYPP, TSE%, DSE%, and SSE%) grouped within PC1 and PC2 helped explain maximum variability and displayed a tendency to cooccur consistently. Therefore, this study provides valuable insights into identifying parameters contributing to variability and selecting suitable lines for breeding, facilitating crop improvement for morpho-floral traits. Furthermore, the investigated advanced backcross population exhibited notable segregation and diversity in morphological and floral characteristics, suggesting its potential for mapping genomic regions associated with yield and out-crossing (stigma exsertion) traits.

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Declaration of competing interest

The authors declare that they have no competing interests related to this study.

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Exploring intra-allelic and inter-allelic gene interactions influencing seed yield and its components in inter-varietal crosses of Mungbean (*Vigna radiata* (L.) Wilczek)

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ABSTRACT

Mungbean (*Vigna radiata* (L.) Wilczek) is a versatile legume widely cultivated for its nutritional value and adaptability. Meeting the increasing global demand for nutritious food requires the development of high-yielding varieties. Therefore, understanding the inheritance of yield and component traits is crucial for defining effective breeding strategies. In the present study, we aimed to investigate the genetic effects and interactions governing inheritance through generation mean analysis. The four crosses *viz.*, IPM409-4×VGG18-002, IPM409-4×WGG42, COGG13-39×VGG16-058 and COGG13-39×VGG18-002 and their five generations (P_1, P_2, F_1, F_2, and F_3) were evaluated for nine yield and yield component traits during 2023 summer season. The significance of additive, dominance, and epistatic components *viz.*,additive×additive [*i*] and dominance×dominance [*l*] of each trait was found to be different among all the crosses. Mungbean is a self-pollinated crop, so only fixable gene effects can be exploited for trait improvement. In the IPM409-4×VGG18-002 cross, all the traits exhibited additive or additive × additive gene action except for plant height and seed yield per plant (dominance). The scaling test was significant in IPM409-4×WGG42 cross for all the traits, except for the number of pods per cluster. Except for the number of branches per plant in which the dominance effect was evident, additive or additive×additive gene effects were observed for the other traits. In COGG13-39×VGG16-058 and COGG13-39×VGG18-002 crosses, all the yield traits recorded fixable (additive and additive×additive) gene effects except for number of pods per plant in COGG13-39×VGG18-002. Considering the results of all four crosses, gene actions that exhibit consistency across crosses revealed that epistatic interaction (additive×additive) significantly influenced the expression of various mung bean traits. Therefore, the

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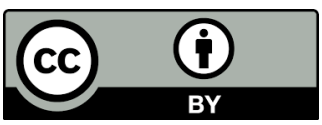

later generation selection of short-duration segregants with high yield, bold seeds, and resistance to yellow mosaic disease from the above populations can be carried out to develop commercially valuable mung bean varieties.

1 Introduction

Mungbean (*Vigna radiata* (L.) Wilczek) is a dynamic crop in Asia, serving significant roles as a food and economic resource. It is consumed in various forms, including whole or split seeds, flour, or sprouts, and serves as a valuable protein source (24 %). Additionally, mungbean offers substantial amounts of dietary fiber (64%), calcium (13%), magnesium (47%), and other vitamins and minerals (Shanthala et al. 2020). Despite its nutritional and economic value, the yield and productivity of mungbean in India remain unstable and volatile, leading to a broadening gap between demand and supply (Varma and Vishwanath 2023). Consequently, developing high-yielding varieties is crucial to meet the growing demands.

Conventional breeding methods in mungbean primarily involve hybridization followed by selection. Adopting an appropriate breeding strategy to enhance selection efficiency is essential based on understanding the gene action related to the yield and yield component traits. These traits are governed by complex gene actions, *viz.*, intra-allelic (additive and dominance) and inter-allelic interactions (epistasis), which are strongly influenced by environmental factors. Additive gene action resulting from the cumulative effects of many alleles can be effectively fixed through selection (Singh and Mahama 2023). Conversely, exploiting dominant gene action through hybrid development is impractical in mungbean due to the self-pollinating nature of the crop and the challenges in maintaining heterosis. Epistasis, or the interaction between genes at different loci, can significantly influence trait expression and complicate the estimation of additive and dominance effects. Although the epistatic component is of low magnitude, it should not be overlooked, as it can lead to biased estimates of additive and dominance variance. The presence or absence of epistasis can be identified through scaling tests. The application of the biometrical tool, generation mean analysis, is crucial for accurately delineating the nature of gene effects and the types of epistasis involved in the trait expression (Yadav et al. 2017). This method involves evaluating parents, F_1, and segregating generations to partition the genetic variance into its constituent components. By doing so, researchers can identify the contributions of additive, dominance, and epistatic effects to the overall phenotype. This approach assists in determining the most appropriate breeding strategy for enhancing various yield-contributing traits in mungbean.

Therefore, the present investigation uses generation mean analysis to estimate the additive, dominance, and epistatic effects of the yield and yield contributing traits in the intra-specific crosses of mungbean. This knowledge will enable the development of mungbean varieties with stable and enhanced yields, ultimately bridging the gap between demand and supply.

2 Materials and Methods

Five mungbean genotypes including IPM 409-4, COGG 13-39, VGG 16-058, VGG 18-002 and WGG 42 were used to generate four segregating populations *viz.*, IPM 409-4 × VGG 18-002, IPM 409-4 × WGG 42, COGG 13-39 × VGG 16-058 and COGG 13-39 ×VGG 18-002. These crosses were decided based on the contrasting nature of the parents for duration and yield attributes. Details of the parental source and characteristics are presented in Table 1. The field experiment was conducted at the Department of Pulses, Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The

Table 1 Parent materials involved in the study with their characteristic features

S. No.	Genotypes	Source	Special Characteristics
		Female parents	
1.	IPM 409-4	ICAR-Indian Institute of Pulses Research, Kanpur	Variety: Early duration (<28 days to 50 % flowering), Shiny seeds, short pod (pod length < 8 cm)
2.	COGG 13- 39	Department of Pulses, Tamil Nadu Agricultural University, Coimbatore	Breeding line: Shiny seeds, high yield
		Male parents	
3.	VGG 16-058	National Pulses Research Centre, Vamban, Tamil Nadu Agricultural University, Coimbatore	Breeding line: Shiny, bold seeds (test weight > 5g/100 seeds) and resistant to yellow mosaic disease
4.	VGG 18-002	National Pulses Research Centre, Vamban, Tamil Nadu Agricultural University, Coimbatore	Breeding line: Shiny, bold seeds (test weight > 5g/100 seeds), long pod (pod length > 10 cm)
5.	WGG 42	Regional Agricultural Research Station, Warangal, Professor Jayashankar Telangana State Agricultural University, Hyderabad	Variety: Shiny seeds, bold seeds (test weight > 5g/100 seeds), long pod (pod length > 10 cm)

Table 2 Details of population size of all the generations of four crosses

S. No.	Crosses	Population size				
		P_1	P_2	F_1	F_2	F_3
1.	IPM 409-4 × VGG 18-002	10	10	10	48	89
2.	IPM 409-4 × WGG 42	10	10	10	99	253
3.	COGG 13- 39× VGG 16-058	10	10	10	84	210
4.	COGG 13- 39× VGG 18-002	10	10	10	152	179

crossing program was carried out in 2022 summer to synthesize hybrids of the above four crosses. During *Kharif* 2022, F_1s were raised, and the true hybrids were morphologically confirmed based on contrasting traits between parents. The true hybrid plants of F_1s were harvested individually, and the F_2 populations were raised during *rabi* 2022 to procure the seeds for F_3 generation.

2.1 Evaluation of Genetic Materials

The four crosses' five generations (P1, P2, F1, F2, and F3) were raised during the summer of 2023 in a row length of 4 m at a 30 × 10 cm spacing. Standard cultivation practices were strictly adhered to ensure a healthy crop. Nine biometrical traits, *viz.*, plant height, number of branches, number of clusters, number of pods per cluster, number of pods per plant, pod length, number of seeds per pod, hundred seed weight, and seed yield per plant were recorded in all five generations of four crosses (Table 2). The mean values of five generations were used to derive the gene effects.

2.2 Statistical analysis

The scaling test (Mather 1949) was used to test the adequacy of a simple additive-dominance model. The simple additive-dominance model was considered inadequate when either of the scales, *viz.*, C or D, were significant, indicating the presence of epistasis. The generation means analysis with a five-parameter model (Hayman 1960) was performed to estimate residual effect [*m*], additive [d], dominance [*h*], and epistatic components, *i.e.*, additive × additive [*i*] and dominance × dominance [*l*]. The five-parameter model lacks insights into additive × dominance [*j*] interactions, and data analysis was conducted using the TNAUSTAT statistical package (Manivannan 2014).

3 Results and Discussion

The precise knowledge of gene effects governing the expression of yield and yield-contributing traits is essential for formulating an efficient breeding and selection strategy. The generation mean analysis was carried out in the four inter-varietal crosses *viz.*, IPM 409-4 × VGG 18-002, IPM 409-4 × WGG 42, COGG 13-39 × VGG 16-058 and COGG 13-39 × VGG 18-002 to estimate the additive, dominant and epistatic effects influencing yield and yield components. Each parent exhibited distinctive characteristics, such as IPM 409-4 with early duration (<28 days for 50 % flowering), COGG 13-39 being high-yielding (>20 g/singe plant), and male parents *viz.*, VGG 18-002, VGG 16-058 and WGG 42 with bold seeds (test weight >5 g/100 seeds) (Table 3). This indicates that variability in mean performance exists for yield components, suggesting the potential for further improvement.

Among the parents, COGG 13-39 outperformed for traits such as number of branches (2.70), number of clusters (15.70), number of pods per cluster (5.00), number of pods per plant (49.90), and seed yield per plant (23.09 g) (Table 3). The male parent, VGG 18-002 was found to be superior for pod length (10.15 cm), number of seeds per pod (12.40), and hundred seed weight (5.54 g) (Table 3). In F_1 generation, mean performance surpassed that of the parental mean value in IPM 409-4 × VGG 18-002 cross for seed yield per plant (18.85 g) and in IPM 409-4 × WGG 42 cross for number of branches (3.50) indicating potential vigor for yield and branching (Table 3). However, major yield components *viz*., number of clusters, number of pods per plant, pod length and hundred seed weight in all the four crosses (IPM 409-4 × VGG 18-002, IPM 409-4 × WGG 42, COGG 13-39 × VGG 16-058 and COGG 13-39 × VGG 18-002) exhibited intermittent values within the range of parental values (Table 3). In F_2 to F_3 generations, an upward trend was observed in the expression of all traits in IPM 409-4 × VGG 18-002 and COGG 13-39 × VGG 18-002 populations except for number of clusters in IPM 409-4 × VGG 18-002 and number of branches, pod length and number of seeds per pod in COGG 13-39 × VGG 18-002 (Table 3).

In all the four inter-varietal crosses evaluated for nine biometrical traits through scaling tests, at least one of the two scales (C and D) consistently exhibited statistical significance except the number of pods per cluster in IPM 409-4 × WGG 42 and the number of pods per plant in COGG 13-39 × VGG 16-058 (Table 4). The significant deviation of scales from zero indicated the presence of epistasis (non-allelic interaction). The presence of epistatic interactions in mungbean for yield and yield-related traits has been substantiated through various studies and research findings. In mungbean, Lenka et al. (2022) and Nainu et al. (2023) have observed complex genetic interactions influencing traits such as yield per plant, number of pods per plant, seed weight, and other

productivity-related parameters. These interactions play a significant role in shaping the phenotypic expression of traits and are critical considerations in breeding programs aimed at enhancing yield in mungbean varieties.

Table 3 Mean and standard errors for yield and yield component traits in various generations of mungbean crosses

Cross	Traits	Generation				
		P_1	P_2	F_1	F_2	F_3
IPM 409-4 × VGG 18-002	Plant height (cm)	60.69±0.97	58.39±0.80	52.16±1.31	32.33±0.72	40.68±0.98
	Number of branches	2.20 ± 0.13	2.40 ± 0.16	2.40 ± 0.24	2.71 ± 0.12	2.88 ± 0.09
	Number of clusters	14.90 ± 0.72	8.40 ± 0.62	11.60 ± 0.93	10.04 ± 0.42	8.38 ± 0.34
	Number of pods per clusters	4.10 ± 0.28	4.20 ± 0.25	3.60 ± 0.24	3.25 ± 0.08	4.07 ± 0.10
	Number of pods per plant	50.50 ± 4.01	29.80 ± 1.05	31.80 ± 1.11	27.31 ± 1.22	31.78 ± 1.54
	Pod length (cm)	7.05 ± 0.11	10.15 ± 0.13	7.88 ± 0.11	7.65 ± 0.12	7.95 ± 0.08
	Number of seeds per pod	11.50 ± 0.29	12.40 ± 0.29	12.20 ± 0.27	10.74 ± 0.12	10.95 ± 0.10
	Hundred seed weight (g)	3.53 ± 0.11	5.54 ± 0.09	4.71 ± 0.07	4.17 ± 0.07	4.25 ± 0.05
	Seed yield per plant (g)	16.48 ± 1.13	15.97 ± 0.84	18.85 ± 0.86	12.55 ± 0.41	14.39 ± 0.42
IPM 409-4 × WGG 42	Plant height (cm)	60.69 ± 0.97	66.76 ± 2.46	48.67 ± 2.32	30.32 ± 0.60	39.11 ± 0.35
	Number of branches	2.20 ± 0.13	2.10 ± 0.10	3.50 ± 0.22	2.33 ± 0.06	2.29 ± 0.03
	Number of clusters	14.90 ± 0.72	12.90 ± 0.5	14.83 ± 1.62	11.01 ± 0.30	9.28 ± 0.2
	Number of pods per clusters	4.10 ± 0.28	4.40 ± 0.37	4.00 ± 0.37	3.79 ± 0.09	3.92 ± 0.05
	Number of pods per plant	50.50 ± 4.01	30.60 ± 0.69	38.00 ± 4.18	35.61 ± 1.18	29.78 ± 0.81
	Pod length (cm)	7.05 ± 0.11	9.56 ± 0.14	7.18 ± 0.16	7.75 ± 0.07	6.80 ± 0.03
	Number of seeds per pod	11.50 ± 0.29	12.73 ± 0.24	10.06 ± 0.23	10.67 ± 0.09	11.26 ± 0.05
	Hundred seed weight (g)	3.53 ± 0.11	5.02 ± 0.17	4.27 ± 0.10	4.09 ± 0.05	3.44 ± 0.02
	Seed yield per plant (g)	16.48 ± 1.13	15.96 ± 0.75	11.30 ± 2.34	15.42 ± 0.53	13.95 ± 0.22
COGG 13-39 × VGG 16-058	Plant height (cm)	66.54 ± 1.60	58.10 ± 0.74	52.34 ± 1.15	54.99 ± 0.63	54.51 ± 0.51
	Number of branches	2.70 ± 0.26	1.70 ± 0.26	2.10 ± 0.10	3.17 ± 0.09	2.76 ± 0.07
	Number of clusters	15.70 ± 0.98	9.00 ± 0.47	8.10 ± 0.60	9.99 ± 0.48	8.44 ± 0.21
	Number of pods per clusters	5.00 ± 0.15	4.00 ± 0.26	5.00 ± 0.26	4.08 ± 0.09	4.03 ± 0.06
	Number of pods per plant	49.90 ± 2.51	28.80 ± 0.71	29.20 ± 1.70	33.95 ± 2.14	33.88 ± 0.96
	Pod length (cm)	8.04 ± 0.1331	8.84 ± 0.147	8.07 ± 0.22	8.55 ± 0.06	8.41 ± 0.05
	Number of seeds per pod	11.63 ± 0.3	11.53 ± 0.14	11.12 ± 0.29	11.37 ± 0.10	11.19 ± 0.06
	Hundred seed weight (g)	4.13 ± 0.03	5.03 ± 0.05	4.28 ± 0.14	4.51 ± 0.05	4.37 ± 0.04
	Seed yield per plant (g)	23.09 ± 0.9	14.24 ± 0.35	15.47 ± 1.22	15.79 ± 0.38	14.32 ± 0.28
COGG 13-39 × VGG 18-002	Plant height (cm)	66.54 ± 1.6	58.39 ± 0.77	53.50 ± 1.79	64.29 ± 1.02	65.13 ± 0.81
	Number of branches	2.70 ± 0.26	2.40 ± 0.16	2.60 ± 0.22	3.11 ± 0.08	3.42 ± 0.08
	Number of clusters	15.70 ± 0.98	8.40 ± 0.62	14.00 ± 0.63	10.55 ± 0.37	12.14 ± 0.38
	Number of pods per clusters	5.00 ± 0.15	4.20 ± 0.25	3.60 ± 0.22	4.15 ± 0.07	4.96 ± 0.07
	Number of pods per plant	49.90 ± 2.51	29.80 ± 1.05	45.30 ± 3.12	37.45 ± 1.31	45.56 ± 1.25
	Pod length (cm)	8.04 ± 0.13	10.15 ± 0.13	8.60 ± 0.12	8.24 ± 0.04	7.85 ± 0.04
	Number of seeds per pod	11.63 ± 0.30	12.40 ± 0.29	11.87 ± 0.16	11.40 ± 0.06	11.24 ± 0.05
	Hundred seed weight (g)	4.13 ± 0.030	5.54 ± 0.09	4.66 ± 0.09	4.21 ± 0.03	3.96 ± 0.02
	Seed yield per plant (g)	23.09 ± 0.90	15.97 ± 0.48	20.97 ± 0.77	16.35 ± 0.53	16.50 ± 0.39

Mean ± Standard error

Table 4 Scaling test and estimates of genetic parameters for yield and yield contributing traits of mungbean crosses

Cross	Trait	Scale		Parameter				
		C	D	*m*	[*d*]	[*h*]	[*i*]	[*l*]
IPM 409-4 × VGG 18-002	Plant height	-94.39** ± 4.09	-21.32** ± 4.38	32.33** ± 0.72	1.00 ± 0.63	-9.05** ± 3.12	0.48 ± 2.97	97.42** ± 8.53
	Number of branches	1.43* ± 0.72	1.49** ± 0.49	2.71** ± 0.12	-0.10 ± 0.11	-0.65 ± 0.38	-0.95* ± 0.42	0.07 ± 1.26
	Number of clusters	-6.33* ± 2.68	-9.86** ± 1.87	10.04** ± 0.42	3.25** ± 0.48	5.46** ± 1.39	12.01** ± 1.65	-4.70 ± 4.56
	Number of pods per clusters	-2.50** ± 0.70	1.47** ± 0.56	3.25** ± 0.08	-0.05 ± 0.19	-1.95** ± 0.34	-1.50** ± 0.47	5.29** ± 1.05
	Number of pods per plant	-34.65** ± 6.78	-7.82 ± 7.82	27.31** ± 1.22	10.35** ± 2.08	-8.91 ± 4.84	20.14** ± 6.2	35.77** ± 13.10
	Pod length	-2.35** ± 0.54	-0.70 ± 0.44	7.65** ± 0.12	-1.55** ± 0.08	-0.65 ± 0.33	-3.03** ± 0.36	2.20* ± 1.07
	Number of seeds per pod	-5.32** ± 0.85	-1.60* ± 0.63	10.74** ± 0.12	-0.45* ± 0.21	0.42 ± 0.41	-0.72 ± 0.55	4.97** ± 1.34
	Hundred seed weight	-1.82** ± 0.35	-0.39 ± 0.30	4.17** ± 0.07	-1.01** ± 0.07	0.14 ± 0.21	-2.06** ± 0.25	1.90** ± 0.66
	Seed yield per plant	-21.44** ± 2.77	-1.49 ± 2.35	12.55** ± 0.41	-0.5 ± 0.70	-0.71 ± 1.51	-3.57 ± 1.96	26.59** ± 4.62
IPM 409-4 × WGG 42	Plant height	-103.53** ± 5.85	-31.64** ± 3.23	30.32** ± 0.6	-3.04* ± 1.32	-11.22** ± 2.17	-2.23 ± 2.88	95.84** ± 8.04
	Number of branches	-1.97** ± 0.53	0.19 ± 0.24	2.33** ± 0.06	0.05 ± 0.08	0.90** ± 0.21	-0.35 ± 0.28	2.87** ± 0.77
	Number of clusters	-13.43** ± 3.57	-12.71** ± 1.33	11.01** ± 0.3	1.00* ± 0.44	7.17** ± 1.34	8.24** ± 1.69	0.95 ± 5.05
	Number of pods per clusters	-1.35 ± 0.94	-0.39 ± 0.54	3.79** ± 0.09	-1.15* ± 0.23	-0.21 ± 0.34	-	-
	Number of pods per plant	-14.68 ± 10.43	-33.18** ± 5.71	35.61** ± 1.18	9.95** ± 2.04	17.13** ± 4.24	39.58** ± 6.37	-24.67 ± 15.23
	Pod length	0.02 ± 0.48	-4.88** ± 0.27	7.75** ± 0.07	-1.26** ± 0.09	2.13** ± 0.20	0.74** ± 0.27	-6.54** ± 0.75
	Number of seeds per pod	-1.65* ± 0.71	-0.54 ± 0.48	10.67** ± 0.09	-0.62** ± 0.19	-1.98** ± 0.28	-1.15* ± 0.46	1.48 ± 1.01
	Hundred seed weight	-0.73* ± 0.34	-2.97** ± 0.23	4.09** ± 0.05	-0.75** ± 0.10	1.85** ± 0.13	0.36 ± 0.21	-2.99** ± 0.48
	Seed yield per plant	4.61 ± 5.31	-9.55** ± 1.94	15.42** ± 0.53	-0.77 ± 0.68	1.19 ± 1.98	5.60* ± 2.58	-18.88* ± 7.64

Cross	Trait	Scale		Parameter				
		C	D	*m*	[*d*]	[*h*]	[*i*]	[*l*]
COGG 13-39 × VGG 16-058	Plant height	-9.36* ± 3.84	-16.59** ± 2.97	54.99** ± 0.63	4.22** ± 0.88	-0.48 ± 2.00	17.94** ± 2.71	-9.64 ± 6.49
	Number of branches	4.07** ± 0.54	0.31 ± 0.50	3.17** ± 0.09	0.50* ± 0.18	0.37 ± 0.27	1.47** ± 0.41	-5.00** ± 0.83
	Number of clusters	-0.95 ± 2.5	-10.92** ± 1.67	9.99** ± 0.48	3.35** ± 0.54	2.87* ± 1.18	13.82** ± 1.72	-13.30** ± 4.29
	Number of pods per clusters	-2.67** ± 0.71	-1.03* ± 0.43	4.08** ± 0.09	0.50** ± 0.15	0.74* ± 0.31	1.24** ± 0.39	2.18* ± 1.08
	Number of pods per plant	-1.29 ± 9.59	-11.10 ± 6.32	33.95** ± 2.14	10.55** ± 1.30	-2.97 ± 5.12	-	-
	Pod length	1.17* ± 0.55	-0.33 ± 0.31	8.55** ± 0.06	-0.40** ± 0.10	0.05 ± 0.23	-0.39 ± 0.30	-2.00* ± 0.82
	Number of seeds per pod	0.10 ± 0.78	-1.17* ± 0.46	11.37** ± 0.10	0.05 ± 0.17	0.33 ± 0.32	1.89* ± 0.49	-1.68 ± 1.16
	Hundred seed weight	0.32 ± 0.34	-0.70** ± -0.19	4.51** ± 0.05	-0.45** ± 0.03	0.22 ± 0.17	-0.37* ± 0.18	-1.36* ± 0.56
	Seed yield per plant	-5.12 ± 3.03	-11.60** ± 1.67	15.79** ± 0.38	4.43** ± 0.48	3.69** ± 1.35	15.73** ± 1.74	-8.64 ± 4.70
COGG 13-39 × VGG 18-002	Plant height	25.24** ± 5.71	7.01 ± 4.22	64.29** ± 1.02	4.08** ± 0.89	-9.43** ± 3.2	7.68* ± 3.70	-24.31* ± 10.39
	Number of branches	2.15** ± 0.63	2.37** ± 0.46	3.11** ± 0.08	0.15 ± 0.15	-1.18** ± 0.3	-0.93* ± 0.42	0.30 ± 0.97
	Number of clusters	-9.89** ± 2.27	3.35 ± 2.04	10.55** ± 0.37	3.65** ± 0.58	-1.93 ± 1.32	3.42* ± 1.69	17.66** ± 3.97
	Number of pods per clusters	0.21 ± 0.60	2.34** ± 0.42	4.15** ± 0.07	0.4* ± 0.15	-2.53** ± 0.27	-0.73* ± 0.34	2.85** ± 0.90
	Number of pods per plant	-20.51* ± 8.59	27.66** ± 6.27	37.45** ± 1.31	10.05** ± 1.36	-16.41** ± 4.72	-1.76 ± 5.42	64.23** ± 14.95
	Pod length	-2.42** ± 0.36	-3.29** ± 0.25	8.24** ± 0.04	-1.06** ± 0.09	1.3** ± 0.16	-0.32 ± 0.23	-1.16* ± 0.53
	Number of seeds per pod	-2.18** ± 0.58	-1.87** ± 0.48	11.4** ± 0.06	-0.38 ± 0.21	0.73** ± 0.21	0.12 ± 0.42	0.41 ± 0.71
	Hundred seed weight	-2.17** ± 0.24	-2.25** ± 0.16	4.21** ± 0.03	-0.7** ± 0.05	0.96** ± 0.11	-0.27* ± 0.13	-0.10 ± 0.39
	Seed yield per plant	-15.59** ± 2.82	-5.76** ± 2.15	16.35** ± 0.53	3.56** ± 0.51	2.69 ± 1.58	8.36** ± 1.91	13.11* ± 5.16

* & ** Significance at 0.05 and 0.01 probability levels, respectively; C- Scale C; D- Scale D; m- Residual; [d]- Additive; [h]- Dominance; [i]- Additive × Additive; [l]- Dominance × Dominance; (-)- Absence of epistasis

3.1 Gene action

On dissection of the generation mean into five different genetic components, the effect of residual mean was highly significant, indicating variation across the generations in all crosses. For each trait, the significance and relative strength of additive, dominance, and epistatic components *viz.*, additive × additive [*i*] and dominance × dominance [*l*] were different from each other in all four crosses (Table 4). The observed disparity can be attributed to the involvement of genes with varying frequencies and contrasting or synergistic effects in the parental genotypes. Therefore, a targeted selection strategy tailored to specific crosses could be developed for trait improvement, followed by a more generalized strategy. However, as a highly self-pollinated crop, mungbean, only the fixable gene effects, *viz.*, additive and additive × additive, could be exploited for trait improvement. Therefore, in the present study, for deciding the selection criteria, the non-fixable gene effects (dominance and dominance × dominance) were ignored when the fixable gene effects for the traits were significant.

In IPM 409-4 × VGG 18-002 population, various traits such as number of clusters, number of pods per plant, pod length, and hundred seed weight displayed additive and additive × additive gene actions (Table 4). This indicates that the expression of these traits is influenced by individual gene effects (additive) and interactions between genes (epistasis), specifically additive × additive interactions. In contrast, traits like the number of seeds per pod exhibited additive gene action, and the number of branches and pods per cluster exhibited additive × additive gene action (Table 4). These findings suggest that selection strategies for traits exhibiting epistatic interactions should be deferred to later generations to harness these interactions for trait improvement effectively. Previous studies by Latha et al. (2018) and Kanwade et al. (2019) have reported similar genetic findings in mungbean, corroborating the presence of fixable gene effects for traits such as number of branches, number of clusters, number of pods per cluster, number of pods per plant and hundred seed weight. This implies that these traits can be improved through selective breeding efforts targeting additive and epistatic gene interactions. Conversely, plant height and seed yield per plant displayed nonfixable gene effects (dominance and dominance × dominance) (Table 4). Latha et al. (2019) and Lenka et al. (2022) have also reported the predominance of dominance × dominance gene interaction for plant height and Kanwade et al. (2019) and Patel et al. (2012) for seed yield per plant in mungbean. However, in mungbean, non-fixable gene effects cannot be exploited through selection.

The outcomes of the scaling test in the IPM 409-4 × WGG 42 population revealed that epistasis was significant for all the traits except for the number of pods per cluster (Table 4). Notably, additive gene action was observed for the number of pods per cluster, indicating the feasibility of early-generation selection for trait improvement. The additive gene action for the number of pods per cluster corroborates with the findings of Pathak et al. (2014) in mungbean, validating the role of additive effects in shaping this trait. Simultaneously, additive and additive × additive gene effects influenced the inheritance of the number of clusters, number of pods per plant, pod length, number of seeds per pod, and hundred seed weight, while the predominance of additive gene effect for plant height and additive × additive gene effects for seed yield per plant were observed (Table 4) underscoring their complex genetic inheritance. With fixable gene effects and epistasis, the above traits can be improved through selection in later generations. The results of fixable gene effects were reported for plant height, number of clusters, and number of pods per plant by Latha et al. (2018), for pod length by Lenka et al. (2022), and number of seeds per pod and hundred seed weight by Yadav et al. (2017) and Narasimhulu et al. (2018) in mungbean. On the other hand, the absence of significant fixable gene effects for the number of branches indicated that trait improvement through selection is not feasible in this population.

In this study, COGG 13-39 × VGG 16-058 population of mungbean, we investigated the genetic effects influencing various agronomic traits (Table 4). Epistasis was notably present in all traits except for the number of pods per plant, which displayed primarily additive gene action, suggesting its suitability for early-generation selection. This finding is consistent with previous research by Kumar et al. (2015), further supporting the role of additive genetic effects in influencing the number of pods per plant. Whereas, for most traits *viz.*, plant height, number of branches, number of clusters, number of pods per cluster, hundred seed weight, and seed yield per plant were recorded as additive and additive × additive gene effects (Table 4). Further, pod length and number of seeds per pod exhibited additive and additive × additive gene actions, respectively (Table 4). Consequently, the above traits with fixable epistatic gene effects can be effectively improved by postponing the selection process to later generations. This indicates a complex genetic architecture where trait expression is influenced by interactions between alleles at different loci, highlighting the need for strategic breeding approaches. The findings of Pathak et al. (2014) suggested a selection procedure involving delayed selection that fixes the additive × additive genetic effect for the above traits in later generations. The selection procedure of delayed selection, which fixes the additive × additive genetic effect for the number of branches, number of clusters, number of pods per cluster, and hundred seed weight, was also reported by Narasimhulu et al. (2018) and for pod length and number of seeds per pod by Lenka et al. (2022) in mungbean. Kumar et al. (2015) have also reported additive and additive × additive gene actions for seed yield per plant in mungbean. These studies advocate for a

delayed selection strategy that stabilizes additive × additive genetic effects in subsequent generations, providing insights for optimizing breeding programs to improve mungbean productivity traits through genetic manipulation and selection strategies tailored to trait-specific genetic architectures.

In COGG 13-39 × VGG 18-002 population, additive, and additive × additive gene effects were reported for plant height, number of clusters, number of pods per cluster, hundred seed weight, and seed yield per plant (Table 4). The findings of significant fixable genetic components for the above traits in mungbean were in accordance with Latha et al. (2018), Narasimhulu et al. (2018), and Nainu et al. (2023). Whereas, additive gene action was prevalent for expressing the number of pods per plant and pod length, and additive × additive gene action was prevalent for the number of branches (Table 4). Additive × additive gene action for the number of branches (Kumar et al. 2015; Narasimhulu et al. 2018) and additive gene effects for the number of pods per plant and pod length (Alam et al. 2014) were reported in mungbean. The presence of significant additive and additive × additive gene actions suggests that selecting for these traits should be deferred to later generations to exploit their genetic potential fully. This strategic selection delay aligns with current literature recommendations, ensuring stabilization and enhancement of desired traits through cumulative genetic effects over generations. On the contrary, the predominance of dominance gene action in the expression of the number of seeds per pod indicates that trait improvement is not achievable through selection. This finding is corroborated by recent studies of Narasimhulu et al. (2018) and Latha et al. (2019), highlighting the challenge of improving number of seeds per pod through traditional selection approaches due to the nature of non-fixable genetic effects. The complex interplay of additive, additive × additive, and dominance gene actions in trait inheritance is evident. These insights underscore the importance of tailored breeding strategies that leverage genetic interactions to optimize mungbean cultivars for enhanced productivity.

Conclusion

For formulating a selection program for mungbean, emphasis was placed on identifying gene actions that exhibit consistency in most of the crosses. Epistatic interactions played a crucial role in the inheritance of the number of branches, number of clusters, number of pods per cluster, hundred seed weight, and seed yield per plant. However, these traits were under the influence of additive × additive (fixable) gene effects, and hence, improvement through selection in later generations is feasible when desirable recombinants become available. As these populations were derived from early-maturing, bold-seeded, and high-yielding parents, selecting high-yielding segregants, combined with traits such as early maturity and bold seeds, becomes feasible in subsequent generations. In addition, due to the involvement of MYMV-resistant male parents (VGG 16-058), the selection strategy coupled with MYMV screening will be rewarding.

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Conflict of Interest

The authors declare no conflict of interest

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Inheritance pattern of Qualitative traits, Genetic analysis and association of yield attributes in F_2 populations of Rice (*Oryza sativa*)

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ABSTRACT

Understanding the extent of genetic variability within the segregating generations is crucial for identifying superior segregants with high yield and better market acceptability. Thus, the present study was carried out to quantify the extent of genetic variation available in the segregating population of rice. Three crosses, *viz.*, CO 55 × IC 457996, CO 55 × IC 464685, and CO 55 × IC 115439 were evaluated using a non-randomized experimental design for six yield attributing and two physical grain quality traits in F_2 generation. The inheritance pattern of basal leaf sheath colour and grain colour in CO 55 × IC 115439 indicate digenic complementary gene interaction (9:7), whereas grain colour in CO 55 × IC 464685 exhibits inhibitory gene action (13:3). The positively skewed nature of productive tillers per plant and single-plant yield in the F_2 segregants emphasizes the need for intensive selection to facilitate rapid improvement due to the influence of complementary gene action. Moderate to high GCV with high heritability and GAM for traits such as plant height, productive tillers per plant, hundred seed weight, grain width, and single-plant yield in the F_2 segregants underscore the prevalence of additive gene action and thus provide the most effective condition for simple phenotypic selection. Moreover, productive tillers per plant and single-plant yield showed a strong positive association in all the crosses. Therefore, productive tillers per plant can be considered an indicator trait when selecting high-yielding segregants for grain yield improvement.

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1 Introduction

Rice (*Oryza sativa* L.) is a primary dietary food source that sustains half of the world's population. As the world's population is projected to reach approximately 9.7 billion in 2050, the demand for food is expected to surge by 70% (Bin Rahman and Zhang 2021). However, to keep pace with the growing population, the current annual rice production levels are expected to increase by approximately 5.8 million tons (Santhiya et al. 2024). Over the past decades, the annual growth rate of rice yield remained ≤ 1%, failing to keep pace with the escalating demand due to various constraints (Khush 2013). In the present era, the importance of quality parameters is increasingly recognized, particularly in areas where rice production is self-sustaining. This underscores the importance of developing rice cultivars that yield high and exhibit superior quality. In light of the presently recognized resource limitations and changing climatic conditions, boosting rice production through sustainable practices remains a significant challenge. Thus, creating new variability through hybridization is one among several genetic approaches aimed at overcoming the yield barrier and enhancing rice productivity.

Genetic variability is crucial for effectively selecting superior segregants from the segregating population (Thúy et al. 2022). The genetic variability parameters, such as genotypic and phenotypic coefficient of variation, provide an insight into the relative amount of variation present in the segregating generations. However, the knowledge of the degree of heritable proportion of variation from the parent to its progeny is of paramount importance in deciding the traits to be selected (Bassuony et al. 2022). Third and fourth-degree statistics, specifically skewness and kurtosis, offer valuable insights into the type of gene action and the number of genes influencing traits within segregating generations (Fisher et al. 1932; Robson 1956). Therefore, understanding the extent of genetic variability and the distribution pattern of the F_2 segregates would be effective tools to identify superior segregates with high yield and better market acceptability (Bassuony et al. 2022). Grain yield is a complex trait governed by polygenic loci, which in turn depends upon many independent contributing characteristics such as the number of productive tillers (Kalaivani et al. 2023), panicle length (Muthuvijayaragavan and Jebaraj, 2022), panicle weight (Nofal et al. 2024), thousand seed weight (Kalaivani et al. 2023), and filled grains per panicle (Bassuony et al. 2022). Therefore, direct selection for yield is often not effective. A better understanding of their association with single plant yield would be essential for formulating an effective breeding strategy, attributing traits as effective indicators in selection. In this context, the present study was designed to analyze the statistics and quantify the level of genetic variation present within the F_2 (segregating) generation for yield, yield-attributing, and grain quality traits.

2 Materials and Methods

The study was conducted at the Department of Rice, TNAU, Coimbatore, Tamil Nadu, India (11° N latitude and 77° E longitude). Hybridization was carried out between a popular high-yielding fine grain rice variety, CO 55, with three drought-tolerant landraces *viz.*, IC 457996, IC 464685, and IC 115439 during *Rabi* 2022, and the resulting F_1s were evaluated during Summer 2023. True hybrids were distinguished based on their distinct morphological characteristics, such as the pigmentation of basal leaf sheath in CO 55 (green) × IC 115439 (purple) (Figure 1), as well as grain colour in CO 55 (white) × IC 464685 (red) and CO 55 (white) × IC 115439 (red). Subsequently, all identified hybrids were further subjected to molecular confirmation using polymorphic marker RM1 between CO 55 and IC 464685 and RM3412 between CO 55 and IC 115439. Due to the lack of a visual morphological marker in CO 55 × IC 457996, polymorphic marker RM208 was used for identifying the true F_1s between CO 55 and IC 457996 (Figure 1). The heterozygous plants obtained from each cross CO 55 × IC 457996 (cross I), CO 55 × IC 464685 (cross II), and CO 55 × IC 115439 (cross III) were advanced to F_2 generation during *Kharif* 2023. Seedlings were transplanted on twenty-one days, with a single seedling per hill, while maintaining a spacing of 30 cm between the rows and 20 cm between the plants. The crop was grown adopting recommended agronomic practices. Qualitative characteristics like basal leaf sheath colour were individually assessed in the F_2 segregants of cross III. Likewise, grain colour was observed in crosses II and III using a single panicle from each plant. Six yield attributing traits *viz.*, days to first flowering (DFF in days), plant height (PH in cm), productive tillers per plant (NPT), panicle length (PL in cm), hundred seed weight (HSW in g) and single plant yield (GYPP in g) along with two physical grain quality traits, grain length (KL in mm) and grain width (KB in mm) were recorded from 380, 391 and 217 F_2 plants in crosses I, II and III, respectively.

The inheritance pattern for qualitative traits was assessed through the χ^2 (Chi-square) test (Fisher 1936). Shapiro-Wilks' W test was carried out to test the normality of the F_2 populations using R software (Shapiro et al. 1968). Skewness and kurtosis were computed to understand the extent of the distribution pattern for yield and quality attributes in the segregating population by using SPSS Statistics version 29.0.2.0 (Snedecor and Cochran 1989). The Genotypic and Phenotypic Coefficient of Variation (GCV and PCV) were calculated as per the formula given by Burton, 1952. The broad sense heritability (h^2) and genetic advance as a percentage of the mean (GAM) were calculated as per Lush (1940) and Johonson et al. (1955). Pearson's pairwise correlation among variables was computed to analyze their mutual linear relationship using the '*corrplot'* statistical package in R software.

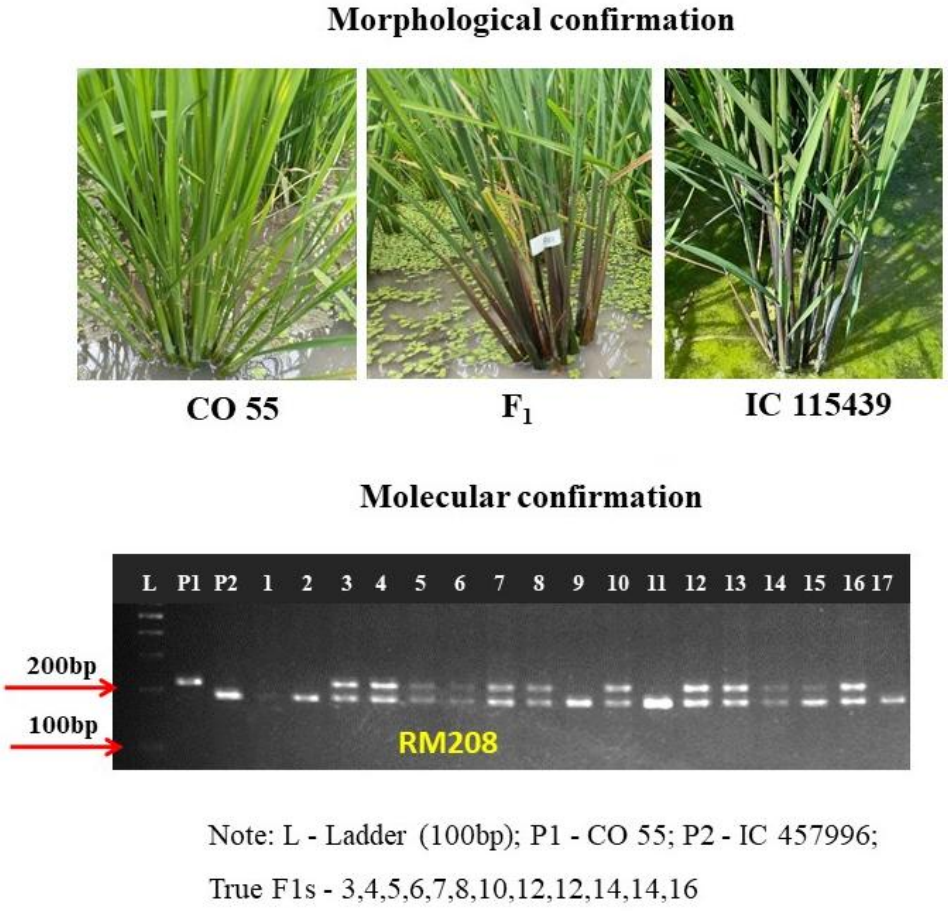

Figure 1 Hybrid confirmation using morphological and molecular markers

Table 1 Segregation pattern of the progenies for qualitative traits in F_2 generation

Basal leaf sheath colour							
Name of the cross	P_1/P_2	F_1 Phenotypes	Number of F_2 plants		χ^2 ratio	χ^2 value	p-value
			Purple	Green			
CO 55 × IC 115439	Green/Purple	Purple	119	98	9:7	0.18	0.6752
Grain colour							
Name of the cross	P_1/P_2	F_1 Phenotypes	Number F_2plants		χ^2 ratio	χ^2 value	p-value
			Red	White			
CO 55 × IC 115439	White/Red	Red	175	42	13:3	0.05	0.8194
CO 55 × IC 464685	White/Red	Red	208	183	9:7	1.48	0.2236

3 Results and Discussion

3.1 Inheritance pattern of qualitative traits

True hybrids were identified by their distinct morphological characteristics, such as the pigmentation of basal leaf sheath in CO 55 × IC 115439 and grain colour in CO 55 × IC 464685 and CO 55 × IC 115439. All true F_1s resulting from CO 55 × IC 115439 exhibited red grain colour and purple pigmentation in their leaf sheath (Figure 1). Likewise, in CO 55 × IC 464685 the true F_1s had red grain colour. Besides conferring disease resistance, basal leaf sheath colour is an important morphological marker for distinguishing off types at an early stage. The segregation pattern of the progenies of the cross between green and purple pigmented

leaf sheath in the F_2 generation closely fitted to the 9:7 ratio (χ^2 = 0.68) (Table 1). The result herein emphasizes that two genes with complementary gene interaction jointly govern the expression of basal leaf sheath colour in rice (Pandey et al. 2016). Likewise, the proportion of plants exhibiting red and white grain colour in F_2 generation of the cross CO 55 × IC 464685 closely followed a 9:7 ratio (χ^2 = 0.22) (Table 1). Thus, it confirms that the red grain colour in rice is determined by digenic complementary gene interaction. Meanwhile, a segregation ratio of 13:3 (red:white) gave the best fit for grain colour in the cross CO 55 × IC 115439 (χ^2 = 0.82) (Table 1). This indicates that the red grain colour in the cross CO 55 × IC 115439 might be due to dominant suppression epistasis, i.e., two genes with an inhibitory effect jointly govern the expression of the trait (Waghmode et al. 2017).

3.2 *Per se* performance of yield-attributing traits

The *per se* performance of the studied traits in the F_2 segregants indicated a wide range of variation (Table 2, 3, and 4), providing ample opportunities to identify and utilize the most promising segregants in future breeding programs. Days to first flowering in the F_2 segregants of the cross I (CO 55 × IC 457996) ranged from 75.00 to 114.00 days (Table 2), while it was from 76.00 to 105.00 days in cross II (CO 55 × IC 464685) (Table 3) and 79.00 to 117.00 days in cross III (CO 55 × IC 115439) (Table 4). Transgressive segregants with lower values than the early parent are desirable for days to first flowering. Nearly 20% of plants in cross I, 27.59% in cross II, and 5.53% in cross III were identified as early flowering compared to their parent CO 55 (86.4 days). Transgressive segregants with higher values than the high-yielding parent are desirable for yield-attributing traits. The transgressive segregants with higher value of productive tillers per plant were more in the F_2 generation of cross I (93 plants) followed by cross III (55 plants) and cross II (23 plants). Similarly, single plant yield in the F_2 segregants of cross I, varied from 8.97 to 74.52 g, while in cross II, it ranged from 8.11 to 61.96g and in cross III, it ranged from 10.08 to 61.86 g. The wide range of variation for these traits in all three crosses underscores the presence of transgressive segregation. The number of plants in F_2 that outperformed the mean single plant yield of parents was 176 in cross I, 116 in cross II, and 96 in cross III. Koide et al. (2019) indicated

Table 2 Genetic variability estimates of yield and quality attributes in the F_2 population of CO 55 × IC 457996

Traits	W-test	Probability	Mean	Range	Skewness	Kurtosis	PCV	GCV	h^2	GAM
DFF	0.981	0.001	95.26	75.00 - 114.00	-0.08	-0.74	9.68	9.59	97.96	19.54
PH	0.968	0.000	147.11	70.00 - 200.00	-0.51	-0.26	15.23	14.81	94.56	29.66
NPT	0.968	0.000	14.15	5.00 - 28.00	0.55**	-0.12	35.18	30.99	77.61	56.24
PL	0.992	0.039	27.27	20.00 - 35.60	-0.03	-0.02	9.82	8.62	76.94	15.57
HSW	0.996	0.501	2.07	1.43 - 2.84	0.09	-0.05	11.96	11.22	87.97	21.68
KL	0.805	0.000	8.40	7.20 - 9.80	0.17	-0.28	6.61	6.54	97.74	13.31
KB	0.247	0.000	2.44	1.90 - 3.80	0.60**	1.52**	10.45	10.08	92.97	20.02
GYPP	0.963	0.000	34.27	8.97 - 74.52	0.70**	0.12	37.39	34.75	86.35	66.52

**1% level of significance; *5% level of significance; DFF - Days to First Flowering; PH - Plant Height (cm); NPT - Productive Tillers per plant; PL - Panicle Length (cm); HSW - Hundred Seed Weight (g); GYPP - Single Plant Yield (g); KL - Grain Length (mm); KB - Grain Breadth (mm); PCV - Phenotypic Coefficient of Variation; GCV - Genotypic Coefficient of Variation; h2 - Heritability in broad sense; GAM - Genetic Advance as per cent of Mean

Table 3 Genetic variability estimates for yield and quality attributes in the F_2 population of CO 55 × IC 464685

Traits	W-test	Probability	Mean	Range	Skewness	Kurtosis	PCV	GCV	h^2	GAM
DFF	0.965	0.000	91.30	76.00 - 105.00	-0.27	-0.22	6.21	6.01	93.82	11.99
PH	0.936	0.000	122.43	80.00 - 184.00	0.43**	-0.98	19.02	18.39	93.50	36.63
NPT	0.960	0.000	12.53	4.00 - 35.00	0.80**	1.77**	36.77	28.81	61.40	46.51
PL	0.973	0.000	26.08	19.00 - 37.00	0.52**	0.99**	10.17	8.32	67.05	14.04
HSW	0.939	0.000	1.76	1.26 - 2.55	0.99**	0.35	17.64	17.05	93.46	33.96
KL	0.972	0.000	8.41	7.00 - 9.60	-0.58	0.38	5.55	5.44	96.36	11.01
KB	0.984	0.000	2.35	1.40 - 3.50	0.11	-0.54	16.48	16.07	95.07	32.28
GYPP	0.908	0.000	28.78	8.11 - 61.96	0.52**	-0.76	38.53	34.06	78.14	62.03

**Significance at 1% level; *Significance at 5% level

Table 4 Genetic variability estimates for yield and quality attributes in the F_2 population of CO 55 × IC 115439

Traits	W-test	Probability	Mean	Range	Skewness	Kurtosis	PCV	GCV	h^2	GAM
DFF	0.981	0.005	100.65	79.00 - 117.00	-0.34	-0.12	7.32	7.19	96.50	14.56
PH	0.976	0.001	138.82	90.00 - 184.00	-0.39	-0.40	13.99	12.70	82.38	23.75
NPT	0.972	0.000	12.28	4.00 - 28.00	0.58**	0.72*	34.73	30.18	75.52	54.04
PL	0.972	0.000	26.76	18.00 - 32.00	-0.40	0.48	8.42	6.75	64.24	11.14
HSW	0.972	0.000	2.05	1.12 - 2.95	0.18	1.40**	14.18	13.70	93.30	27.26
KL	0.980	0.004	8.29	7.10 - 9.80	0.33	0.37	5.04	4.97	97.37	10.10
KB	0.970	0.000	2.90	2.20 -3.80	0.37*	-0.25	10.77	10.39	93.18	20.67
GYPP	0.980	0.004	31.01	10.08 - 61.86	0.45**	0.47	32.05	26.90	70.43	46.50

**Significance at 1% level; *Significance at 5% level

that transgressive segregation is more common in the F_2 population derived from parents with more proximal phenotypes. Among the obtained high-yielding F_2 segregants, 28 plants in cross I, 16 in cross II, and 4 in cross III were found to be high-yielding and early flowering. Therefore, the promising transgressive segregants thus identified offer significant potential for employing them in breeding programs for evolving short-duration high-yielding rice cultivars.

3.3 Skewness and Kurtosis

Shapiro-Wilks test (W-test) revealed that except for hundred seed weight in Cross I, none of the traits exhibited a normal distribution, suggesting the skewed nature of the segregating population (Table 2, 3, and 4). The nature of gene action governing any trait can be identified based on the sign of skewness and its distribution pattern (Fisher et al. 1932; Robson 1956). Cross I noticed significant positive skewness for productive tillers per plant, grain width, and single plant yield (Table 2). Similarly, in cross II, the traits productive tillers per plant, plant height, panicle length, hundred seed weight, and single plant yield (Table 3) were positively skewed (Figure 2, 3). Likewise, productive tillers per plant, grain width, and single plant yield in cross III were identified to have significant positive skewness (Table 4 and Figure 4). The skewed nature underscores the significance of complementary gene action in determining the expression of these traits. Thus, intensive selection is needed to improve the genetic gain of these traits, as mild selection could lead to slow progress (Riyanto et al. 2021; Bassuony et al. 2022). However, the non-significant values for skewness for the remaining traits in the crosses indicate the existence of additive gene action, implying that early-generation selection will be more effective for these traits. Kurtosis indicates the shape of the distribution curve, and it helps determine the number of genes governing the traits. The significant and positive kurtosis observed for traits such as grain width (in cross I), productive tillers per plant, panicle length (in cross II) as well as productive tillers per plant and hundred seed weight (in cross III) indicate their leptokurtic distribution. This suggests that only a few genes are involved in governing their expression. However, the non-significant kurtosis values for the remaining traits in the crosses suggest a mesokurtic distribution, implying that a larger number of genes will likely determine these traits.

3.4 Estimation of variance component

Understanding the extent of genetic variability within breeding materials is essential for optimizing selection processes in any breeding program. Thus, understanding genetic parameters allows breeders to enhance the effectiveness of selection. For all traits investigated across the three crosses, the Phenotypic Coefficient of Variation (PCV) was consistently higher than the Genotypic Coefficient of Variation (GCV) (Tables 2, 3, and 4). However, a narrow difference between PCV and GCV in the F_2 segregants for all the studied traits signifies a pronounced genetic influence on the phenotypic expression with minimal environmental impact. In cross I, PCV values ranged from 6.61% (grain length) to 37.39% (single plant yield), while in cross II, it varied from 5.55% (grain length) to 38.53% (single plant yield). Likewise, in cross III, PCV values ranged from 5.04% (grain length) to 34.73% (productive tillers per plant). Similarly, GCV values in cross I varied from 6.54% (grain length) to 34.75% (single plant yield), while in cross II, they varied from 5.44% (grain length) to 34.06% (single plant yield). Further, GCV values in cross III varied from 4.97% (grain length) to 30.18% (productive tillers per plant). High GCV estimate (>20%) was observed for productive tillers per plant and single-plant yield in all the crosses. High GCV estimates indicate these traits' inherent high degree of variability, which could augur well for rice improvement programs (Riyanto et al. 2021; Kumar et al. 2023). Plant height, hundred seed weight, and grain width in all crosses displayed moderate GCV estimates (10% to 20%). Conversely, the low estimate of GCV (<10%) was observed for days to first flowering, panicle length, and grain length across all crosses. Low GCV estimates indicate the presence of a limited range of variability for these traits within the population (Thúy et al. 2022; Kalaivani et al. 2023; Kumar et al. 2023). Therefore, selection based on these traits is expected to be less effective.

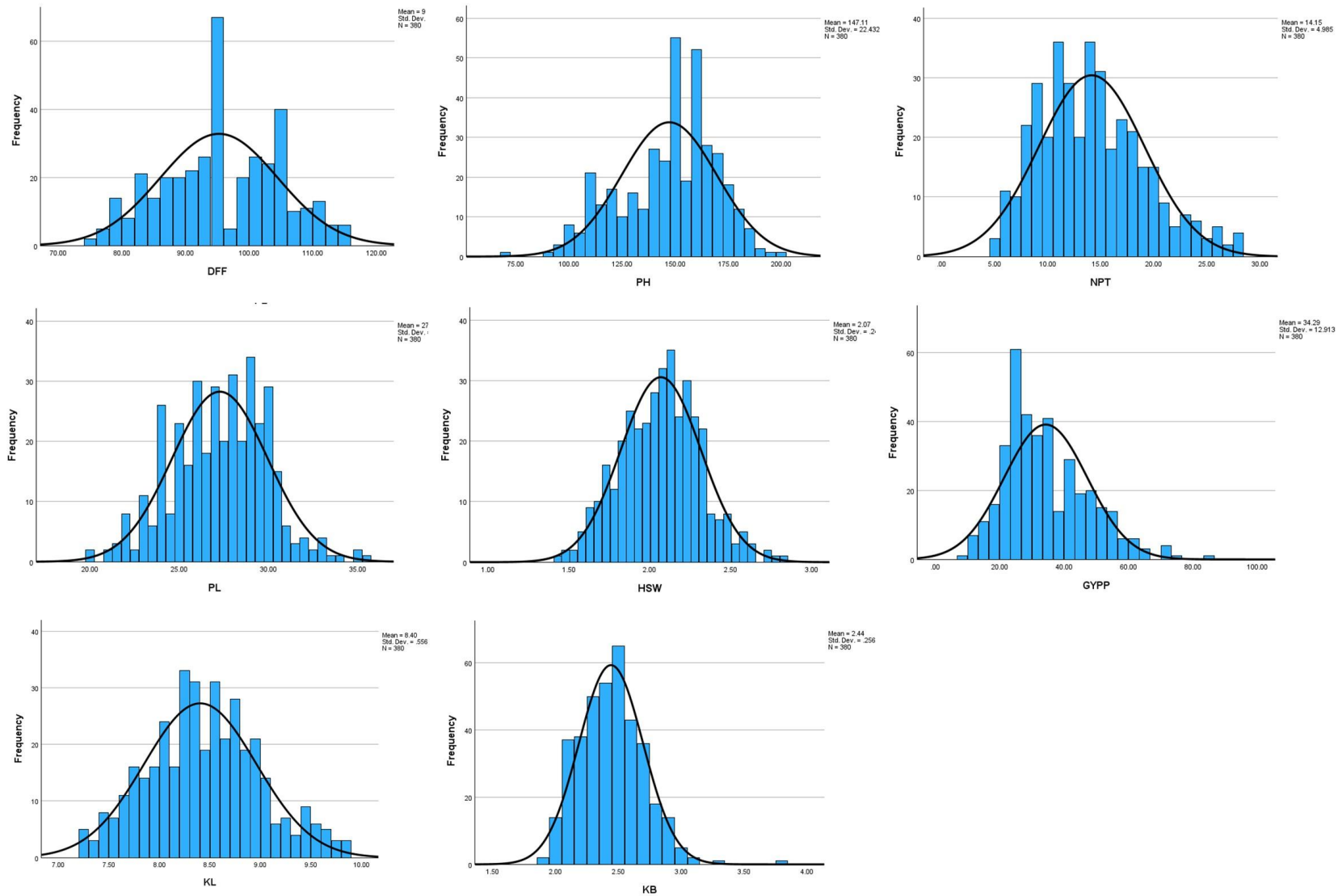

Figure 2 Distribution pattern of yield and quality attributes in the segregating population of CO 55 × IC 457996

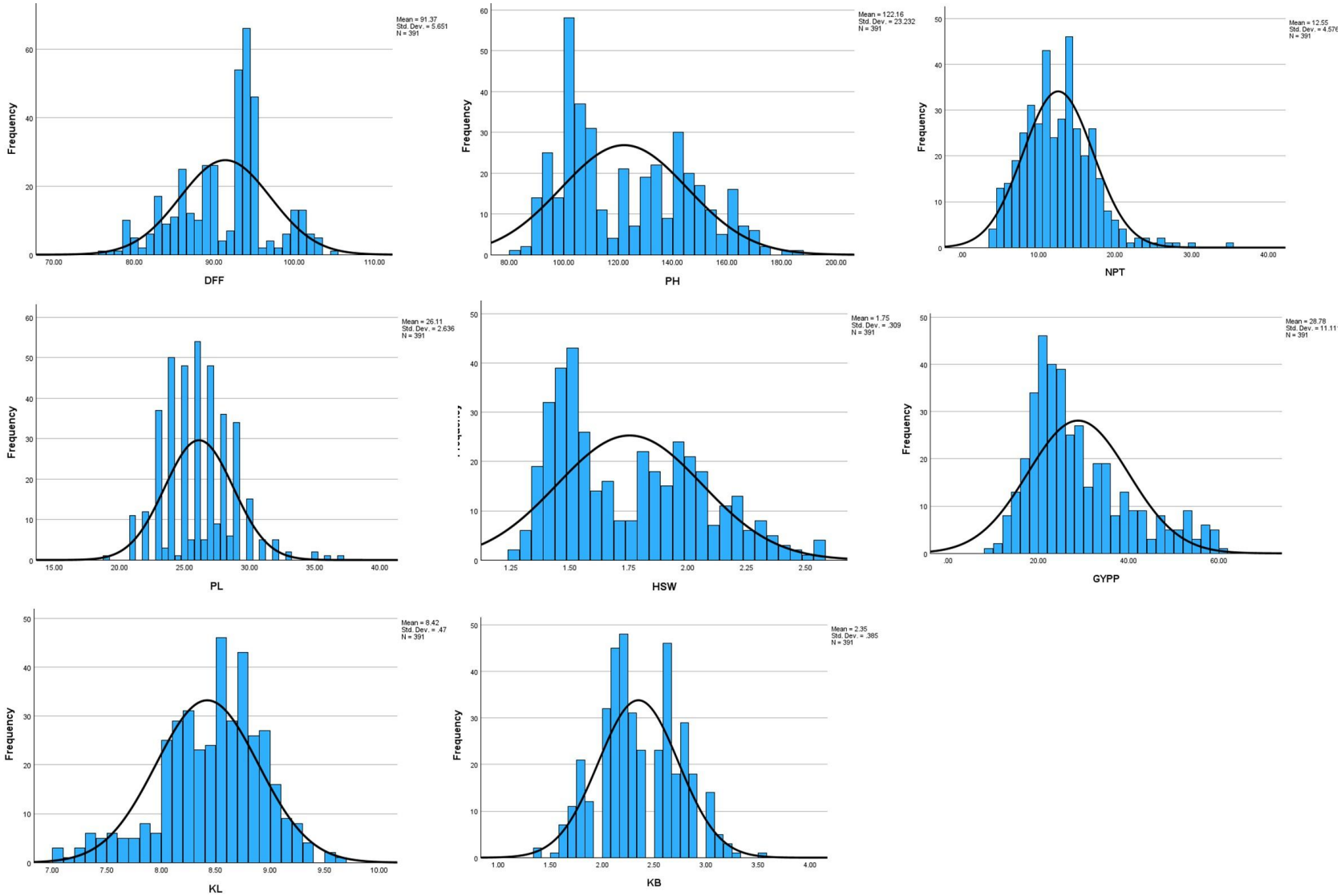

Figure 3 Segregation pattern of yield and quality attributes in the segregating population of CO 55 × IC 464685

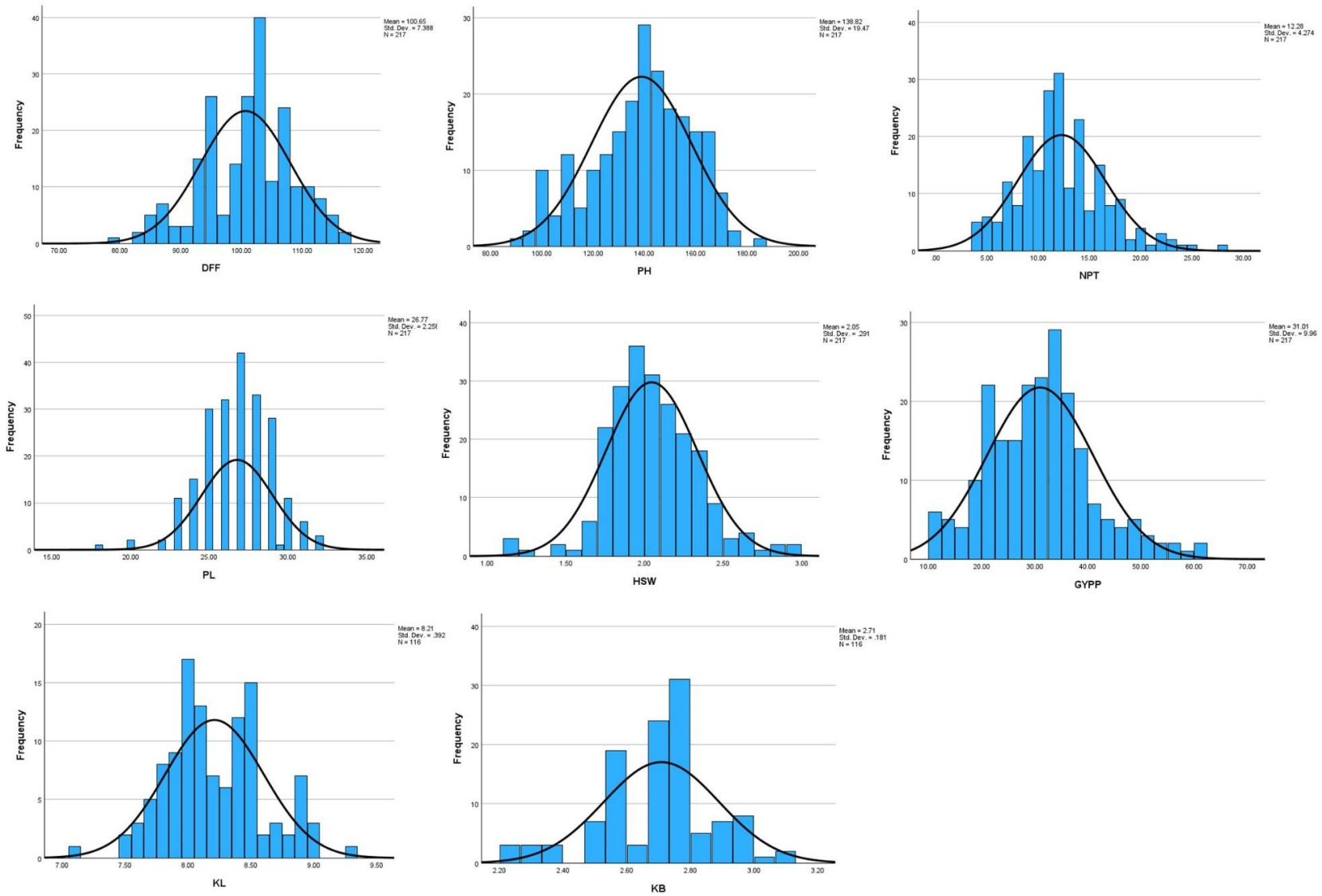

Figure 4 Segregation pattern of yield and quality attributes in the segregating population of CO 55 × IC 115439

3.5 Broad sense heritability and Genetic advance as percent of mean

Although GCV provides insights into the genetic variability present within the population, it alone does not suffice to ascertain the heritable portion of the variation. The efficiency of exploiting the residing genetic variability through selection depends upon the heritability of individual traits. In cross I (Table 2), broad sense heritability ranged from 76.94% (panicle length) to 97.96% (days to first flowering), with genetic advance as a percent of the mean (GAM) varying from 13.31% (grain length) to 66.52% (single plant yield). Similarly, in cross II (Table 3), heritability ranged from 61.40% (productive tillers per plant) to 96.36% (grain length), with GAM ranging from 11.01% (grain length) to 62.03% (single plant yield). In cross III (Table 4), heritability ranged from 64.24% (panicle length) to 97.37% (grain length), with GAM ranging from 11.14% (panicle length) to 54.04% (productive tillers per plant). High broad sense heritability (H^2) observed for all the examined traits across three crosses suggests that environmental factors have minimal influence, with genetic factors predominantly governing these traits. Lestari et al. (2015) suggested that early-generation selection is possible for traits with high heritability as genetic factors predominantly influence the plant phenotype. For effective selection, heritability in consonance with GAM could be useful in predicting the genetic gain under selection (Bassuony et al. 2022). Across all the crosses, high H^2 (>60%) and GAM (>20%) were noticed for single plant yield, productive tillers per plant, plant height, hundred seed weight, and grain width. High broad sense heritability coupled with high GAM emphasis that these traits are predominantly controlled by additive gene action, making them highly amenable to simple phenotypic selection (Prajapati et al. 2022; Kalaivani et al. 2023; Kumar et al. 2023). Furthermore, traits like days to first flowering, panicle length, and grain length exhibited high H^2 (>60%) and moderate GAM (10% to 20%) across all the F_2 segregants. High broad sense heritability coupled with moderate GAM emphasizes that both additive and non-additive gene action play a major role in determining their expression (Yaseen et al. 2020; Prathiksha et al. 2022; Kumar et al. 2023). However, the high heritability in these crosses might be due to environmental factors rather than genotype, rendering selection less rewarding. Consequently, enhancing the genetic gain of these traits can be achieved by intermating superior genotypes within the segregating population.

Table 5 Phenotypic correlation among yield and quality attributes in the segregating population

Traits	Cross	DFF	PH	NPT	PL	HSW	KL	KB
PH	1	0.33**						
	2	0.26**						
	3	0.24**						
NPT	1	0.01	0.24**					
	2	0.11	0.36**					
	3	0.03	0.08					
PL	1	0.37**	0.42**	0.01				
	2	0.23**	0.38**	0.23**				
	3	0.22**	0.38**	0.18**				
HSW	1	0.08	0.11*	-0.01	0.08			
	2	0.15**	0.50**	0.23**	0.11*			
	3	0.00	0.17**	-0.18**	-0.01			
KL	1	0.06	0.03	0.01	0.05	0.29**		
	2	0.06	-0.26**	-0.11*	0.05	-0.27**		
	3	0.13*	-0.08	0.05	-0.02	0.15*		
KB	1	0.04	0.02	-0.07	0.05	0.10	-0.54**	
	2	0.02	0.44**	0.20**	0.03	0.64**	-0.29**	
	3	0.04	0.04	0.04	0.04	0.02	0.13*	
GYPP	1	0.19**	0.42**	0.65**	0.21**	0.12*	0.07	-0.12*
	2	0.15**	0.49**	0.66**	0.20**	0.38**	-0.22**	0.34**
	3	0.43**	0.17**	0.87**	0.27**	0.18**	0.09	0.01

3.6 Correlation analysis

Correlation provides a better insight into the mutual relationship among the traits. Thus, a better understanding of their association with grain yield would make the selection more precise and accurate with the attributing traits as effective indicators in selection. Across all crosses, productive tillers plants (0.65, 0.66, and 0.87) exhibited a strong positive correlation with single plant yield (Table 5). Similarly, the traits *viz*., plant height (0.42, 0.49 and 0.17), panicle length (0.21, 0.20 and 0.27), and hundred seed weight (0.12, 0.38 and 0.18) showed a positive significant association with single plant yield across all the crosses. These results herein show that more tillers might produce more panicles and increased synthesis and translocation of photo-assimilates from the source to sink could significantly improve yield through enhanced grain filling and seed set (Thúy et al. 2022; Kalaivani et al. 2023). In crosses I and II, days to first flowering and single plant yield showed a positive association (0.19 and 0.15). Likewise, within cross II, single plant yield positively correlated with grain width (0.34) while negatively correlated with grain length (-0.22). In general, selecting indicator traits can target high yield through potential traits directly. The results herein show that productive tillers per plant, panicle length, and hundred-seed weight can be indicator traits while selecting high-yielding segregants for grain yield improvement.

Conclusion

The findings of this study emphasize that productive tillers per plant can be used as a reliable trait for direct selection when identifying high-yielding segregants for enhancing grain yield in rice as it exhibits high estimates for PCV and GCV, heritability, and GAM. The positively skewed distribution and leptokurtic nature of the productive tillers per plant suggests the involvement of a limited number of genes governing its expression, and thus, an intense selection from existing genetic variability could significantly enhance the genetic gain of this trait. Moreover, correlation analysis emphasizes that grain yield improvement in rice can be attained by indirectly selecting F_2 segregants with more productive tillers and longer panicles with high hundred seed weights.

Conflict of interest

The authors declare no conflict of interest.

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CRISPR driven Cyanobacterial Metabolic Engineering and its role in metabolite production

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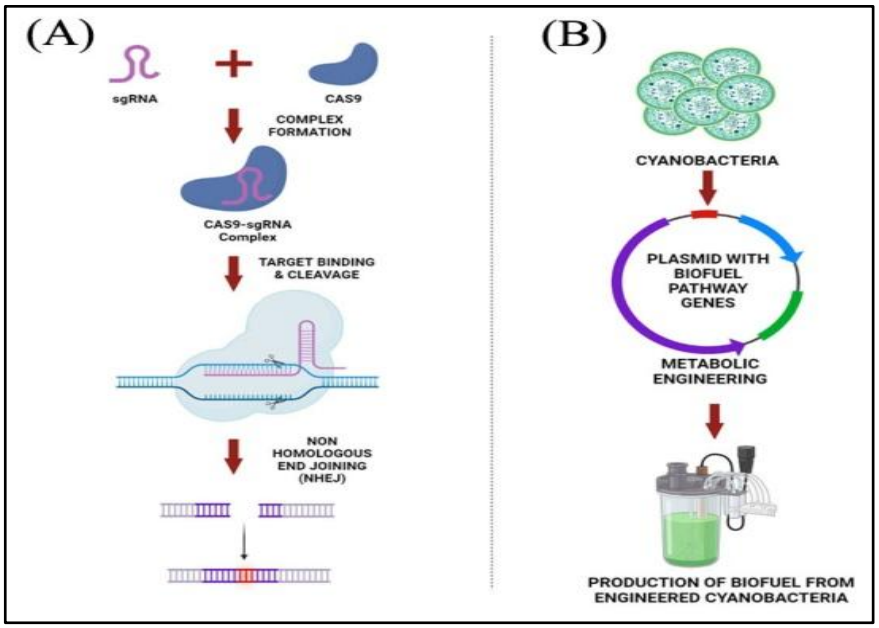
GRAPHICAL ABSTRACT



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KEYWORDS

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ABSTRACT

Recently, the advancement in sustainable methods for fabricating novel metabolites is one of the prime challenges in metabolic engineering. The current increase in fuel prices and its limited supply made the scientific community more concerned about finding an alternate source of fuel generation. Scientists are now interested in biofuel because of its low cost and ease of production. An intriguing area of research in metabolic engineering is using imaginative manipulation of microbes to manufacture chemicals or molecules of commercial importance. One such bacterium whose commercial potential is rapidly attracting the attention of the scientific fraternity is Cyanobacteria, which are either single-celled or multi-cellular filamentous photosynthetic organisms that can also fix CO_2. The generation of biofuel has been transformed by the use of CRISPR (clustered regularly interspaced short palindromic repeats) technology in cyanobacteria, which allows for precise genetic alterations to improve their metabolic processes. Scientists can effectively modify the cyanobacterial genome using CRISPR to increase lipid accumulation, maximize photosynthetic efficiency, and enhance stress tolerance. Cyanobacteria have gained attention in the scientific community as a potential source for biofuel production due to several advantageous characteristics like photosynthetic capacity, genetic manipulation, lack of dependency on fertile land, high biomass yield, versatile biofuel production etc. which our present manuscript aims to catalogue. Cyanobacteria play a pivotal role in developing environmentally friendly energy solutions by converting CO_2 into renewable energy sources, serving as a flexible platform for producing different types of biofuels and reducing greenhouse gas emissions.

1 Introduction

Through metabolic engineering, cyanobacteria (a varied group of photosynthetic Gram-negative bacteria) have gained attention due to their potential for the sustainable production of biochemicals, biofuels, and pharmaceuticals. However, many unanswered questions remain about the effective and scalable use of CRISPR in cyanobacteria to produce important metabolites. For instance, the genome complexity and strain availability of cyanobacteria are limited, there is a lack of an efficient CRISPR delivery mechanism in cyanobacteria, and the metabolic pathways of several cyanobacteria remain poorly understood. Finally, the environmental impact, safety, and ethical issues must be addressed during metabolic engineering in cyanobacteria by CRISPR technology (Carroll et al. 2018).

Although freshwater cyanobacteria are important components of ecosystems, their potential to create cyanotoxins presents significant environmental and public health risks (Nugumanova et al. 2023). In contrast, marine and terrestrial cyanobacteria are the sites for synthesizing promising new drugs. Focusing on the natural CRISPR – Cas (clustered regularly interspaced short palindromic repeats) system in cyanobacteria, cyanobacterial CRISPR loci studies demonstrated that cas1/cas2 genes were discovered in 86 out of 126 cyanobacterial genomes besides the marine cyanobacteria *Synechococcus* which grows in a setting without cyanophages (Pattharaprachayakul et al. 2020). Until now, many cyanobacterial secondary metabolites have been genetically and biochemically elucidated (Babele et al. 2023; Satta et al. 2023; Bashir et al. 2023). About 30 gene clusters in charge of producing cyanobacterial secondary metabolites have been discovered due to the development of genomic data on the genome of cyanobacteria and potent bioinformatic methods (Méjean and Ploux 2013). Recent advancements in CRISPR-based approaches have improved the metabolic engineering of cyanobacteria (Satta et al. 2023). CRISPR is a short DNA segment with small base sequence repetitions that have a role in viral defence mechanisms in bacteria and are dispersed by spacer sequences belonging to the foreign DNA element. Cas (CRISPR-associated) genes code for Cas proteins adjacent to the CRISPR array. Short RNA molecules (crRNAs) are synthesized from the transcribed and processed CRISPR arrays and interact with specific Cas protein complexes to create ribonucleoproteins (RNP). The crRNAs and/or tracrRNA (transactivating small RNA) also function as guide RNAs (gRNA) to specifically target and degrade invading DNA or RNA molecules (Choi and Lee 2016). The advantages of CRISPR-based methods include the need for minimal prior knowledge, the ability to carry out several genetic alterations simultaneously, the cheaper cost of custom synthesizing guide RNA molecules, and the potential to carry out several rounds of genomic modifications in a week by changing the guide RNA's sequence. These characteristics have hastened the establishment of multiplex and markerless properties of CRISPR-based techniques. Because of these factors, a CRISPR-based strategy replaces other genome-editing tools for cyanobacteria (Behler et al. 2018). Further, Cas9 (type II) and Cas12a (type V) endonucleases are the most often utilized Class 2 Cas proteins for cyanobacteria in synthetic biology for deleting a target gene without a selection marker. CRISPRi generated from

dCas12a (or dCpf1) possesses a more impactful suppression procedure than dCas9, in addition to being less toxic to cyanobacteria as compared to that of dCas9 (Ratner et al. 2016; Pattharaprachayakul et al. 2020). The field of genome editing and genetic engineering tools are both expanding very rapidly. Therefore, these genome engineering tools successfully assisted the development of cyanobacterial hosts for the construction of effective bio-solar cell factories for the consumption of CO_2 (Santos-Merino et al. 2019).

Biofuels have gained increasing attention in recent years as a potential alternative to fossil fuels for several reasons. One of the most significant reasons is that they are renewable and can be produced from various biological sources such as crops, algae, and waste products, which can be replenished relatively quickly. Biofuels are also believed to be more environmentally friendly than fossil fuels, producing lower greenhouse gas emissions and pollutants when burned (Bessou et al. 2011; Farrokh et al. 2019). Additionally, the production of biofuels can create new jobs and stimulate economic growth in rural areas where agricultural resources are abundant (Gheewala et al. 2013).

However, it is important to note that the use of biofuels also has some drawbacks and challenges. For instance, there are concerns that the production of biofuels could lead to deforestation, land-use change, and competition for food resources, which could negatively impact the environment and society (Weng et al. 2019). Therefore, it is crucial to carefully evaluate the potential benefits and drawbacks of biofuel production and use and to develop sustainable and responsible practices to ensure that the benefits of biofuels outweigh the negative impacts. This can involve promoting the use of advanced biofuels made from non-food crops or waste materials and implementing policies and regulations that incentivize sustainable biofuel production practices (Searchinger et al. 2008).

Photosynthetic organisms such as plants, algae, and cyanobacteria are considered promising sources for biofuel production because they can use sunlight as energy to convert atmospheric carbon dioxide into organic compounds, including those used as biofuels. Photosynthetic organisms can capture light energy and use it to drive a series of chemical reactions that produce organic molecules, such as sugars and lipids, that can produce biofuels. This process, known as photosynthesis, is a natural and sustainable way to produce biofuels that can reduce greenhouse gas emissions and decrease dependence on fossil fuels (Shen 2014). Additionally, photosynthetic organisms have the potential to be grown on non-arable land or in closed systems, such as photobioreactors, which can minimize competition with food crops and reduce the environmental impact of biofuel production. However, there are also challenges associated with using photosynthetic organisms for biofuel production, such as efficient and cost-effective methods for biomass harvesting, processing, and conversion into biofuels. Additionally, there is ongoing research to improve the efficiency of photosynthesis and to develop new strains of photosynthetic organisms that can produce higher yields of biofuels (Schenk et al. 2008; Sitther et al. 2020).

Cyanobacteria are an up-and-coming group of photosynthetic organisms for biofuel production due to their ability to fix nitrogen, grow rapidly, tolerate extreme conditions, and be amenable to genetic manipulation. Cyanobacteria can be genetically modified to enhance their ability to produce specific biofuels, such as ethanol or hydrogen, or to increase their overall productivity (Khan et al. 2019; Sitther et al. 2020). Genetic manipulation can also optimize gene expression in photosynthesis, carbon fixation, and other metabolic pathways relevant to biofuel production. Additionally, cyanobacteria have the potential to be used in industrial-scale bioreactors, which can improve the scalability and efficiency of biofuel production (Díaz-Santos 2019).

The introduction of genetically engineered cyanobacteria into the environment may have some possible risks (like ecological imbalance, horizontal gene transfer to other microorganisms, toxin production, may impact on carbon and nutrient cycles), like any other genetically modified organism (Chorus et al. 2021; Sebesta et al. 2022). So, it is important to carefully assess and manage the potential risks associated with genetic modification to ensure that the benefits of using cyanobacteria for biofuel production outweigh any potential negative impacts. Thus, using genetically modified cyanobacteria for biofuel production holds great potential for sustainable and renewable energy production, but continued research and development is necessary to optimize their productivity and ensure their safe and responsible use (Srivastava et al. 2022).

The mechanism of the CRISPR/Cas9 system was discovered by two scientists, Doudna and Charpentier (Javed et al. 2019). CRISPR has been used in cyanobacteria to knock out genes responsible for inhibiting biofuel production and introduce genes that enhance biofuel production or modify metabolic pathways (Verma 2020; Satta et al. 2023). Previous researchers have used CRISPR to knock out genes that encode for enzymes involved in the production of glycogen, a storage molecule that competes with the production of biofuels (Quintana et al. 2011; Satta et al. 2023). Cyanobacteria can divert more resources towards biofuel production by eliminating glycogen production, leading to higher yields. Additionally, CRISPR has been used to introduce genes that increase the production of specific biofuels, such as ethanol or hydrogen. Previous researchers, such as Khan et al. (2019), have introduced genes encoding enzymes that increase ethanol production in cyanobacteria, resulting in strains that produce higher ethanol yields than wild-type cyanobacteria. In this review, we aimed to showcase research that has used CRISPR-based techniques to manipulate cyanobacteria's metabolism for enhanced biofuel production. Although CRISPR editing offers a viable method for genetically modifying cyanobacteria to produce

biofuel, further study is required to maximize the effectiveness and safety of this strategy.

2 Importance and advantage of Cyanobacteria as a metabolic cell factory

Cyanobacteria exemplify a potential system for the synthesis of secondary metabolites from plants. Secondary metabolites are derivatives of primary metabolites that offer resistance against varying environmental stress, infections, UV irradiation, ozone and wounds (Korkina 2007). The potential outcomes of plant secondary metabolites as anticancer, antioxidant, antiviral, and anti-inflammatory agents on human health have drawn intense study attention. It is expensive and challenging to either extract secondary metabolites from plants or produce them chemically; as a result, cyanobacteria are chosen as the site of synthesis for these metabolites (Xue and He 2015). Cyanobacteria are extensively produced in phototrophic conditions due to less contamination, cheaper approach and CO_2 consumption (Chen et al. 2011). Intriguing photosynthetic hosts for chemical synthesis, cyanobacteria can be genetically modified to shift the intrinsic metabolic flow toward desired target molecules. Photosynthetic activity is a significant factor in cyanobacteria's metabolism, making it capable of producing secondary metabolites. Cyanobacteria use solar energy to steer CO_2 to produce organic compounds in the presence of water. A system of internal membranes called the thylakoids is responsible for energy conversion (thy). CO_2 fixation occurs in specialized compartments called carboxysomes (Cx) (Behler et al. 2018; La Rocca et al. 2018). Cyanobacteria are excellent for this task due to their ability to express plant cytochrome P450 monooxygenase enzyme, which plays crucial roles in the manufacture of numerous plant secondary metabolites like phenylpropanoids, alkaloids, terpenoids, cyanogenic glycosides, and glucosinolates are produced via biosynthetic gene clusters (BGCs) (Mizutani and Ohta 2010). The carbon skeleton is subjected to several oxidative changes by these organisms that use NADPH or NADH as reducing equivalents. Most known cyanobacterial species have P450 sequences in their genomes, citing examples of *Anabaena* sp. PCC 7120, which has six P450 genes, while *Synechocystis* sp. PCC 6803 only has one (Xue and He 2015). BCGs are gene clusters that are located relatively close to each other for the synthesis of chemicals. Due to their abundance in BGCs, cyanobacteria can produce numerous secondary metabolites. In an experiment utilizing antiSMASH, 196 full cyanobacterial genome sequences that were accessible through the NCBI genome portal were checked for BGCs in order to learn more about the secondary metabolites that cyanobacteria manufacture (Jeong et al. 2020; Leao et al. 2017). BGCs were categorized into 33 different categories (Jeong et al. 2020). Following the phylogenetic tree, the 196 complete cyanobacterial genome sequences in the BGC search were organized. Based on the heat map of the number of each type of BGC observed in each cyanobacterium, those belonging to the same genera had similar quantities and classes of BGCs. Several BGCs with several instances in a single genome were evident. In particular, cyanobacteria comprised a significant portion of the anticipated BGCs (n = 2119), accounting for 74.4% (Jeong et al. 2020). These BGCs included bacteriocin, terpene, and non-ribosomal peptide synthetase (NRPS) BGCs (Jeong et al. 2020). Cyanobacteria are a superior option for this purpose to other organisms because of their high photosynthetic efficacy and ease of genetic manipulation. The enhancement of product titers, bioprocess scale-up, and material restoration are some of the hurdles that cyanobacteria still face in engineering applications (Xue and He 2015). One of the main benefits of CRISPR editing is that it allows for the targeted modification of specific genes involved in biofuel production. Scientists can use CRISPR to delete or modify genes that limit the efficiency of the photosynthetic process, increase the production of enzymes involved in biofuel synthesis, or improve the tolerance of cyanobacteria to environmental stressors. By doing so, researchers can create strains of cyanobacteria that are more efficient at producing biofuels and better adapted to growth in challenging conditions. In addition, CRISPR editing is a relatively quick and inexpensive process compared to traditional methods of genetic modification. This makes it an attractive option for researchers who want to develop new strains of cyanobacteria for biofuel production.

3 Need for Metabolic Engineering in cyanobacteria

Cyanobacteria offer an attractive platform for sustainable bioproduction because of their capacity to absorb carbon dioxide and sunlight. Cyanobacterial metabolic engineering has revolutionized with the introduction of CRISPR-Cas systems, which provide accurate, effective, and adaptable tools to modify cyanobacterial metabolism for increased synthesis of essential metabolites. CRISPR editing in cyanobacteria is a powerful tool that can be used to enhance biofuel production. Further, CRISPR editing allows for precise genetic modifications to be made to cyanobacteria, which can improve their biofuel production capabilities.

Currently, industries depend on the availability of fossil fuels, which leaves behind a large ecological footprint, but the sector of metabolic engineering offers sustainable and eco-friendly solutions. Photosynthetic organisms such as cyanobacteria are being utilized to produce biofuels as they can use the carbon from the atmosphere to derive energy from light and direct these for the biosynthesis of the biofuels (Behler et al. 2018).

Genetic mutations in cyanobacteria can arise in various ways, such as exposure to mutagens, mistakes in replication, or due to environmental stresses. Genetic variety from these mutations can help cyanobacterial populations adapt to various environmental

circumstances. This tendency for genetic alteration can also make it challenging to keep strains steady for biofuel production. By taking advantage of this feature, scientists may accurately alter the cyanobacterial genome using cutting-edge genetic technologies like CRISPR. By adding or fixing advantageous mutations, they can increase the cyanobacterium's capacity to produce biofuel while maintaining higher uniformity and efficiency (Behler et al. 2018; Cassier-Chauvat et al. 2021; Mehdizadeh and Peerhossaini 2022).

Since certain cyanobacteria are polyploid or oligoploid, genetic engineering in such species becomes time-consuming and critical. For example, *Synechococcus elongates* can accommodate 2 copies of chromosomes per cell, while *Synechocystis spp* can accommodate up to 53 copies per cell (Watanabe et al. 2015). To develop a homozygous mutant in a polyploid or oligoploid strain, a separation procedure is compulsory to ensure that all the chromosome copies in the transformants contain the same fragments of the enhanced genetic material. This requires several rounds of sub-culturing aided by primary selection, thereby taking sufficient time to complete the procedure(Zerulla et al. 2016).

The emergence of CRISPR-based technologies has transformed the way that genomes are being engineered. CRISPR-associated Cas systems and homologous recombination-based technologies are employed for cyanobacterial genetic engineering. CRISPR/Cas induces a double-stranded break, which stimulates the homology-directed repair. It accentuates the genome editing process (Knoot et al. 2018). Wendt et al. (2022) were the first to bring this efficiency to cyanobacteria using the CRISPR/Cas9 system (Wendt et al. 2022). They chose the non-bleaching protein A (nblA) gene as their target to ensure that a mutation would result in phenotypic alterations like depigmentation, making it a great modification reporter. Finally, the researchers found that cyanobacteria's genome-editing efficiency was significantly enhanced when CRISPR/Cas systems were correlated with metabolic engineering (Wendt et al. 2016; Lee et al. 2023).

The association between the expression levels and enzyme kinetics in inherent and engineered metabolic matrices is poorly understood. This constitutes a major barrier in the sector of metabolic engineering. Due to the prevalence of such gaps, data-driven methods, high-throughput screening, OMICS, and machine learning have gained importance in place to improve strain. Therefore, more engineering techniques must be developed to accentuate such data-driven methods. This can be achieved by coupling new tools for CRISPRa with the existing tools of CRISPRi, which will ultimately optimize the biosynthesis of engineered cyanobacteria (Fontana et al. 2020). Thus, the need for metabolic engineering increases exponentially with the increasing population. This can only be achieved by employing CRISPR-driven technologies that involve the genetic knock-outs, knock-ins, and regulation of transcriptional activity of the target genes. Other methods that can be further researched are the upregulation of transcription activity using CRISPRi and the modulation of mRNA levels using Cas13a systems (Abudayyeh et al. 2016). Moreover, the characterization of CRISPR units from diversified photosynthetic organisms such as cyanobacteria can also be achieved by CRISPR-based technologies. Therefore, the multiplexing attribute of CRISPR-based technologies makes it an essential component in the metabolic optimization of regulatory and genetic factors required for the metabolic engineering of cyanobacteria (Yao et al. 2016).

4 Application of CRISPR Cas-based genome editing in Cyanobacteria

4.1 Overview of CRISPR/Cas9-based gene editing

Genome Editing Using Engineered Nucleases (GEEN) is a successful genetic engineering technique that targets and digests DNA at specified sites in the genome using "molecular scissors," or artificially created nucleases (Osakabe and Osakabe 2015; Rafeeq et al. 2023). Double-stranded DNA breaks (DSBs) are caused by the designed nucleases at the target location, which are then repaired by natural processes such as homologous recombination (HR) or non-homologous end-joining (NHEJ) (Martin et al. 2016). Site-directed mutagenesis possibly uses the NHEJ-mediated mechanism, which produces varied insertion or deletion mutations. Further, the targeted gene replacement uses HR with double-stranded donor DNAs to produce exact nucleotide substitutions or insertions (Zhu 2015). CRISPRs were first identified in *E. coli* at the beginning of 1987, and afterwards, it was identified in numerous additional bacterial species (Ishino et al. 2018). By causing RNA-guided DNA cleavage, these sequences contributed to the adaptive immunological defence of bacteria and archaea against invading foreign DNA (Tadić et al. 2019). The CRISPR/Cas9 system functions mechanically by combining a Cas9 endonuclease and a single-stranded guide RNA (sgRNA). A distinct 20 base-pair (bp) sequence is frequently included in the sgRNA in order to complement the target DNA site in a sequence-specific way (Wang et al. 2020). The "protospacer adjacent motif" (PAM) is a crucial short DNA region upstream required for compatibility with the Cas9 protein utilized after this. Watson-Crick base pairing helps the sgRNA attach to the target sequence, and after this, Cas9 carefully cleaves the DNA to create a DSB (Wang et al. 2014; Uniyal et al. 2019). Multi-protein effector complexes comprise Class 1 CRISPR-Cas systems, while only one effector protein is present in Class 2 systems. Till now, six CRISPR-Cas types and 29 subtypes have been described, and this number has grown in recent years (Barakate and Stephens 2016). The type II CRISPR/Cas9 system is the most widely utilized subtype of CRISPR systems. It relies on a single Cas protein from *Streptococcus pyogenes* (SpCas9) targeting specific DNA sequences and is a desirable gene editing tool. DNA-DSB repair mechanisms start the genome repair process after the DSB (Konstantakos et al. 2022). The

irrevocable, permanent alteration of the genome's information that results from DNA editing also has ethical and security concerns. This is how the molecular scissors cut the faulty DNA so that the right gene may replace it (Gosavi et al. 2020).

4.2 Recent studies in Cyanobacteria

Recent advancements in synthetic biology have opened up new possibilities for modifying and editing heterologous hosts, leading to increased productivity and yield of biofuels at an industrial scale. Synthetic biology combines principles from biology, engineering, and computer science to design and construct new biological systems or modify existing ones to perform specific functions. By employing techniques such as genetic engineering, metabolic engineering, and directed evolution, scientists can manipulate organisms' genetic makeup and metabolic pathways to optimize their ability to produce biofuels.

Synechococcus elongatus PCC 7942, a strain of cyanobacteria, has been used in research to produce isobutanol and isobutyraldehyde directly from carbon dioxide (CO_2) (Khan et al. 2019). Isobutanol and isobutyraldehyde are valuable compounds that can serve as biofuels or chemical precursors. Researchers can achieve light-dependent expression of these genes by integrating the PDC and ADH genes at the psbA2 locus under the control of the PpsbA2 promoter (Miao et al. 2017). This approach allows for regulating biofuel production in response to light availability, as cyanobacteria primarily carry out photosynthesis in the presence of light. This type of genetic modification enables the cyanobacteria to produce isobutanol, specifically when exposed to light, harnessing the energy of photosynthesis for biofuel synthesis. It provides a light-controlled system for optimizing productivity and preventing unnecessary energy consumption when light is unavailable. Integrating gene sets into specific loci and using light-inducible promoters are strategies commonly employed in synthetic biology to precisely control gene expression and metabolic pathways in cyanobacteria and other organisms (Singh et al. 2016).

The potential of cyanobacteria as a source of DAG and TAG is an active area of research. If lipid production can be optimized in cyanobacteria, it could have applications in biofuel production and other valuable lipid-based products. However, it is important to note that commercial-scale production of DAG and TAG from cyanobacteria is not yet a reality, and further research and development are needed to improve lipid yields and cost-effectiveness (Radakovits et al. 2010; Sheng et al. 2011).

Despite several advantages of genetically engineered cyanobacteria, there are several disadvantages, such as a fragile lag phase that has been reported during the study of the growth curve of genetically engineered cyanobacteria, which could reduce the yield of fatty acids in an industrial bioreactor.

In a recent study, it has been observed that photosynthetic *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803 are the two strains that could convert inorganic carbon to free fatty acids (Santos-Merino et al. 2022). Another approach towards it was inserting an acyl-acyl carrier protein (ACP) thioesterase gene into Synechosystis, producing a high yield of free fatty acids (183-211 mg/L). In addition to constraining the metabolic flux for producing free fatty acids (FFA), the acetyl-CoA carboxylase (ACC) was over-expressed. Also, fatty acid-activating genes were knocked out to prevent the degradation of free fatty acids (Liu et al. 2011). Recently, it was identified that *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 strains can utilize the exogenous fatty acids and secrete endogenous fatty acids (FA) into the culture medium (Kaczmarzyk and Fulda 2010). In the future, these kinds of approaches can be further expanded, and more modified and optimized strains of cyanobacteria can be produced, which will increase the production of free fatty acids.

4.3 SEVA-Cpf1, a CRISPR Cas 12a vector

The *CRISPR*-associated protein 12a or the Cas protein, previously known as the Cpf 1, is a mechanism present in bacteria that destroys the genetic material of the viruses. This is a Rna-guided endonuclease in bacteria (Sun et al. 2018). This is highly selective and only occurs when DNA is adjacent to certain nucleotides. The Cpf 1 enzyme is guided to a specific location in the genome, cutting the dsDNA by the guide RNA and CRISPRCpf 1 of the Cas12a/Cpf 1 enzyme. NHEJ/homologous directed recombination is used to repair the break following the cleavage (Baldanta et al. 2022). Among other aspects, CRISPR-Cpf1 (Cas12a), a single RNA-guided endonuclease, differs from Cas9 in that: Cpf1 recognizes targets with a 5′ T-rich protospacer-adjacent motif (5′-TTN-3′), in contrast to G-rich Cas9 PAM. Because Cpf1-associated CRISPR arrays have both nuclease activity and ribonuclease activity, which enables them to convert the precrRNA array into mature crRNAs, they do not require an additional trans-activating crRNA. They have a unique property which helps in the detection of the disease (Pasin et al. 2017; Wang et al. 2023). Cas 12 cuts the DNA at the defective site; after cutting, it binds to the complementary strand, which turns on the trans cleavage. Cas12 ligates to the structure and chops the DNA if it binds (Martin-Pascual et al. 2021). Although SEVA vectors have been successfully used in Gram-negative bacterial CRISPR editing operations, cyanobacterial gene editing has not yet been investigated. In one strain, *Synechocystis* 6803, natural transformation, electroporation, and conjugation have all been shown to successfully transform SEVA vectors with RSF1010 or RK2 origins to express heterologous genes. Although it will be necessary to demonstrate that they can be employed for transformation processes in several cyanobacterial strains, the prospective application of SEVA vectors for gene editing in cyanobacteria is extremely intriguing (Pasin et al. 2017).

CRISPR technology appears to be a more sophisticated and reliable approach to increasing the accuracy and efficiency of gene manipulation in cyanobacteria (Liu et al. 2011). This approach includes genome editing without markers, quick modification of numerous genes, and the quick transcriptional regulation of various genes, which helps modify bacterial strains (Behler et al. 2018). The production of biofuel in cyanobacteria using CRISPR technology is still in progress. Until now, several scientific approaches have been made toward it, such as the overexpression of genes. Pyruvate decarboxylase and alcohol dehydrogenase are the two most efficient enzymes in biofuel production in cyanobacteria. Pyruvate enzyme, a product of the glycolytic pathway, is converted to acetaldehyde, and alcohol dehydrogenase reduces carbon dioxide and acetaldehyde. Using CRISPR/Cas9, the activity of two enzymes, i.e. alcohol dehydrogenase and pyruvate decarboxylase, can increase. Overexpression of these two enzymes will increase biofuel production (Shanmugam et al. 2023).

4.4 Inactivation of aas (acyl-acyl carrier protein synthase)

Acyl-acyl carrier protein synthase shuttles the growing fatty acid chain into fatty acid synthase, ultimately converting it to lipids (Currie et al. 2020). Free fatty acid production can be enhanced by deactivating the acyl-acyl carrier protein synthase using dcas9 (Kaczmarzyk et al. 2018).

4.5 Engineering promoters

The promoter is an integral part of the cell that plays a significant role in gene expression and regulation (Singh et al. 2016). Promoters are responsible for recruiting the RNA polymerase to start the transcription process. By engineering the promoter region, its activity can be increased, which will directly affect the activity of transcription factors and the RNA polymerase, and these will bind more readily to the promoter region. This will increase the transcriptional activities of the genes responsible for biofuel production.

4.6 Optimizing Ribosomal Binding Sites

Ribosomal binding sites are specific sequences that are present in mRNA. The translation of the target gene in the downstream region is initiated upon binding with the ribosome. The binding affinity with ribosome is always highest with a strong RBS, while the lowest binding affinity indicates the presence of a weak RBS. The core Shine Dalgarno sequence of the ribosomal binding site interacts with 3' terminal sequence of 16s rRNA by complementary pairing (Singh et al. 2016). The translation rate also depends on the rate at which the ribosome is recruited to RBS. The nucleotide sequences between the Shine Dalgarno sequence and the start codon (AUG) form a secondary structure, and the efficiency of RBS also depends on it (Chen et al. 1994; Singh et al. 2016). By optimizing the ribosomal binding sites, the efficiency of translation and expression of a gene can be increased towards large-scale biofuel production for industries.

5 Recent advancements and Future Prospects

In order to increase biofuel yields, recent developments in the use of CRISPR in cyanobacteria for biofuel production have concentrated on improving genetic efficiency and accuracy. Through the effective use of CRISPR, researchers have introduced genes that improve the metabolic pathways unique to biofuel synthesis and eliminate genes that prevent the generation of biofuels. Recent research has focused on blocking the mechanisms that produce glycogen to redirect cellular resources to synthesizing ethanol and lipids. Furthermore, advancements in CRISPR technology, including CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi), have made it possible to precisely regulate gene expression, providing more precise control over metabolic processes. These developments further optimize cyanobacterial strains for large-scale biofuel production by increasing their resistance to environmental challenges and increasing biofuel output. CRISPR-based technologies are also being utilized for the metabolic engineering cyanobacterial strains like *Synechococcus sp*, *Synechocystic sp*, and *Anabaena sp*. Selection markers are being replaced as these cause unnecessary pressure on the cell, allowing the manipulation of genes numerous times. Moreover, this leads to a significant decrease in the time required for the downstream processes, and the introduction of CRISPRi technology has opened up multiple opportunities for tuning metabolic pathways even without disturbing the cell viability (Behler et al. 2018).

CRISPR has been only utilized for knock-ins, knock-outs, and down-regulation of transcriptional activities. However, attempts can be made in the future to moderate upregulation of transcriptional activities or regulate mRNA using CRISPRi, especially the Cas13a system. Derepression of pathways for enzyme production or disruption of the genes encoding for the specific enzymes can also open up immense opportunities for the bioengineering of cyanobacteria (Abudayyeh et al. 2016). For example, deleting genes such as cyAbrB2, which act as transcription regulators, can accentuate free fatty acid production. Moreover, manipulating the native metabolic flux can also prove beneficial for the efficacy of the target product (Georg et al. 2014). Alternatively, CRISPRg RNAs can be utilized for synthetic screening methods for the modulation of organisms meant for industrial use and for designing genes for regulatory factors such as promoters, terminators, and ribosome binding sites (Cho et al. 2018). However, CRISPR-based systems can only be used temporarily as their extensive use may prove toxic to the cell. Thus, the expression of Cas9 protein needs to be regulated using inducible promoters, and more research should be conducted in this sector for a better understanding and utilization of this

technology for bioengineering the strains and paving a path for enhancing its industrial application.

Conclusion

The bacterial CRISPR-Cas system is an adaptive immune system prevailing naturally in the bacterial cell and has the potential to be immensely exploited as a remarkable genetic platform in multiple sectors of biotechnological studies. Cyanobacteria have an inbuilt CRISPR-Cas system (generally Type I and Type III) that is a defence mechanism against viral invasion. However, the creation of various tools for genetic engineering in cyanobacteria, which involves the application of CRISPR-Cas systems that are synthetic, for instance, genetic engineering tools which are based on Cas12a and Cas9, has been established because of the complexities in the regional CRISPR-Cas system of cyanobacteria and also due to the absence of enough functional knowledge on the topic.

Till now, several studies have demonstrated the effectiveness of CRISPR-based techniques in modifying the genetic makeup of cyanobacteria. Conventional CRISPR-based techniques include engineering a point mutation, insertion, or deletion in cyanobacteria by double homologous recombination between the host genome and a suicide vector. These techniques also need substituting a selection marker for the targeted gene. There are numerous advantages of CRISPR-based engineering in cyanobacteria over traditional methods. Selection markers are not required because, based on the viability of the cell, selective pressure plays a crucial role in the CRISPR-based technique. Therefore, producing knock-outs and knock-ins without markers using editing techniques based on CRISPR increases the probability and is a way to delete or introduce an infinite number of genes in multiplex processes. With the help of CRISPRi, it is possible to modify metabolic pathways and lessen cellular fluxes towards undesirable byproducts without significantly compromising cell survival.

Numerous strategies, including knock-ins, knock-outs, and downregulated transcription of specific genes, have been employed in cyanobacteria. Furthermore, given the many benefits of CRISPR-based methods for metabolic engineering, characterizing CRISPR components from diverse species would be appropriate for researchers to pursue soon. Looking into the recent cyanobacterial CRISPR-Cas system, current developments in prime editing and base editing technologies have expanded the possibilities for metabolic engineering in the CRISPR-Cas system now used by cyanobacteria. The field of genome editing and metabolic engineering tools is both expanding and growing at a faster pace. Therefore, these metabolic engineering tools successfully assisted the development of cyanobacterial hosts for the construction of efficient factories for the production of bio-solar cells that help in the consumption of CO_2. Increasing the variety of CRISPR-based tools and making them available to researchers would encourage more scientific expeditions to achieve more novel approaches and methods, not only for the engineering of cyanobacteria but also in the field of other biotechnologically significant species. Therefore, CRISPR editing in cyanobacteria can greatly enhance biofuel production's efficiency and scalability. By creating genetically modified strains of cyanobacteria that are better adapted to growth in challenging conditions and produce higher yields of biofuels, we can move closer to a sustainable and environmentally friendly energy future.

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The underlying factors of occurrence of Mucormycosis in post-COVID-19 patients – A meta-analysis of case histories

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ABSTRACT

Mucormycosis is a life-threatening fungal infection caused by fungi of the order Mucorales. It usually affects people with weakened immune systems, such as those with uncontrolled diabetes, acquired immunodeficiency syndrome, iatrogenic immunosuppression, and hematological malignancies, as well as individuals who have had organ transplants. The type of mucormycosis a person suffers from is often determined by their underlying conditions. The most common types are rhino-cerebral mucormycosis, pulmonary mucormycosis, cutaneous mucormycosis, cerebral mucormycosis, gastrointestinal mucormycosis, and disseminated mucormycosis. The incidence of mucormycosis has been increasing over the years, with an overall mortality rate of 54%. Recent cases have shown a correlation between COVID-19 and mucormycosis. Using anti-inflammatory drugs to combat the cytokine storm associated with COVID-19 can weaken the immune system, making individuals more susceptible to opportunistic fungal infections like mucormycosis. Underlying health conditions further exacerbate the condition. This study reviewing 198 cases of mucormycosis and conducting a meta-analysis found that post-COVID-19 patients most commonly developed rhino-orbital-cerebral mucormycosis, followed by pulmonary and gastrointestinal mucormycosis. The study also identified diabetes as the most common underlying factor contributing to the development of mucormycosis in post-COVID-19 patients, followed by hypertension and obesity. The study also examined the influence of age, affected organs, and

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the use of certain drugs on the development of mucormycosis. Age was found to be a significant factor in the infection. This meta-analysis is one of the first to compare post-COVID mucormycosis cases with those from the pre-COVID era. The hope is that this study and analysis will help identify the determinants of mucormycosis in post-COVID patients and aid the scientific community in finding a solution to this problem.

1 Introduction

Black fungus, also known as dematiaceous fungus, refers to fungi that produce dark melanoid pigments on the cell walls of vegetative cells and conidia, giving them a distinctive blackish-grey hue in their colonies (Chowdhary et al. 2014). There are numerous black fungi in the environment, many of which are pathogenic to humans and animals (de Hoog et al. 2000). While some species, such as *Auricularia auricula* and *Pestalotiopsis* sp. have been studied for their nutritional benefits (Yao et al. 2019; dos Santos et al. 2020), others like *Trichophyton schoenleinii* and *Microsporum audouinii* are known to cause diseases and infections in humans (Matsumoto et al. 1994; Revankar 2012). These fungi are commonly found in soil and are widespread, with some associated with a few specific diagnoses and others occupying specialized ecological and geographical niches. They have increasingly been recognized as significant pathogens in both immunocompromised and healthy individuals. The treatment for these uncommon and occasionally refractory illnesses is not standardized, necessitating further research for better understanding. Between 2019 and 2021, the term "black fungus" gained sudden prominence after the COVID-19 pandemic. This term refers to a rare but serious infection known as Mucormycosis, caused by fungal spores of the class Mucormycetes (Petrikkos et al. 2012). These organisms are commonly found in the environment and seldom cause illness in healthy individuals. The most likely transmission routes in susceptible individuals are inhalation of spores or direct inoculation into the skin or mucosa during interventional procedures or trauma (Petrikkos et al. 2003).

2 Mucormycosis

2.1 Types of Mucormycosis

Mucormycosis can manifest in various types, and the clinical symptoms of infections are influenced by the site of the organisms' infection and the method of transmission. Pulmonary mucormycosis has a fatality rate of 40-70% (Khan et al. 2020; Muthu et al. 2021). The infection begins when fungal spores are inhaled into the body. The individual's immune system determines the severity and progression of the infection. The infection is more common in immunocompromised individuals with transplants and hematological malignancies. The most common fungal species associated with pulmonary mucormycosis are *Rhizopus* spp., *Mucor*, and *Rhizomucor* species (Novais et al. 2020). Gastrointestinal mucormycosis is exceptionally rare, accounting for approximately 2 to 11% of all cases (dos Santos et al. 2023). The stomach and intestine are the most commonly affected organs, and in some cases, the infection can spread to other parts of the digestive system. Infections are typically moderate, but in rare cases, they can be severe. The intestinal infection begins when spores are ingested with food or other substances and then pass through the gastrointestinal tract (Clemente-Gutiérrez et al. 2019). Cutaneous mucormycosis can manifest as a localized infection or progress to affect deeper tissues and potentially spread to other body parts. This infection is caused by the pathogen entering the body through skin injuries or wounds from surgery, natural disasters, or contact with soil and other contaminated sources. The infection can rapidly progress from the skin's surface to the subcutaneous layer, fascia, and bone. *Apophysomyces* and *Saksenaea*, widespread soil saprophytes, are the most prevalent species associated with Cutaneous mucormycosis. Cutaneous mucormycosis infections can be categorized into two main types: primary and secondary. Primary infections occur when the organism is present on the skin and directly invades through breaches in the skin barrier, such as wounds, burns, surgical sites, or areas of trauma. Secondary infections occur when the organism spreads to the skin from another site of infection, often through the bloodstream. The most affected regions in cutaneous mucormycosis are the legs and arms, with uncommon incidences in the scalp, face, back, thorax, breast, neck, and genitals (Zuglian et al. 2019). Disseminated mucormycosis is the rarest form and mainly occurs in neutropenic individuals with hematologic malignancies or post-transplant recipients. Despite its rarity, it has a high fatality rate of roughly 90% due to the invasive nature of the infection (Alqarihi et al. 2023). The tendency to invade endothelial cells within the vascular system contributes to the high rate of dissemination associated with mucormycosis. Infections can spread to various organs, such as the lung, pancreas, brain, and spleen, causing moderate to severe infections in some or all of these areas. Extremely immunocompromised individuals who have received deferoxamine, a medication that binds iron and aluminium, are at risk of developing the disease (Araf et al. 2022). The disseminated mucormycosis symptoms and presentations are often unclear, leading to delayed diagnosis and further infection. Direct inoculation of the fungus is the most common route of transmission, which can lead to cutaneous, subcutaneous, muscular, and skeletal tissue infections (Alqhamdi et al. 2019).

Rhino cerebral mucormycosis is a rare disease caused by a filamentous fungus of the order Mucorales. This fungus affects the

paranasal sinuses, nose, and brain. When the fungus develops rapidly and aggressively, it may become chronic. The most common form of mucormycosis is rhino cerebral mucormycosis, and the incidence of infections varies depending on the presence of certain high-risk groups. The infection starts in the nasal cavity and then spreads to the nearby paranasal sinuses. The fungus then adheres to the surface of the sinus and begins multiplying due to the moist environment, promoting its proliferation and invasion. In the early stage of the infection, a fungus ball grows in the maxillary sinus with no evidence of bone degradation (Aggarwal et al. 2022). Depending on the length of the infection, the host's immunity, and the severity of the infection, the condition may worsen. Several virulence factors drive the disease's progression. It starts with blood vessel invasion and destruction of endothelial cells, leading to ischemia and tissue necrosis. The invasion of the sphenopalatine and internal maxillary arteries results in the invasion of the brain and orbits of the brain. Diabetic individuals with diabetic ketoacidosis and hyperglycemia are more likely to develop rhino cerebral mucormycosis (Dong et al. 2022). The generic symptoms of the illness make it difficult to diagnose the infection early. Common symptoms include one-sided pain behind the eyes and fatigue. Other symptoms encompass face discomfort, numbness, nasal discharge, sinusitis, convulsions, altered mental state, and gait issues (Martínez-Herrera et al. 2021).

2.2 Affected groups

Studies have revealed that specific groups of individuals are more vulnerable to mucormycosis. Hyperglycemia creates an optimal environment for fungal growth. *Mucor* and *Rhizopus* species possess an active ketone reductase system, enabling them to thrive in an acidic pH and glucose-rich environment (Ibrahim et al. 2012). Furthermore, hyperglycemia fosters fungal proliferation and impacts neutrophil chemotaxis, leading to frequent occurrences in patients with diabetic ketoacidosis. *Rhizopus* species thrive in iron-rich environments and are commonly found in patients undergoing deferoxamine therapy (an iron-chelating agent) (Pathak et al. 2018). Mucorales also rely on iron for growth, leading to phagocytic activities and dissemination into the vasculature (Thomas et al. 2020). Studies have also demonstrated that individuals with leukemia, particularly those who have undergone hematologic bone marrow transplants, are prone to various types of mucormycosis. Immune deficiencies associated with hematological malignancies may heighten susceptibility to opportunistic fungi, as neutrophils play a crucial role in the protective host response (Cheong et al. 2017). Long-term use of glucocorticoids and corticosteroids also significantly contributes to mucormycosis development. Corticosteroids are often prescribed for the treatment of various disorders, including severe allergies or skin conditions, asthma, arthritis, or autoimmune diseases such as Systemic Lupus Erythematosus (SLE) (Arnold et al. 1988; Durcan and Petri 2016; Daley-Yates et al. 2021; Reynolds et al. 2024). Furthermore, abnormal levels of iron and aluminum play a substantial role in mucormycosis development, particularly in patients undergoing dialysis or iron-chelating therapy (Reyes et al. 2008).

2.3 Pathology

2.3.1 Route of Infection

Mucormycetes, also known as black fungi, are spores of molds that typically thrive in organic matter such as soil or compost. These spores can enter the human body through inhalation, ingestion, or direct inoculation via skin wounds. Inhalation of spores is the primary route of infection, causing severe conditions like rhinocerebral and pulmonary mucormycosis in immunocompromised individuals (Prakash and Chakrabarti 2019). Ingestion of spores can lead to gastrointestinal mucormycosis, especially in neonates and malnourished individuals (Roden et al. 2005). Direct inoculation through traumatic injuries or burns can result in cutaneous mucormycosis, commonly observed in disaster settings (Pilmis et al. 2018).

2.3.2 Colonization

Mucormycetes are commonly found but typically do not cause significant issues because our immune system is equipped to defend against such infections. Iron is essential for the growth and development of mucormycetes, and the fungus has developed various strategies to obtain it from the host (Ibrahim et al. 2012). A higher concentration of iron provided by the host's serum increases the likelihood of fungal proliferation. Unbound free iron can be toxic and create an unguarded platform for infecting pathogens. It triggers harmful reactive oxygen species, causing damage to cellular components. Simultaneously, pathogens exploit free iron for growth and proliferation. Excess unbound iron overwhelms the body's defense mechanisms, facilitating pathogen colonization and infection. This highlights the crucial role of iron balance in maintaining health and preventing disease. To counter this, humans have a defense mechanism that involves binding iron to proteins such as transferrin, ferrin, and lactoferrin. Mucorales cannot thrive in iron-low serum unless it is externally added (Ibrahim et al. 2012). Mucorales utilize various strategies to acquire iron from the host. One method involves using xenosiderophores, such as deferoxamine, which strips iron from transferrin proteins and transports it intracellularly after converting it into a soluble form. Another mechanism involves the utilization of high-affinity iron permease, including ferrous reductase, multicopper oxidase, and ferrous permease, to actively convert ferric to soluble ferrous iron and facilitate its uptake. Additionally, Mucorales use heme-oxygenase to sequester iron from heme proteins found in the host's blood, further enhancing their ability to acquire this essential nutrient (Ibrahim et al. 2012).

During the COVID-19 pandemic, mucormycetes have been labelled as "opportunistic black fungus" because they exploit the situation where the immune system is weakened by the use of corticosteroids administered to COVID patients to address the exaggerated release of cytokines mediated by angiotensin-converting enzyme 2 (ACE2). This leads to vascular inflammation, shock, and ultimately results in multi-organ failure and death (Singh et al. 2020).

2.3.3 Immunopathology of COVID-19

Infection with the SARS-CoV-2 virus compromises the integrity of the respiratory passages, providing a pathway for the invasive Mucorales (Zhang et al. 2023). The overexpression of GRP78 in COVID-19 may facilitate Mucorales hyphal invasion and spore germination (Alqarihi et al. 2020). COVID-19 disrupts macrophage function by directly infecting them, making them prone to hyperglycemia and generating neutrophil extracellular traps, which affects innate defenses against Mucorales. Thrombocytopenia, a decline in natural killer (NK) cells, and infected dendritic cells are all linked to the COVID-19 virus, leading to a reduced immune response to Mucorales. Cytokines generated during COVID-19 induce leucocyte oxidative damage, disrupt mitochondria, and lead to a build-up of reactive oxygen species. Additionally, COVID-19 infection results in hyperferritinemia, which inhibiting the hematological growth of B- and T-lymphocytes (Dave et al. 2022).

2.3.4 Treatment

The FDA has only approved three antifungal drugs for treating mucormycosis: amphotericin B, posaconazole, and isavuconazole lipid formulations. Surgical debridement is also performed if needed. However, the mortality rate for mucormycosis remains over 50% when only antifungal drugs are used for treatment (Gebremariam et al. 2019). Surgical removal of necrotic tissue is the primary therapy for mucormycosis (Sipsas et al. 2018). Patients with pulmonary mucormycosis show significant improvement after surgical intervention combined with antifungal drugs compared to treatments relying solely on antifungal drugs. In some cases, localized surgery may be sufficient. Magnetic Resonance Imaging (MRI) is used for rhino-orbital-cerebral mucormycosis to determine the extent and exact location of the necessary surgical intervention. Surgical intervention is crucial in these cases (Sipsas et al. 2018).

Additionally, adjunctive therapy plays a crucial role in treatment, aiming to reactivate the suppressed immune system (Sipsas et al. 2018). Patients often succumb to the disease due to poor recovery of bone marrow function. One approach is to reverse neutropenia in hematologic patients by incorporating hematopoietic growth factors into the host's system or by white cell transfusion (Whitsett 1995). In HIV/AIDS patients, immunity restoration can be achieved through antiretroviral therapy. Aggressive hyperglycemia in individuals with uncontrolled diabetes should be addressed and can be treated with sodium bicarbonate to block the invasion of endothelial cells by the fungus, restoring host iron chelation and neutrophil function. Another effective strategy to inhibit the growth of fungal infection is through iron chelation, reducing iron availability in the host's blood. Deferasirox was tested on mice, and the survival rate increased. Neutrophil functionality can be enhanced by increasing oxygen pressure with hyperbaric oxygen (HBO), mediating the improvement of neutrophil functionality and wound healing. This is particularly helpful for diabetic patients with sinusitis or cutaneous mucormycosis (Sipsas et al. 2018).

Certain protocols were issued by the European Confederation of Medical Mycology (ECMM) and mycoses Study Group Education and Research Consortium in 2019 (Bhatt et al. 2021). According to these protocols, immediate surgical intervention is advised whenever possible in cases of fungal infection. The primary approach to managing these infections involves using antifungal drugs, with a combination of liposomal *amphotericin B*, *isavuconazole*, and *posaconazole* being the preferred initial treatment. In cases where patients have undergone graft transplants and are experiencing graft vs host reactions, *prophylaxis* involving *posaconazole* may be recommended (Bhatt et al. 2021). For CAPA (SARS-CoV-2) associated pulmonary *aspergillosis*, the recommended drugs are classified into two main categories: allergic *aspergillosis* and invasive aspergillosis (Bhatt et al. 2021). The most common strain responsible for this type of Black fungal infection is *Aspergillus fumigatus*, which commonly affects immunosuppressed individuals, patients who have undergone hematopoietic transplantation, or those receiving corticosteroid treatment for COVID-19-related lung injury.

Allergic *aspergillosis* is typically treated with *triazoles* (such as *itraconazole*, *voriconazole*, *posaconazole*, and *isavuconazole*) in combination with *corticosteroids*. As for invasive *aspergillosis* observed in post-COVID-19 patients, treatment may involve *voriconazole*, lipid *amphotericin* B formulations, *posaconazole*, *isavuconazole*, *itraconazole*, and *echinocandins* (*micafungin* or *caspofungin*). Triazoles can also be administered but require close Therapeutic Drug Monitoring (TDM) to monitor for potential side effects in patients due to *azole* (Bhatt et al. 2021). For *Cryptococcus neoformans* variant infections in immunosuppressed patients post-COVID-19, the recommended treatment protocol includes *trimethoprim*, sulfamethoxazole, and TDM, as outlined by Hoagland et al. (1961). Severe lung infections or central nervous system infections may require the use of *Amphotericin* B in combination with *flucytosine*, as recommended by the CDC (Center for Disease Control and Prevention). Following this, *flucytosine* alone is administered until the patient fully recovers (Bhatt et al. 2021).

In cases of *Candida auris* infections, which are responsible for hospital outbreaks and commonly observed in COVID-19 patients, treatment typically involves *echinocandins* (*caspofungin*, *micafungin*, *anidulafungin*), *azoles* (*fluconazole*, *voriconazole*, *itraconazole*), and *Amphotericin* B, including its liposomal formulations. Identifying *C. auris* poses a challenge due to its multidrug resistance (Bhatt et al., 2021). Salvage options include *Deferasirox* and *Posaconazole* (Spellberg et al., 2009). *Deferasirox* should not be administered for more than 4 weeks due to a substantial increase in toxicity, while *posaconazole* can be administered for longer durations.

In a study by Gebremariam et al. (2019), a new approach to combat mucormycosis was demonstrated. The study revealed that mucormycosis targets human umbilical vein endothelial cells (HUVEC) by using the fungal cell surface protein cot-H3, which specifically binds to another protein on the surface of HUVEC called glucose-regulated protein-78 (GRP-78). When the levels of glucose, iron, and ketone bodies in the bloodstream are higher than normal, the expression of GRP-78 and Cot-H3 is increased, enabling fungal cells to gain access to endothelial cells through endocytosis. This discovery suggests that selectively silencing Cot-H3 expression can halt fungal infection, as Cot-H3 is highly conserved within the order Mucorales and is a promising target for therapeutic interventions. Additionally, the study found that polyclonal antibodies synthesized against two peptides forming the GRP-78 binding region on Cot-H3 effectively prevented invasive and hematogenous dissemination of the fungus.

Another study by Watkins et al. (2018) demonstrated that inhibiting the epidermal growth factor receptor (EGFR) signaling could be a potential strategy against mucormycosis. Through RNA-seq analysis, the researchers investigated the host transcriptional response to *Rhizopus delemar*, a fungal strain, and found that the EGFR pathway was activated by *mucorale* species, facilitating invasion of host cells. Using Gefitinib, an FDA-approved drug, the study showed that it could prevent invasion by modulating gene expression related to EGFR signaling. This research was conducted using human alveolar epithelial cells and *R. delemar*. The study illustrated that EGFR plays a central role in the uptake of *mucorale* and subsequent lung cell damage. By blocking EGFR signaling using Gefitinib and the monoclonal antibody Cetuximab, the research team prevented the fungus from gaining control over the host's body.

2.3.5 Challenges in the treatment of mucormycosis

Despite advancements in fungal infection management strategies, the overall mortality rate remains at 90% in cases of disseminated mucormycosis (Boutin and Luong 2024; Katragkou et al. 2014). Different strains show varying responses to different drugs. Further, *R. oryzae* was found resistant to *posaconazole* in vitro, while *Mucor circinelloids* were susceptible to *posaconazole*. When the *R. oryzae* strain was sequenced, it was revealed that it had genetically adapted to survive in unfavorable environments with antifungal agents. As a result, *itraconazole* and *posaconazole* need to be administered in higher doses for their efficacy, which exceeds the safely achievable drug concentration. Recurring infections by *R. oryzae* in *voriconazole*-administered patients suggest its lack of activity. Similarly, when prescribed liposomal amphotericin B, it is administered at the maximum tolerated dose with a risk of nephrotoxicity (Katragkou et al., 2014).

2.4 Disease burden and demographic characteristics of mucormycosis

According to epidemiological studies, mucormycosis's frequency and characteristics may vary among countries (Prakash and Chakrabarti 2019; Skiada et al. 2020). However, most European countries experienced lower incidences, and after this, haematological malignancies and transplantation emerged as primary risk factors. In contrast to this, India exhibited the highest estimated incidence of mucormycosis, and uncontrolled diabetes is reported as the predominant risk factor. A French study that employed data extraction codes from the French hospital information system and the International Classification of Diseases (ICD) to classify diseases was the primary source of the current countrywide population-based incidence statistics (Sacconi et al. 2018). From 1997 to 2006, there was a rising trend in the incidence of mucormycosis. The incidence rate in France was frequently utilized as a benchmark for evaluating the burden of mucormycosis in other nations (Corzo-León et al. 2015; Pegorie et al. 2017; Sabino et al. 2017). To determine the public health measures and the diagnostic and therapeutic needs of the population, a thorough evaluation of the disease burden and the identification of the group at risk must be performed. However, large-scale epidemiological studies on mucormycosis are still rare in Asia and are mostly restricted to reviews of single- or multicenter studies conducted in India (Prakash and Chakrabarti 2021). Taiwan's National Health Insurance Research Database (NHIRD) can be used as a population-based database for extensive healthcare research (Hsieh et al. 2019). Studies using NHIRD showed an increase in the number of people with diabetes and immunocompromised people over time. A significant rise in the incidence of invasive pulmonary *aspergillosis* from 2002 to 2011 was also observed based on these data (Sheen et al. 2019; Sun et al. 2016). The incidence of mucormycosis was not quantified. Therefore, using *aspergillosis* as a comparator, this study sought to outline the temporal trend of disease burden and demographic characteristics of mucormycosis in Taiwan from 2006 to 2017 based on the NHIRD

3 Previous Case History

The first recorded case of mucormycosis was documented by Furbinger from Germany in 1876 after a cancer patient passed away and scans revealed hemorrhagic infarct of fungal hyphae and

sporangia in the right lung (Skiada et al. 2020). Subsequently, in 1885, Arnold Paltauf reported the first case of disseminated mucormycosis, which he named "mucosismucorina." His illustrations depicted structures resembling rhizoids and sporangiospores, identifying the causative organism as *Lichtheimia corymbifera* (Skiada et al. 2020). Since then, the incidence of mucorales fungal infections has risen, making it the second most common disease-causing mold after *Aspergillus*. Initially deemed incurable, the first documented recovery occurred in the United States in 1955, when a young girl with diabetes was successfully treated with *amphotericin* B (Skiada et al. 2020).

The understanding and findings of mucormycosis advanced when, in 1957-1958, two cases led to the prescription of various antibiotics due to uncertainty regarding the cause of the disease, including *penicillin* and *chloramphenicol* (Hoagland et al. 1961). Once it became evident that the fungus was the underlying cause of the malignancy and was aggressively progressing, the antibiotics were discontinued, and *nystatin* and potassium iodide were administered instead. Similarly, in 1960, *amphotericin* B was used to combat the infection, coupled with surgical intervention to excise the necrotic tissue (Hoagland et al. 1961). A literature review revealed that between 1970 and 1993, 203 cases of mucormycosis were documented (Hendrickson et al. 1999).

In a study discussing the treatment of rhinocerebral mucormycosis with hyperbaric oxygen therapy, Couch et al. (1988) highlighted the main forms of mucormycosis, including pulmonary, rhinocerebral, and intestinal. They noted that individuals at high risk of infection included those with weakened immune systems, diabetes, burns, and those undergoing corticosteroid therapy. The overall mortality rate associated with rhinocerebral mucormycosis was 70% until the 1960s, before the availability of *amphotericin* B and radical surgical debridement. Following the introduction of these treatments, the mortality rate decreased to approximately 40%. The study suggested that hyperbaric oxygen therapy showed potential in treating fungal infections, although the specific mechanism for controlling the infection was not fully understood then. Additionally, the exact duration and dosage of *amphotericin* B required for eradicating the fungus were not yet established (Couch et al. 1988).

In a separate review of cases of rhino-orbito-cerebral mucormycosis, Hendrickson et al. (1999) noted that the fungus could infect individuals of any age or occupation, including seemingly healthy individuals. They also highlighted that individuals with leukemia and lymphomas were particularly susceptible to fungal infection. Other underlying conditions that could potentially lead to mucormycosis included renal disease transplantation, malnutrition, and patients undergoing deferoxamine treatment. It was identified that *deferoxamine* led to alterations in transferrin levels, which enabled the fungus to thrive more easily while decreasing cellular immunity. The study provided general treatment guidelines for rhinocerebral mucormycosis, clearly outlining the dosage of *amphotericin* B and introducing newer formulations, such as liposomal encapsulated and lipid complex *amphotericin* B, which had fewer side effects. The study also indicated that the mode of action of hyperbaric oxygen therapy was being further understood (Hendrickson et al. 1999).

A review by Roden et al. (2005) found that mucormycosis can also target a broader and more diverse population, unlike other fungal infections that mainly affect immunocompromised patients. The review documented that over 50% of the cases were observed in individuals with either diabetes mellitus or no underlying conditions. Additionally, the study noted that while most fungal infections spread from other organs to the skin, mucormycosis exhibited the opposite pattern in cutaneous mucormycosis. The highest percentage of disseminated infection was found in patients taking *deferoxamine* as part of their treatment, indicating iron's importance in the infection's virulence. Some researchers suggested that *estrogen* may be behind the observed higher susceptibility of males to the infection compared to females, but this remains unexplained. Roden et al. (2005) reported a mortality rate of over 54% due to mucormycosis infection. Bitar et al. (2009) studied mucormycosis cases in France from 1997 to 2006, revealing an increase from 0.7 cases per million in 1997 to 1.2 cases per million in 2006. The number of cases was particularly high in patients with hematological malignancies and those who had undergone bone marrow transplants. The study also found a higher incidence of the infection in diabetic patients.

After compiling data from various sources, Danion et al. (2015) reported that the species causing infection varies depending on the geographic area. For example, in North America, most cases were caused by *Rhizopus* spp., followed by *Mucor* spp., *Rhizomucor* spp., and *Lichtheimia* spp. In France, *Rhizopus arrhizus* was the most common, followed by *Leichtheima* and *Rhizopus* microspores, while in India, *Rhizopus arrhizus* and *Apophysomyces elegans* were the most commonly isolated species.

4 Black Fungus Infections in Post-Covid-19 Patients in India: A brief Case Study

As of July 15, 2021, there have been 45,432 reported cases of mucormycosis or black fungus in India, resulting in more than 4,300 deaths. 84.4% of the black fungus patients had previously COVID-19. Among these cases, rhino cerebral mucormycosis is the most common (77.6%), followed by cutaneous (4.3%) and pulmonary (3%) (Dutta 2021; “Mucormycosis: India Records More than 4,300 ‘Black Fungus’ Deaths,” 2021). This "opportunistic black fungus" infects COVID-19 patients by taking advantage of weakened immune systems caused by factors such as

diabetes, cancer, or the prolonged use of medications like steroids. These reports led to a review of 198 case histories related to mucormycosis from literature databases. A meta-analysis was also conducted to identify the determining factors of the black fungus infection, which will be discussed in the upcoming sections.

5 Data Analysis

5.1 Methods of Data Acquisition

Search engines like PUBMED (https://pubmed.ncbi.nlm.nih.gov) and Elsevier (https://www.elsevier.com) have been used to retrieve data using keywords such as "Mucormycosis" or "Black Fungus" in conjunction with "COVID-19" (Table 1) and "Mucormycosis" and "Cases" (Table 2). Each report was thoroughly examined to confirm the presence of both COVID-19 and mucormycosis. Furthermore, we considered cases reported from 1944 to 2021 during our analysis. A mathematical analysis explored the correlation between different co-factors, potentially linking COVID-19 and mucormycosis. Frequency was calculated using Microsoft Excel, where Frequency % = (n/N)×100, with "n" being the total number of observations in each category and "N" being the total number of categorical observations across all categories. A total of 16 entries were documented for Table 1, and 198 entries were accounted for Table 2.

5.2 Information Collected

Data from the database were initially screened using titles and abstracts, followed by full-text reviews. Information was extracted from articles, tables, and graphs, including the year of publication, patient demographics, underlying conditions, infection sites, and prevalence of mucormycosis types. Clinical manifestations were categorized based on affected body locations and infection severity at diagnosis, as determined by histopathological and radiological findings. The data analysis primarily focused on correlating underlying diseases and medications with the occurrence of mucormycosis, although limited information was available on prior medication history. The study also investigated the majorly affected organs and types of mucormycosis.

6 Results and Discussion

A study of post-COVID-19 mucormycosis cases discovered that most cases were rhino-orbito-cerebral mucormycosis (69%), followed by pulmonary mucormycosis (25%). This may be due to the prevalence of diabetes among the patients, which commonly leads to rhino-orbital-cerebral mucormycosis. The study also aimed to establish a correlation between underlying diseases and all types of mucormycosis. It was observed that the most common underlying disease was diabetes (56%), followed by hypertension (50%). In diabetic individuals, the altered immunogenic response to infections creates a favorable environment for fungal proliferation (Figure 1). Diabetes ketoacidosis mucormycosis is generally caused by *Rhizopus oryzae*, which thrives in the patient's ketone bodies. The study revealed that the most vulnerable age group to mucormycosis was 60-69 years (31%), followed by 40-49 years (25%). Higher age groups are more vulnerable due to immunological changes and the administration of immunosuppressive antibiotics (Figure 1). The study also examined the influence of drugs administered for underlying diseases and found that insulin was the most commonly administered drug (50%). It was observed that underlying conditions such as diabetes and hypertension had a greater involvement in certain types of mucormycosis. However, the total number of post-COVID-19 mucormycosis cases studied was only 16, so a meta-analysis was performed using a larger dataset (198 studies) to understand the underlying factors better.

Upon analyzing Table 2, the data revealed that most cases were associated with specific types of mucormycosis. Rhino-orbito-cerebral mucormycosis accounted for 42.42% of the cases, followed by pulmonary mucormycosis (17.68%) and cutaneous mucormycosis (13.13%) (Figure 2A). It is important to note that several other types of mucormycosis, such as renal, intestinal, orbital, ENT, orbito-facial, and hepatic, were also documented. These additional types were grouped under the main five categories of mucormycosis to simplify data analysis and to facilitate the generation of general conclusions. The symptoms of rhino-orbito-cerebral mucormycosis included fever, eye discomfort, facial swelling, and headache. Pulmonary mucormycosis presented with symptoms such as fever, fatigue, cough, and chills, while cutaneous mucormycosis manifested as lesions and ulcerations in the affected area.

It was observed that the most common underlying condition preceding mucormycosis was diabetes (31%), followed by leukemia (20%) (Figure 2B). Leukemia, especially in combination with neutropenia (> 500 neutrophils per mm^3 for more than 10 days), prolonged use of broad-spectrum antibiotics for over 96 hours, extensive chemotherapy, and prolonged use of corticosteroids, was found to be a significant contributing factor to mucormycosis (Bhatt et al. 2011).

Analysis of affected body parts before the diagnosis of mucormycosis revealed that the most affected body part was the bone marrow (17%), followed by the pancreas (15%) and the kidneys (12%) (Figure 2C). This could be attributed to therapeutic strategies such as bone marrow transplantations in leukemia patients, as well as the increased vulnerability of diabetic patients, leading to a higher risk for kidney and pancreas complications. The use of various drugs for the underlying conditions was also examined. While this data was often unavailable (45%), it was noted that *prednisone* (11%), *insulin* (7%), and chemotherapy (6%) were the most commonly used drugs before the diagnosis of mucormycosis

Table 1 Correlation of post-COVID-19 Mucormycosis patients with underlying diseases

No of death	Age	Gender	Type of Mucormycosis	Any disease before COVID + Mucormycosis	Drug administration for that disease (Generic name)	Class of drug	Drug administration for COVID	Class of Drug administered for COVID	Organs infected pre-Mucormycosis	Probable infected organs	Infected regions during Mucormycosis	Complications faced	Reference
1	60	M	Rhino-orbital	Diabetis Mellitus	N/A	Antihypoglycemic Medication	Meropenem , oral oseltamivir , Methylprednisolone, Dexamethasone	Carbapenem antibiotic, Antiviral neuraminidase inhibitor, Corticosteroid, Glucocorticoid	N/A	Kidney	Lungs, Brain, Eyes,	Breathlessness, Pyrexia, Tachypnea, Malaise	Mehta and Pandey 2020
1	22	M	Pulmonary	Obesity, Hypothyroidism	N/A	N/A	N/A	N/A	N/A		Lungs	N/A	Hanley et al. 2020
1	49	M	Pulmonary	None	None	None	Remdesivir, Tocilizumab, Dexamethasone	Nucleoside analog, IL-6 receptor inhibitor	N/A		Lungs	Fever, cough, and shortness of breath	Placik et al. 2020
1	86	M	Gastrointestinal	Arterial hypertension	N/A	N/A	Ceftriaxone, Azithromycin, Oseltamivir, Hydrocortisone	Cephalosporin antibiotic, Macrolide antibiotic, Antiviral neuraminidase inhibitor, Glucocorticoid	N/A	Heart	Lungs, Intestine	Acute diarrhea, Cough, dyspnea, fever	Monte Junior et al. 2020
1	66	M	Pulmonary	Arterial hypertension	N/A	ACE-inhibitors	Hydroxychloroquine and lopinavir-ritonavir	Anti-rheumatic drug, HIV-1 protease inhibitor,	N/A	Heart	Lungs	Rapid deterioration of oxygenation	Pasero et al. 2020
1	24	F	Rhino-orbital	Obesity	N/A	Data not provided	Not Administred	Not administered	N/A		Nose, Lungs	Pain in left midface region, left lid swelling, Maxillary hypoesthesia	Waizel-Haiat et al. 2021
1	55	M	Pulmonary	Diabetes mellitus, Hypertensio, and Ischemic cardiomyopathy	N/A	Oral hypoglycemic drugs	Dexamethasone, remdesivir	Glucocorticoid, nucleosideanalog	N/A	Heart, Kidney	Lungs	Fever, Dry cough, and Progressive breathlessness	Garg et al. 2021

No of death	Age	Gender	Type of Mucormycosis	Any disease before COVID + Mucormycosis	Drug administration for that disease (Generic name)	Class of drug	Drug administration for COVID	Class of Drug administered for COVID	Organs infected pre-Mucormycosis	Probable infected organs	Infected regions during Mucormycosis	Complications faced	Reference
0	46	M	Rhino-orbital-cerebral	Diabetes Mellitus	N/A	Insulin	Not Administred	Not Adminitstered	N/A	Kidney	Eyes	Pain, Redness, Periocular swelling, Progressive drooping of eyelids, Limitation of ocular movements, Painful loss of vision	Sen et al. 2021
0	60	M	Rhino-orbital-cerebral	Diabetes Mellitus, hypertension	N/A	Insulin	Methylprednisolone, Oral prednisolone	Corticosteroid	N/A	Kidney, Heart	Eyes	Pain, Redness, Periocular swelling, Progressive, Drooping of Eyelids, Limitation of ocular movements, Painful loss of vision	Sen et al. 2021
0	73	M	Rhino-orbital-cerebral	Diabetes Mellitus, hypertension, Coronary artery disease	N/A	Insulin	Methylprednisolone, Oral prednisolone	Corticosteroid	N/A	Kidney, Heart	Eyes	Pain, Redness, Periocular swelling, Progressive, drooping of eyelids, Limitation of ocular movements, Painful loss of vision	Sen et al. 2021
0	72	M	Rhino-orbital-cerebral	Diabetes Mellitus	N/A	Insulin	Methylprednisolone, Oral prednisolone	Corticosteroid	N/A	Kidney	Eyes	Pain, Redness, Periocular swelling, progressive, Drooping of eyelids, Limitation of ocular movements, Painful loss of vision	Sen et al. 2021

No of death	Age	Gender	Type of Mucormycosis	Any disease before COVID + Mucormycosis	Drug administration for that disease (Generic name)	Class of drug	Drug administration for COVID	Class of Drug administered for COVID	Organs infected pre-Mucormycosis	Probable infected organs	Infected regions during Mucormycosis	Complications faced	Reference
0	62	M	Rhino-orbital-cerebral	Diabetes Mellitus, hypertension	N/A	Insulin	Methylprednisolone, Oral prednisolone	Corticosteroid	N/A	Kidney, Heart	Eyes	Pain, Redness, Periocular swelling, progressive, Drooping of eyelids, Limitation of ocular movements, Painful loss of vision, Headache	Sen et al. 2021
0	47	M	Rhino-orbital-cerebral	Diabetes Mellitus, Coronary artery disease	N/A	Insulin	Methylprednisolone, Oral prednisolone	Corticosteroid	N/A	Heart, Kidney	Eyes	Pain, Redness, Periocular swelling, Progressive, drooping of eyelids, Limitation of ocular movements, Painful loss of vision	Sen et al. 2021
0	41	M	Rhinocerebral	Diabetes mellitus	N/A	Insulin	Hydroxychloroquine	Anti-rheumatic drug	N/A	Kidney	Nose	Deep aching pain in nose and throat	Alekseyev et al. 2021
1	60	M	Rhino-orbital	Diabetes, Asthma, Hypertension, Hyperlipidemia	N/A	Insulin	Remdesivir	Nucleoside analog	N/A	Lungs, Kidney, Heart	Nose	Dyspnea and hypoxia	Mekonnen et al., 2021
1	33	F	Rhino-orbital-cerebral	Hypertension and asthma	N/A	N/A	N/A	N/A	N/A	Heart, Lungs	Brain, Lungs	Vomiting, cough, and shortness of breath	Werthma-Ehrenreich 2021

Various post-COVID-19 cases of Mucormycosis had been taken into account. A total of 17 entries, from the year 2020 to 2021, had been used to perform data analysis. Susceptibility of the disease due to age, most frequent type of mucormycosis and the most common underlying medical comorbidities were studied. Medication and class of drugs taken for the underlying disease as well as COVID-19 were documented.

Table 2 Correlation of Mucormycosis patients with underlying diseases

Year	Place	No of patients affected	No of survival	No of death	Type of Mucormycosis	Any disease before Mucormycosis	Drug administration for that disease /Other Treatment(if any)	Organs infected pre-Mucormycosis	Symptoms prior to diagnosis of mucormycosis	Organs infected during Mucormycosis	Reference
1944	U.S	1	0	1	Rhino cerebral	N/A	N/A	N/A	Immovable left arm and leg, Right pupil larger than right with inflammation, Purulent discharge	Eyes, Lungs, Brain	Lecompte and Meissner 1947
1951	U.S	1	0	1	Rhino cerebral	N/A	N/A	N/A	Vitreous humor filled with dust like opacities of rhomboid, Refractile body, Pale gray, Elevated area in lower temporal region	Eyes	Chikley et al. 2019
1953	Georgia	1	0	1	Rhino cerebral	Diabetic mellitus	Protamine-zinc insulin		Spinal cord was grey, Pooled, Turbid with flattened convolution	Brain , Meningitis	Bauer et al. 1955
1954	England	2	2	0	Rhino cerebral	Diabetic	Insulin		Loss of vision, Loss of sensation in the eye, Ulcer in the roof of mouth	Brain	Baker 1957
1954					Rhino cerebral	Diabetic acidosis	N/A		Left eye swelling, Left ethmoid sinusitis	Brain	Baker 1957
1954	U.S	1	0	1	Rhino cerebral	Diabetic	N/A		Retrobulbar space white granular material enveloped the eye muscle, Extended to posterior aspect of the globe	Brain, Eyes	Bauer et al. 1955
1955	U.S	2	2	0	Rhino Orbital	N/A	N/A	N/A	Corneal ulcer, Pain in left eye	Left eye	Barsky 1959
1955					Rhino Orbital	Chronic simple glaucoma	Pilocarpine	Left eye	Severe pain in left eye	Left eye	Barsky 1959
1956	England	1	0	1	Rhino cerebral	Portal hypertension	Portacaval shunt operation	Liver	Fever, Orbital pain	Brain	Baker 1957
1957	Georgia	1	1	0	Rhino cerebral	Diabetic,pancreatitis	Streptomycin	Pancreas	Pain in right malar area, Swelling and redness in right side of the face with decreased sensation	Right eye	Hoagland et al. 1961
1957	U.S	1	1	0	Rhino cerebral	N/A	N/A	N/A	Proptosis of right eye	Eyes,Brain	Faillo et al. 1959
1957	U.K	4	4	0	Rhino Orbital	N/A	N/A	N/A	Pain, Injection, Irritation, Lacrimation, Loss of visual activity of right eye	Right eye	Anderson et al. 1959
1957					Rhino Orbital	Both eye burn	Steroids	Eyes	Swelling, Pain in operated area	Eyes	Anderson et al. 1959
1957					Rhino Orbital	N/A	N/A	N/A	Injection , Decrease in vision, Pain in right eye	Right eye	Anderson et al. 1959

Year	Place	No of patients affected	No of survival	No of death	Type of Mucormycosis	Any disease before Mucormycosis	Drug administration for that disease /Other Treatment(if any)	Organs infected pre-Mucormycosis	Symptoms prior to diagnosis of mucormycosis	Organs infected during Mucormycosis	Reference
1957					Rhino Orbital	Conjunctivitis and Corneal ulceration	Sodium sulfacetamide	Eyes	N/A	Eyes	Anderson et al. 1959
1957	U.S	2	2	0	Rhino Orbital	Corneal abscess of left eye	Erythromycin, Procaine penicillin G, Neohydreltasol	Left eye	Perforated cornea	Left eye	Barsky 1959
1957					Rhino Orbital	Diabetic mellitus	N/A	N/A	Pain, Inflammation, Cormeal ulcer in left eye, Blindness	Left eye	Barsky 1959
1958	U.S	4	0	4	Pulmonary	Leukemia, Steroid induced Diabetic	prednisone (meticorten) and 6-mercaptopurine	Bone marrow	Cushingoid features, Fever	Heart, Lungs	Hutter 1959
1958	U.S				Pulmonary	Leukemia	6-chloropurine	Bone marrow	Weakness, Fatigue	Lungs, Spleen	Hutter 1959
1958	U.S				Disseminated	Lymphosarcoma, Metabolic acidosis	X-ray and meticorten	Bone marrow	Respiratory difficulty	Lungs, Vocal cord, Heart, Esophagus , Stomach and small intestine	Hutter 1959
1958	U.S				Disseminated	Acute leukemia, Hepatosplenomegaly, Steroid induced Diabetic	6-Mercaptopurine	Bone marrow	Vesicular eruption on face, Neck, Right arm, Thigh	Brain, Lungs, Stomach, Small intestine	Hutter 1959
1958	U.K	1	1	0	Rhino Orbital	N/A	N/A	N/A	Itching , Burning, Inflammation of left eye	Left eye	Anderson et al. 1959
1958	U.S	2	2	0	Rhino Orbital	N/A	N/A	N/A	Corneal ulcer, Pain in left eye	Eyes	Barsky 1959
1958	U.S				Rhino Orbital	Chronic corneal ulcer	N/A	Eyes	N/A	Eyes	Barsky 1959

Year	Place	No of patients affected	No of survival	No of death	Type of Mucormycosis	Any disease before Mucormycosis	Drug administration for that disease /Other Treatment(if any)	Organs infected pre-Mucormycosis	Symptoms prior to diagnosis of mucormycosis	Organs infected during Mucormycosis	Reference
1959	Canada	1	0	1	Rhino cerebral	N/A	N/A	N/A	Fever, Vomiting, Diarrhoea, Left cheek pain, Anorexia	Liver, Spleen, Eyes, Brain	Dolman and Herd 1959
1959	U.S	1	0	1	Rhino cerebral	Diabetic mellitus, Pneumonia, Right renal necrotizing papillitis	Insulin, right kidney removal	Kidney, Lungs	Pain in left upper jaw, Numbness, Swelling in left cheek	Eyes, Nose, Brain	Dwyer and Changus 1958
1959	U.S	1	0	1	Dissemi nated	Diabetic mellitus	N/A	N/A	Dehydration, Shock, Coma	Brain, Lungs, Genital tract, Kidney	Long and Weiss 1959
1961	U.S	2	0	2	Dissemi nated	Extensive third degree burn with 64% burn	Novocain, Chloramphenicol, Penicillin	Skin	Occasional fever	Face, Nasal cavity, Brain,Menin ges, Kidney, Stomach	Rabin et al. 1961
1961					Dissemi nated	Extensive third degree burn with 45% burn	Penicillin, Chlortetracycline, Streptomycin, oxytetracyclin	Skin	Fever	Face, Nasal cavity, Kidney, Heart	Rabin et al. 1961
1962	U.S	1	1	0	Dissemi nated	Diabetic mellitus	Tolbutamide	N/A	Severe frontal headache, Chills, Fever, Sore throat, Weakness	Eyes, Brain, Face	Prockop and Silva-Hutner 1967
1965	U.S	1	0	1	Gastroin testinal	N/A	N/A	N/A	Abdominal pain, Swelling, Chills, Hot sweats	Diaphragm, Abdomen, Lungs	Calle and Klatsky, 1966
1966	U.S	2	2	0	Rhino cerebral	Diabetic mellitus	Tolbutamide	N/A	Cough with yellow sputum, Lethargy, Difficulty in breathing	Brain , Eyes, Lungs	Battock et al. 1968
1966	U.S			0	Rhino cerebral	Diabetic mellitus	Insulin	N/A	Frontal headache, Stiff neck, Protrusion of left eye, Progressive double vision	Left eye, Left side of mouth	Battock et al. 1968

Year	Place	No of patients affected	No of survival	No of death	Type of Mucormycosis	Any disease before Mucormycosis	Drug administration for that disease /Other Treatment(if any)	Organs infected pre-Mucormycosis	Symptoms prior to diagnosis of mucormycosis	Organs infected during Mucormycosis	Reference
1966	U.S	1	1	0	Cutaneous	Leukemia	Chemotherapy with Prednisolone, Vincristine, L-asparaginase, Cytosine arabinoside	N/A	Erythema in right arm	Lungs, Right arm	Wirth et al. 1997
1967	U.S	1	0	1	Rhino cerebral	Diabetic mellitus	N/A	N/A	Sore throat, Bifrontal headache, Poluuria, Excessive thirst, Lethargy	Right eye, Brain	Price et al. 1971
1968	U.S	3	0	3	Rhino cerebral	Anaemia and uremia	N/A	N/A	Double vision	Brain, Nose, Eyes, Cheek	Price et al. 1971
1968	U.S				Rhino cerebral	Diabetic mellitus, Diabetic retinopathy, Hypertension	Tolbutamide	N/A	Protrusion, Numbness, Painless swelling	Nose , Eyes	Price et al. 1971
1968	U.S				Rhino cerebral	Diabetic mellitus, Nephropathy , Recurrent bacterial infection	NPH insulin	N/A	Afebrile, Unresponsive	Brain, Meninges	Price et al. 1971
1970	U.S	1	1	0	Other	Progressive renal failure	Hemograft renal transplant, Azathioprine, Prednisone, Antilymphocytic globulin	Kidney	Purulent nasopharyngeal discharge, Dysosmia	Nose	Stevens et al. 1972
1970	U.S	1	1	0	Pulmonary	Acute lymphocytic leukemia	Prednisone, chlorambucil	Lymph node	Short of breath, Cough, Fever	Lung	Medoff and Kobayashi 1972
1970	U.S	1	0	1	Rhino Orbital	N/A	N/A	Eyes, Brain	Diarrhoea, Vomiting, Fever, Draining ears	Eyes, Face	Hale 1971

Year	Place	No of patients affected	No of survival	No of death	Type of Mucormycosis	Any disease before Mucormycosis	Drug administration for that disease /Other Treatment(if any)	Organs infected pre-Mucormycosis	Symptoms prior to diagnosis of mucormycosis	Organs infected during Mucormycosis	Reference
1972	U.S	1	0	1	Pulmonary	Diabetic,renal failure requiring peritoneal dialysis	Cephalothin, Chloramphenicol, Gentamicin, Isoniazid, Rifampin	Lungs	N/A	Lungs	Murray 1975
1972	England	1	0	1	Rhino cerebral	None	None	None	Frontal headache in right side, Decreasing vision in right eye, Swelling in right periorbital area	Eyes	Lowe and Hudson, 1975
1973	U.S	1	0	1	Pulmonary	Acute promyelocytic leukemia	Daunorubicin	Bone marrow	N/A	Lungs	Murray 1975
1973	U.S	2	2	0	Rhino Orbital	Diabetic	N/A	N/A	Decreased vision in right eye followed by total loss of perception	Left eye, Brain	Bullock et al. 1974
1973	U.S				Rhino Orbital	Alcohol withdrawal seizure	N/A	N/A	Chemosis, Blepharoptosis in right eye	Right eye, Brain	Bullock et al. 1974
1975	U.S	1	1	0	Cutaneous	Chronic myelogenous leukemia,Diabetic mellitus	Busulfan	Spleen	Pain in right deltoid area	Right arm	Jain et al. 1978
1976	U.S	1	0	1	Pulmonary	Knee problem	Bilateral knee surgery , Cephalothin sodium prophylaxis	N/A	Fever, Chills, Cough with yellow blood tinged sputum	Lung	Record and Ginder 1976
1977	South Africa	1	1	0	Gastrointestinal	Steven-johnson syndrome	N/A	Skin	Vomiting, Epigastric pain, Loss of weight, Constipation	Stomach	Schulman et al. 1979
1977		30	2	28	Gastrointestinal	Diabetic	N/A	N/A	Bloody diarrhoea (13)*	Stomach (19)	Michalak et al. 1980
1977					Gastrointestinal	Diabetic	N/A	N/A	Diarrhoea (7)	Colon (14)	
1977					Gastrointestinal	Leukemia	N/A	N/A	Clinical obstruction (3)	Small bowel (8)	

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1977					Gastroin testinal	Leukemia	N/A	N/A	Perforation (5)	Esophagus (6)	
1977					Gastroin testinal	Typhoid fever	N/A	N/A			
1977					Gastroin testinal	Typhoid fever	N/A	N/A			
1977					Gastroin testinal	Malaria	N/A	N/A			
1979	U.S	1	0	1	Rhino Orbital	Acute stem cell leukemia, Diabetic mellitus	Bone marrow smears, Thioguanine and Cytosine arabinoside	N/A	Small dark gangrenous lesion in right superior gum	Eyes, Brain	Albert et al. 1979
1980	U.S	2	2	0	Cutaneo us	Acute lymphocytic leukemia	Anti-leukemic chemotherapy	bone marrow	Diffusely swollen area in upper right extremity , Lower right extremity, Left lingual area, Left auxillary area	Skin	Ryan et al. 1982
1980					cutaneou s	Acute lymphocytic leukemia	Chemotherapy	bone marrow	Elevated, Dark, Painful lesion with rim of Erythema in inner aspect of right lower leg	Right leg tibia, Skin	Ryan et al. 1982
1981	U.S	1	0	1	Dissemi nated	Refractory anaemia, Dyserythropoie sis	Prednisone, Pyridoxine, Multiple transfusion, Splenectomy	N/A	Fever , Chills, Confusion	Lung, Kidney	Ingram et al. 1989
1982	U.S	1	0	1	Dissemi nated	Hairy-cellleukemia	Prednisone, Chlorambucil	N/A	Fever, Cough with bloody sputum, Hypotension	Lung, Liver, Brain	Ingram et al. 1989
1983	U.S	2	0	2	Dissemi nated	Lymphocytic leukemia, Diabetic mellitus and splenectomy	Vincristine, Prednisone, Chlorambucil	N/A	Fatigue, Shortness of breath	Lungs, Liver, Kidney, Stomach, Lymph nodes	Ingram et al. 1989

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1983	U.S				Disseminated	Polycystic renal disease	Hemodialysis and deferoxamine	Kidney	Headache, Numbness on right side of the body	Brain , Lungs, Kidney	Ingram et al. 1989
1984	U.S	1	1	0	Pulmonary	Diabetic ketoacidosis	N/A	N/A	N/A	Right lung	Christenson et al. 1987
1984	U.S	1	0	1	Disseminated	Uremia	Hemodialysis	Kidney	Hypercalcemia, Jaundice	Lungs, Jejunum, Ileum	Eiser et al. 1987
1984	U.S	1	1	0	Gastrointestinal	Uremia and hypertension	Hemodialysis and bilateral nephrectomy	N/A	Pericarditis	Intestine	Eiser et al. 1987
1985	Israil	1	1	0	Cutaneous	Hypertension	Thiazides	N/A	Unconsciousness	Skin, Lungs	Koren et al. 1986
1986	India	1	0	1	Disseminated	Acute renal disorder	N/A	Kidney	Hiccups, Vomiting , Tarry stools	Lungs, Kidney, Pancreas	Gupta et al. 1987
1986	U.S	1	1	0	Cutaneous	Meningioma	Dexamethasone	Right thigh	Painless, Non-erythematous weeping ulcer in right thigh	Right thigh	Umbert and Su 1989
1986	India	1	1	0	Cutaneous	Tuberculoid granuloma	Streptomycin, isoniazid	N/A	Painful swelling of left foot, Multiple discharging sinuses, Low grade fever	Left foot	Padhye et al. 1988
1986	Australia	1	1	0	Pulmonary	Pneumothoraces in left lung	Surgical pleurodesis	Left lung	Weight loss, Persistent cough , Fatigue	Left lung	Lake et al. 1988
1987	U.S	1	1	0	Cutaneous	Myelogenous leukemia	Intrathecal chemotherapy	Right leg	Hemorrhagic necrotic ulcer in right leg, Area surrounded by edema	Right foot	Umbert and Su 1989
1987	Australia	1	0	1	Disseminated	Acute hepatic failure,encephalopathy,coagulopathy	Charcoal hemoperfusion, orthotopic liver transplantation	Liver	Fever, General deterioration after hepatic transplant	Liver, Heart, Lungs, Brain, Thymus	Nimmo et al. 1988
1988	Italy	1	0	1	Rhino cerebral	Drug addict	N/A	N/A	N/A	Meninges, Brain	Oliveri et al. 1988

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1990	U.S	1	1	0	Rhino cerebral	Cervical squamous cell carcinoma, HIV	Estrogen hormone treatment	Pelvis	Bifrontal headache, Inability to speak clearly	Brain, Lungs, Stomach, Small intestine	Cabot et al. 1990
1991	U.S	1	1	0	Cutaneous	Acute myelocytic leukemia	Chemotherapy with cytarabine, Duanorubicin, Ecotoposide	Bone marrow	Fever, Pleuritic pain, Cough with hemoptoic sputum	Lungs, Right hand	Lopes et al. 1995
1991	Netherlands	1	1	0	Cutaneous	N/A	N/A	N/A	Swelling in lateral right eyebrow that gradually extended	Skin of face	Prevoo et al. 1991
1992	U.S	1	1	0	Pulmonary	Megakaryoblasti cleukemia	Chemotherapy involving duanomycin, Cytosine arabinoside	N/A	Fever, Irritability in behaviour	Left lung	Cohen-Abbo et al. 1993
1992		1	0	1	Pulmonary	Lymphocytic leukemia	Chemotherapy involving mitoxantrone, Cytosine arabinoside	N/A	Intermittent cough, Loose stools , Decreased appetite, Upper abdomen discomfort	Lungs	Cohen-Abbo et al. 1993
1993	Spain	1	1	0	Gastrointest inal	AIDS	Steroids	Immune system	Epigastric pain, Retrosternal discomfort	Stomach, Ileum, Colon	Brullet et al. 1993
1993	U.S	1	1	0	Cutaneous	Aplastic anaemia, Hepatitis A and B, Pneumonia	Antithymocyteglobulin, prednisone, cyclosporin, aminocaproic acid	N/A	Fever, Chills, Bleeding gums, Epistaxis	Thigh	Weitzman et al. 1993
1994	U.S	1	1	0	Cutaneous	Monocytic leukemia	Chemotherapy involving mitoxantrone and diazoquinone	N/A	Lesion in left knee, calf along with palpable lump	Left leg	Fingeroth et al. 1994
1995	U.S	1	0	1	Pulmonary	Bronchial asthma	B2 agonist inhaler	Lungs	Dyspnea, Productive cough	Lungs	Butala et al. 1995
1995	U.S	1	1	0	Pulmonary	Major renal disorder, Diabetic nephropathy	Renal allograft , Prednisone, Solumedrol, Azathioprine	Kidney	Fever, Right foot ulceration	Lungs	Latif et al. 1997
1995	U.S	1	1	0	Other	Diabetic	Clindamycin, Nafcillin	Leg	Necrotic ulcer of proximal right leg, Vomiting, Nausea	N/A	West et al. 1995

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1995	Austria	1	1	0	Gastrointestinal	End stage renal disease	Dialysis, Cyclosporin A , Steroid, Antithymocyte globulin	Kidney	N/A	Stomach	Winkler et al. 1996
1995	U.S	3	2	1	Other	AIDS	Phenobarbital, Nystatin, Aerosolized pentamidine	N/A	Flank pain, Pyuria, Fever	Kidney	Nagy-Agren et al. 1995
1995					Disseminated	AIDS , peptic ulcer disease,anaemia	Trimethoprim Sulfamethoxazole, Fluconazole and Omepraconazole	N/A	Fever, Diarrhoea	kidney, Liver, Spleen, Thyroid, Bone marrow	Nagy-Agren et al. 1995
1995					Disseminated	AIDS, Presumed progressive multifocal leukoencephalopathy	Acyclovir, Dilantin, Aerosolized pentamide	N/A	Recurrent herpes	Small/ Large intestine, Stomach, Blood vessel	Nagy-Agren et al. 1995
1995		1	1	0	Pulmonary	End stage renal disease	Cyclosporin, Azathioprine, Solumedrol	N/A	Soreness of left anterior chest wall, Non-productive cough, Fever	Lungs	Latif et al. 1997
1996	France	1	1	0	Cutaneous	Aplastic anaemia	Antithymocyteglobulin (ATG)	Bone marrow	Febrile neutropenic episode, Fever, Erythema, Tenderness	Heart, Lungs	Leong et al. 1997
1996	China	1	0	1	Pulmonary	Lymphoblastic leukemia	N/A	Bone marrow	N/A	Lungs, Heart	Levy et al. 1996
1996	China	1	0	1	Other	Acute lymphoblastic leukemia	Cytosine arabinoside, Amsacrine	Liver	N/A	Liver, Lungs	Levy et al. 1996
1998	U.S	1	1	0	Rhino cerebral	Diabetic mellitus	Insulin	N/A	Headache, Nasal discharge, Malaise, Lethargy, Fever	Brain	Weprin et al. 1998
1999	Belgium	5	1	4	Pulmonary	Acute lymphoblastic leukemia (ALL)	Vindesine and Methylprednisolone	N/A	Relapse after Bone marrow Transplant	Lung , Oesophagus	Maertens et al. 1999

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1999					Pulmonary	Chronic myelogenous leukemia (CML)	N/A	N/A	Relapse after Bone marrow Transplant	Lung	Maertens et al. 1999
1999					Pulmonary	Acute Myeloblastic Leukemia with maturation (AML- M2)	N/A	N/A	Relapse after Bone marrow Transplant	Lung	Maertens et al. 1999
1999					Disseminated	Acute Myeloblastic Leukemia with maturation (AML- M2)	N/A	N/A	Relapse after Bone marrow Transplant	Lung	Maertens et al. 1999
1999					Disseminated	Relapsed Acute Myeloblastic Leukemia(AML M5)	N/A	N/A	Relapse after Bone marrow Transplant	Liver, esophagus	Maertens et al. 1999
2000	U.S	1	1	0	Other	Chronic renal insufficiency, Hypercholesterolemia , Diabetic	Kidney , Heart transplant	Kidney and heart	Fever, Chills	Heart, Thorax	Tobon et al. 2003
2002	India	1	1	0	Cutaneous	None	None	None	Fever, boil in upper abdomen	Skin of the abdomen	Kumar et al., 2003
2003	Greece	2	2	0	Rhino cerebral	Diabetic mellitus, Hypertension, Heart failure, Chronic obstructive lung disorder	Gliclazide	N/A	Malodorous discharge from mouth, Necrotic lesion in palatal mucosa	Brain	Kofteridis et al. 2003
2003	Greece				Rhino cerebral	Hypertension, Eye problem, Renal dysfunction	Methylprednisolone	N/A	Left eye infection	Left eye, Brain	Kofteridis et al. 2003
2004	U.S	1	1	0	Pulmonary	Uncontrolled Diabetic	N/A	N/A	Dry, Non-productive cough,Dyspnea, Malaise	Upper lobe of lungs	Reid et al. 2004
2005	India	2	2	0	Cutaneous	N/A	N/A	N/A	Abscess in right leg	Right leg	Shah et al. 2006
2005					Cutaneous	Respiratory distress	Corticosteroid, Broad spectrum antibiotics	N/A	Reddish brown area in perineum	Skin	Shah et al. 2006

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2005	U.S	1	1	0	Rhino cerebral	Diabetic mellitus, Allergic rhinitis, Recurrent sinusitis	N/A	N/A	Right sided facial drooping, Numbness, Diplopia, Right eye ptosis	Eye, Sphenoid sinus	Liang et al. 2006
2006		1	0	1	Rhino cerebral	Cerebral thrombosis, Myocardial infarction, Diabetic mellitus, Hypertension	N/A	N/A	Visual loss of right eye, Pain in right side of the headache	Tongue, Nose, Face, Brain	Yang et al. 2006
2006	Sri-lanka	1	1	0	Rhino cerebral	Malaria	N/A	N/A	Swelling in left side of face	Face, Brain	Jayasuriya et al. 2006
2006	Korea	1	1	0	Gastrointestinal	Acute myeloid leukemia	Idarubicin and cytosine arabinoside	N/A	Diffuse abdominal pain	Stomach	Song et al. 2006
2006	South korea	1	1	0	Rhino Orbital	None	None	None	Right sided facial pain	Paranasal sinus	Park et al. 2006
2006	India	2	2	0	Other	None	None	None	Bilateral flank pain,Fever , Vomiting, White flakes in urine	Kidney	Marak et al. 2010
2006	India				Other	N/A	N/A	N/A	Right flank pain, Fever, Chills and Rigours	Kidney	Marak et al. 2010
2007	Germany	4	3	1	Rhino-orbito-cerebral	Diabetic mellitus	N/A	N/A	Rapid loss of vision, Proptosis bulbi, Ophthalmoplegia	Eyes, Nose	Arndt et al. 2009
2007					Rhino-orbital-cerebral	Hodgkin’s lymphoma	N/A	N/A	Irradiation of the skull	Nose	Arndt et al. 2009
2007					Rhino-orbital-cerebral	Acute Myeloidleukemia (AML)	N/A	N/A	Proptosis bulbi	Eyes, Nose	Arndt et al. 2009
2007	Germany				Rhino-orbital-cerebral	Myelodysplastic syndrome	Cyclosporin A, Anti Myocyte-globuline	N/A	Paranasal sinusitis	Eyes	Arndt et al. 2009
2007	Turkey	2	2	0	Rhino-orbito-cerebral	Idiopathic thrombocytopenic purpura	Antidiabetic medication	N/A	Left facial / periorbital pain, Paraesthesia, Erythema, Visual loss	Brain, Paranasal sinus, Ethmoid sinus	Haliloglu et al. 2008

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2007					Rhino-orbito-cerebral	Diabetic mellitus , Hypertension	N/A	N/A	Headache, Fever, Vomiting, Swelling in upper eyelid	Brain, Paranasal sinus	Haliloglu et al. 2008
2007	Taiwan	1	1	0	Rhino cerebral	Chronic hepatitis, Liver cirrhosis, Diabetic mellitus, Hypertension	N/A	N/A	Pain, Swelling in left side of face	Brain	Lin et al. 2012
2008	North America	1	1	0	Cutaneous	Progressive pulmonary silicosis	Lung transplant , Methylprednisolone	Lungs	Necrosis at site of membrane ventilator placement	Right inguinal region, Thorax	Page et al. 2008
2008	U.S	1	1	0	Gastrointestinal	Hepatitis-C, Hypertension , End-stage renal disease	Hemodialysis	Kidney, Heart	N/A	Colon, Liver	Mezhir et al. 2009
2008	korea	1	1	0	Pulmonary	Renal damage, Pulmonary mucormycosis	Amphotericin B	N/A	Severe nasal obstruction, Facial tenderness	Brain, Lungs	Kim et al. 2013
2008	Australia	1	1	0	Pulmonary	End stage renal failure, Diabetic, Hypertension	Insulin	N/A	Persistent cough	Lungs	Li et al., 2009
2009	Saudi Arabia	1	0	1	Pulmonary	Diabetic mellitus, Aplastic anaemia	Insulin, Tacrolimus	N/A	Fever, Chest pain, Dyspnea	Lungs	Waness et al. 2009
2009	Greece	1	1	0	Disseminated	Acute myeloid leukemia	Cytosine arabinoside,thioguanine and idarubicin	N/A	Febrile, Fever	Liver, Lungs, Brain	Skiada et al. 2009
2009	Israil	1	0	1	Rhino cerebral	Hypertension	Hydrochlorothiazide	N/A	Left palate pain	Brain	Elinav et al. 2009
2010	France	1	1	0	Rhino-orbito-cerebral	AcidoKetotic coma	N/A	N/A	Mucopurulent rhinorrhea, General deterioration of health	Nasal fossa, Right ethmoid, Left anterior ethmoid	Mimouni et al. 2010
2011	Iran	1	0	1	Disseminated	Diabetic mellitus	N/A	N/A	Fever, Polyuria, Polydipsia, Vomiting	Lungs	Mohammadi et al. 2012
2011	Taiwan	1	1	0	other	Hepatitis-B	Tacrolimus, Mycophenolic acid	Liver	Migraine, Swelling , Right sided visual impairment	Eyes, Ethmoid sinus, Paranasal sinus, Sphenoid sinus	Lin et al. 2011

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2011	Italy	1	1	0	Rhino cerebral	Acute lymphoblastic leukemia	Teicoplanin, Imipenem, Caspofungin	N/A	Fever, Painful movement in right eye, Swelling, Palpatation	Right eye, Brain	Gumral et al. 2011
2011	China	1	1	0	Pulmonary	Renal disease and Diabetic mellitus	Renal transplant, Prednisolone, Cyclosporin A, Mycophenolate	N/A	Fever, Dyspnea, Leukocytosis	Lung	Kwan et al. 2013
2012	Japan	2	2	0	Cutaneous	Chronic lymphocytic leukaemia (CLL)	Prednisolone	N/A	Painful purpura/subcutaneous induration (left palm)	Bronchi, Skin	Kawasaki et al., 2012
2012					Cutaneous	Acute myelocytic leukemia (AML)	Prednisolone, Tacrolimus, Mitoxantrone, Etoposide, Cytarabine, Methotrexate	N/A	Lesions head, Nape of neck, Bilateral forearms, Lower limbs	Skin, Lungs	Kawasaki et al., 2012
2012	Malaysia	1	1	0	Rhino-orbito-cerebral	None	None	None	Painful swelling in left eye, Blurring of vision, Diplopia	Eyes, Brain	Shatriah et al. 2012
2012	Taiwan	1	0	1	Disseminated	Acute myeloid leukemia	Blood transfusion	N/A	Fever, Dyspnea, Cough, Loss of appetite	Lungs, Skin	Hsieh et al. 2013
2013	India	1	1	0	Rhino cerebral	Systemic lupus erythematosus (SLE), Anemia	Methylprednisolone, Prednisolone	N/A	Pallor, Swelling face and legs, Hematuria, Oliguria, Diffuse hair loss	Skin	Kumar et al. 2013
2013	China	1	1	0	Cutaneous	Diabetic, Coronary atherosclerosis heart disease, Atrial fibrillation	N/A	N/A	Painful Erythema in forearm	Skin	Li et al. 2013
2013	Iran	1	1	0	Cutaneous	Diabetic	Metformin	N/A	Severe pain, Swelling / bruising in upper extremity, Necrotizing region in hand	Left arm, Forearm	Ahmadinejad et al. 2013
2013	India	1	1	0	Pulmonary	Diabetic mellitus	Insulin	N/A	Tooth ache on left side of face, Swelling, Pain in face	Airways, Eyes, Nose	Singh et al.,2013

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2013	Italy	1	1	0	Rhino cerebral	Diabetic mellitus	Regulatory insulin therapy	N/A	Dental pain, Facial swelling, Ecchymosis in left periorbital region, Decreased visual activity and colour vision	Brain, Eyes, Tongue, Teeth	Di Coste et al. 2013
2014	India	2	2	0	Rhino-orbito-cerebral	Hyperglycemia, Diabetic ketoacidosis, Ketonuria	N/A	N/A	Fever, Abdominal pain, Acidotic breathing, Multiple furuncles over the face	Sinus and Brain	Kumar et al. 2014
2014					Cutaneous	Deep dermal full thickness 60% TBSA burn	Topical antimicrobials, Aseptic antiseptic precautions	Both thighs	Fever, Tachycardia , Tachypnoea	Both thighs	Kumar et al. 2014
2014	U.K	1	1	0	Rhino-orbito-cerebral	Diabetic, Chronic kidney disease , Hypertension, Chronic pancreatitis	N/A	N/A	Left sided facial hyperesthia, Retro-orbital discomfort, Blurred vision, Persistent headache	Left eye, Kidney,Brain	Chow et al. 2014
2014	U.S	1	1	0	Gastrointestinal	B-cell acute lymphoblastic leukemia, Stage 3 rectal cancer	Cetuximab, Capecitabine	N/A	Hemorrhagic shock, Acute hematochezia	Small intestine, Large intestinal, Left leg, Kidney, Ureter	Cloyd et al. 2014
2014	Greece	1	1	0	Rhino cerebral	Diabetic mellitus, Coronary artery disease, Cerebrovascular episodes	Antidiabetic, Hypertensive, Anticoagulant	N/A	Odorous nasal discharge	Brain	Dimaka et al. 2014
2015	Australia	1	1	0	Cutaneous	Diabetic mellitus, Metastatic non-small cell lung cancer	Prednisolone, Chemotherapy	N/A	Erythema, Ulcer, Skin lesion in right lower leg	Right leg	Gardiner et al. 2015
2015	Mexico	1	0	1	Pulmonary	Non-hodgkin T Cell lymphoma	Etoposide,carboplatin,cytarabine	N/A	Fever, Malaise, Persistent cough	Kidney, intestine, abdomen	Rodríguez-Gutiérrez et al. 2015
2016	India	1	1	0	Pulmonary	Diabetic mellitus	N/A	N/A	Fever and cough	Lungs	Biradar et al. 2016

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2016	Spain	1	0	1	Pulmonary	Diabetic mellitus, Siderosis, Renal agenesis	N/A	N/A	Neutropenic fever	Lungs, kidney, thyroid gland	Mouronte-Roibás et al. 2016
2016	North america	1	0	1	Rhino Orbital	Chronic diarrhoea, Recurrent bronchial neuropathy	N/A	N/A	Fever	Ear, face	Kermani et al. 2016
2016	North America	1	1	0	Rhino Orbital	Diabetic	N/A	N/A	Fever, Poorly systematized left inferior lobe infection, Ketoacidosis decomposition	Maxillary sinus, mouth	Kermani et al. 2016
2016	North America	1	1	0	Cutaneous	Arterial hypertension, Diabetic mellitus	N/A	N/A	Pain, Occasional bleeding of lower back	Skin	Rodríguez-Lobato et al. 2017
2017	India	2	0	2	Rhino-orbito-cerebral	Diabetic mellitus	N/A	N/A	Unconsciousness	Brain, Skin	Pathak et al. 2018
2017					Pulmonary	Chronic Persistent asthma, Diabetic mellitus	N/A	N/A	N/A	Lungs	Pathak et al., 2018
2017	Saudi Arabia	1	1	0	Disseminated	Diabetic mellitus	N/A	N/A	Diarrhea, Cough, Weight loss, Shortness of breath	Lungs, stomach	Alqhamdi et al. 2019
2017	Korea	5	4	1	Rhino cerebral	Chronic lymphocytic leukaemia (CLL)	Rituximab, Fludarabine, Cyclophosphamide	N/A	Fever, odontalgia, submandibular swelling	Ectodermal organ (teeth)	Cheong et al. 2017
2017					Rhino cerebral	Acute myeloid leukaemia (AML)	N/A	N/A	Fever, right submandibular swelling	Ectodermal organ (teeth)	Cheong et al. 2017
2017					Rhino cerebral	Acute promyelocytic leukaemia (APL)	N/A	N/A	Neutropenic fever	Ectodermal organ (teeth)	Cheong et al. 2017
2017					Rhino cerebral	Acute myeloid leukaemia (AML)	N/A	N/A	N/A	Ectodermal organ (teeth)	Cheong et al. 2017
2017					Rhino cerebral	Acute myeloid leukaemia (AML)	N/A	N/A	N/A	Ectodermal organ (teeth)	Cheong et al. 2017
2017	China	1	0	1	Gastrointestinal	Rheumatic heart disease, Mitral stenosis	Oral warfarin	N/A	Fever, Difficulty in eating, Abdominal distension, Nausea, Dispnea	Small intestine	Sun et al. 2017

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2017	Pakistan	1	1	0	Other	ALK negative anaplastic large cell lymphoma	N/A	N/A	N/A	Liver	Yasmeen et al. 2017
2017	Mexico	1	0	1	Rhino cerebral	Liver cirrhosis	Prednisone	Liver	Respiratory tract infection, Lethargy , Nose bleed	Nose, Lips, Lungs, Eye, Brain	Avelar Rodriguez et al. 2017
2017	Australia	1	1	0	Pulmonary	End stage renal failure, Diabetic nephropathy	Antithymocyte globulin, Prednisolone, Mycophenolate, Tacrolimus	N/A	Cough, Fever, Hemoptysis	Lungs	Thomas et al. 2018
2017	Iran	1	0	1	Rhino cerebral	Acute lymphoblastic leukemia	Acyclovir, Fluconazole and Ciprofloxacin	Bone marrow	Lethargy, Fever, Chills ,Nasal discharge, Weakness, Difficulty in swallowing	Lungs, Paranasal sinus, Brain	Sharifpour et al. 2018
2018	Korea	1	1	0	Rhino cerebral	Diabetic mellitus, Asthma	N/A	N/A	Nasal obstruction	Brain	Yeo et al. 2018
2018	Greece	1	1	0	Cutaneous	Hypothyroidism	N/A	N/A	Right thigh lesion	Right leg	Gkegkes et al. 2019
2018	Iran	2	2	0	Rhino cerebral	Diabetic, Ischemic heart disease, Neutropenia	Insulin	N/A	Headache, Blurred vision, Pain, Swelling in right eye	Brain	Gholinejad Ghadi et al. 2018
2018					Rhino cerebral	Diabetic mellitus	N/A	N/A	Blurred vision, Headache, Swelling in left posterior maxilla	Brain	Gholinejad Ghadi et al. 2018
2018	U.S	3	3	0	Pulmonary	Hepatitis associated severe aplastic anaemia	Prednisone, Cyclophosphamide, Voriconazole, ATG	N/A	Fever, Pneumonia	Lungs	Elgarten et al. 2018
2018					Pulmonary	Chronic granulomatous disease	Fludarabine, Cyclophosphamide , ATG, Calcineurin inhibitor	N/A	N/A	Lungs	Elgarten et al. 2018
2018					Gastrointestinal	Kidney / Renal disease	Methylprednisolone, Tacrolimus, Mycophenolate mofetil	Kidney, Liver	Abdominal pain, Dark stool	Stomach	Elgarten et al. 2018

Year	Place	No of patients affected	No of survival	No of death	Type of Mucormycosis	Any disease before Mucormycosis	Drug administration for that disease /Other Treatment(if any)	Organs infected pre-Mucormycosis	Symptoms prior to diagnosis of mucormycosis	Organs infected during Mucormycosis	Reference
2018	India	1	1	0	Other	N/A	N/A	N/A	Fever, Turbid urine, Diffuse abdominal pain, Vomiting , Swelling of the body	Kidney	Mathew et al. 2019
2019	Taiwan	1	0	1	Pulmonary	Systemic lupus erythematosus (SLE)	Rituximab	N/A	Fever, Chills, Shortness of breath	Lungs	Hung et al. 2015
2019	U.S	1	0	1	Cutaneous	Liver / Renal failure, Hypertension, Macrocystic anaemia	Blood transfusion, Antithymocyte globulin, Phenolic acid, Tacrolimus	Kidney, Liver	Abdominal surgical site became necrotic and oozed out serosanguinous fluid	Surgical site of abdomen	Haque et al. 2019
2019	China	1	1	0	Gastrointestinal	End stage renal disease	Methylprednisolone, Basiliximab, Tacrolimus, Mycophenolate mofetil	Kidney	Chest pain, Dark stool	Stomach	Peng et al. 2019
2019	India	1	1	0	Rhino cerebral	Neonatal sepsis, End stage organ failure	N/A	N/A	Increasing size of head, Downward gaze, Unable to hold neck straight	Brain	Gupta et al. 2019
2019	Pakistan	1	1	0	Rhino cerebral	Diabetic Ketoacidosis	N/A	N/A	Ulcers in oral cavity, Facial swelling along, Oral, Nasal discharge	Brain, Skin	Ali Asghar et al. 2019
2019	India	1	1	0	Disseminated	Diabetic mellitus	N/A	N/A	Fever, Cough with expectoration, Anorexia	Lungs, Skin	Ramesh et al. 2020
2019	Mexico	1	1	0	Pulmonary	Diabetic mellitus, Hypertension	Insulin glargine	N/A	Chest pain	Bronchi, Lungs	O et al. 2019
2019	U.S	1	1	0	Gastrointestinal	Diabetic mellitus	N/A	N/A	Chronic cough, Weight loss, Fever	Stomach, Brain, Lungs	Malek et al. 2019
2019	U.S	1	0	1	Pulmonary	Bipolar disorder, Hyperthyroidism	N/A	N/A	Fever, Fatigue, Non-productive cough, Myalgia	Kidney, Brain, Liver	Huang et al. 2020
2019	U.S	1	1	0	Gastrointestinal	Diabetic mellitus, End-stage renal disease, TB	Dexamethasone, ethambutol,isoniazid,pyridoxine	Brain and lungs	Acute abdominal pain	Stomach	Malek et al. 2019

Year	Place	No of patients affected	No of survival	No of death	Type of Mucormycosis	Any disease before Mucormycosis	Drug administration for that disease /Other Treatment(if any)	Organs infected pre-Mucormycosis	Symptoms prior to diagnosis of mucormycosis	Organs infected during Mucormycosis	Reference
2019	Australia	1	1	0	Rhino Orbital	T-cell acute lymphoblastic leukemia	N/A	N/A	Fever, Headache, Nasal congestion, Facial pain	Brain, Eyes, Nose	Lee and Sullivan, 2019
2019	Australia	1	1	0	Rhino Orbital	Diabetic ketoacidosis	N/A	N/A	Febrile, Headache	Brain	Lee and Sullivan, 2019
2019	Australia	1	0	1	Rhino Orbital	Pre-B cell acute lymphoblastic leukemia	chemotherapy and allogeneic bone tranplant	N/A	Nasal pain with bloody discharge, Skin discolouration , Fever, Decreased vision	Brain, Face, Nose	Lee and Sullivan, 2019
2019	Australia	1	0	1	Rhino Orbital	Biphenotypicleukemia	chemotherapy	N/A	Left lower eyelid swelling	Nose, Cheek, Mouth, Brain	Lee and Sullivan, 2019
2019	Australia	1	1	0	Rhino Orbital	High risk pre-B cell acute lymphoblastic leukemia	chemotherapy	N/A	Recurrent headache, Irritability	Brain	Lee and Sullivan, 2019
2019	Australia	1	0	1	Rhino Orbital	Graft vs host disease, B cell acute lymphoblastic leukemia	prednisolone-A,cyclophosphamide,mycophenolate	N/A	Severe headache	Brain, Right arm	Lee and Sullivan, 2019
2020	Australia	1	1	0	Disseminated	Diabetic ketoacidosis, Hypertension, Kidney disease	Ramipril, Amlodipine Insulin aspart, Amitriptyline	Kidney	Central Chest pain, Dyspnea, Pedal edema	Lungs	Thomas et al. 2020
2020	U.S	1	0	1	Pulmonary	N/A	N/A	N/A	Cough, Dyspnea, Respiratory distress	Lungs	Seifert et al. 2020
2020	Iran	5	5	0	Rhino Orbital	N/A	N/A	N/A	Erythema, Edema in left inferomedial canthus	Brain	Amanati et al. 2020
2020					Rhino Orbital	Nasolacrimal duct obstruction	N/A	N/A	Swelling in right inferomedial canthus	Brain	Amanati et al. 2020
2020					Rhino Orbital	N/A	N/A	N/A	Purulent discharge, Eye proptosis	Eye, brain	Amanati et al. 2020
2020					Rhino Orbital	N/A	N/A	N/A	Fever, Edema periorbital area	Brain	Amanati et al. 2020

Year	Place	No of patients affected	No of survival	No of death	Type of Mucormycosis	Any disease before Mucormycosis	Drug administration for that disease /Other Treatment(if any)	Organs infected pre-Mucormycosis	Symptoms prior to diagnosis of mucormycosis	Organs infected during Mucormycosis	Reference
2020					Rhino Orbital	None	None	None	Swelling in periorbital region	Brain	Amanati et al. 2020
2020	U.S	1	1	0	Pulmonary	Diabetic ketoacidosis	N/A	N/A	Fevers, Dyspnea, Rightsubclavicular chest pain	Lungs, Trachea, Bronchi	Elmassry et al. 2020

A collection of 198 cases were accounted for patients having various types of mucormycosis, including Rhino-orbital-cerebral, Pulmonary, Cutaneous, Gastrointestinal, Disseminated and others. The timeline begins from the year 1944 and continues till 2020. The place, type of mucormycosis, status of the patient, underlying diseases and drugs taken due to them, symptoms faced by patient and the organ / region of body affected were documented; N/A: Data not available; *: data in parenthesis represents the number of cases

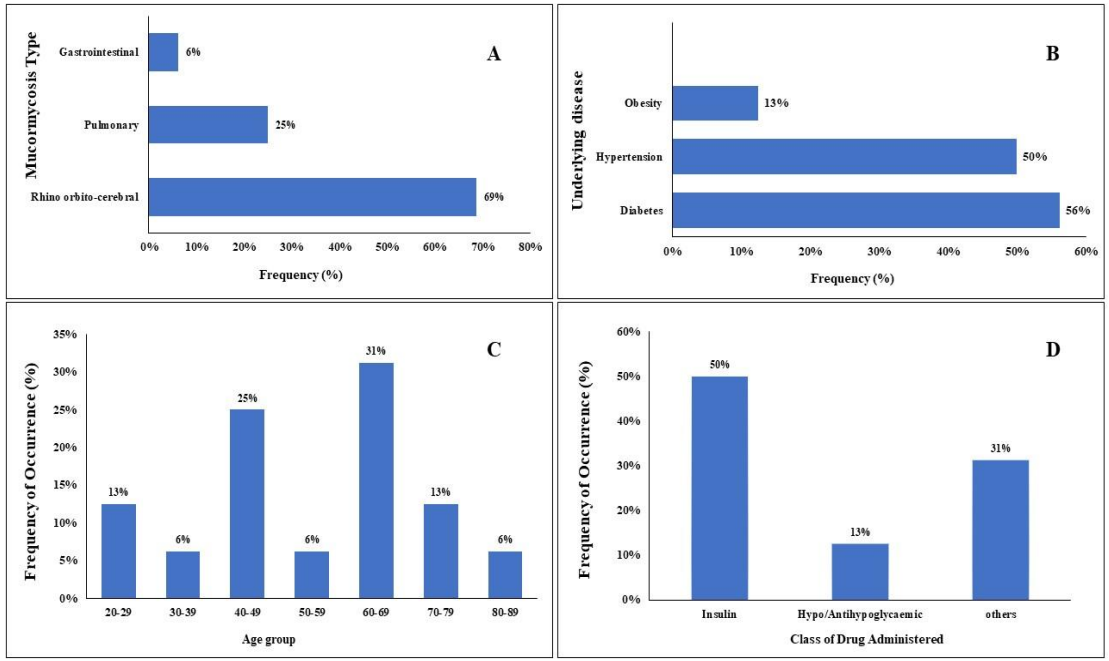

Figure 1 Total number of case histories analysed = 16; (1A) occurrence of mucormycosis in post-COVID-19 patients; (1B) underlying diseases prior to the diagnosis of mucormycosis; (1C) various age groups affected by mucormycosis post COVID-19; (1D) Influence of drugs for underlying diseases on mucormycosis patients.

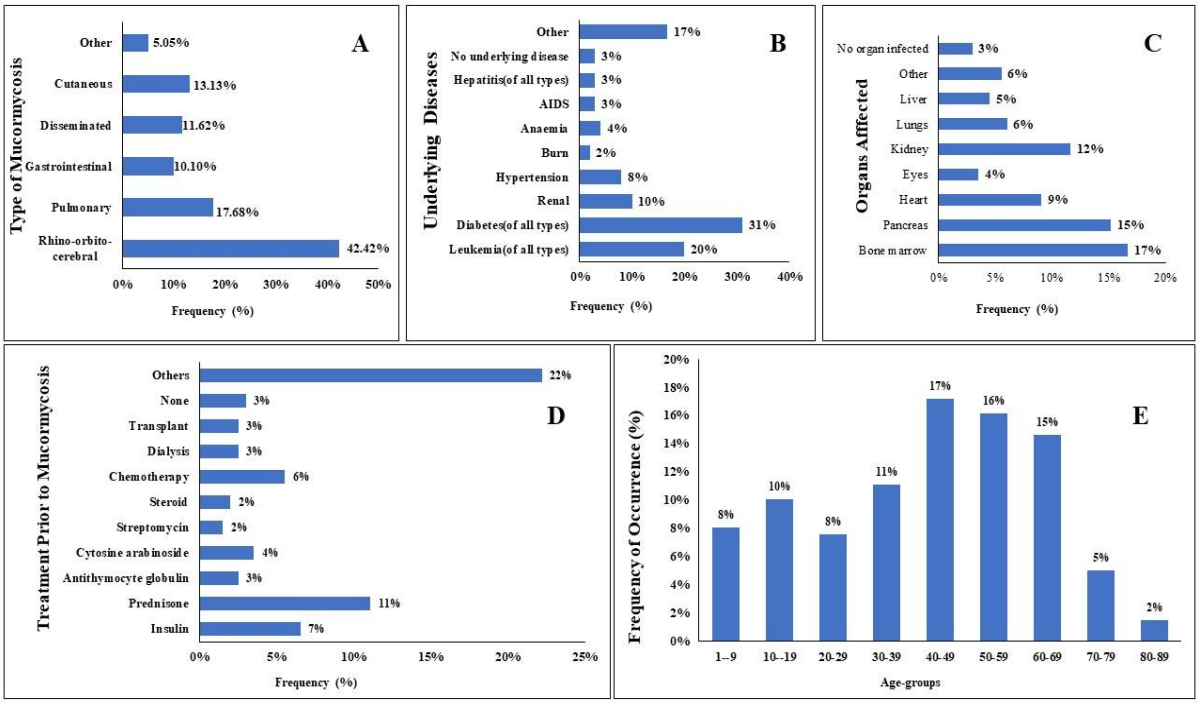

Figure 2 Total number of case histories analysed = 198; (2A) Occurrence of mucormycosis in patients; (2B) Underlying diseases prior to diagnosis of mucormycosis; (2C)Most affected organs before diagnosis of mucormycosis; (2D) Influence of drugs for underlying diseases on mucormycosis patients; (2E) Various age groups affected by mucormycosis

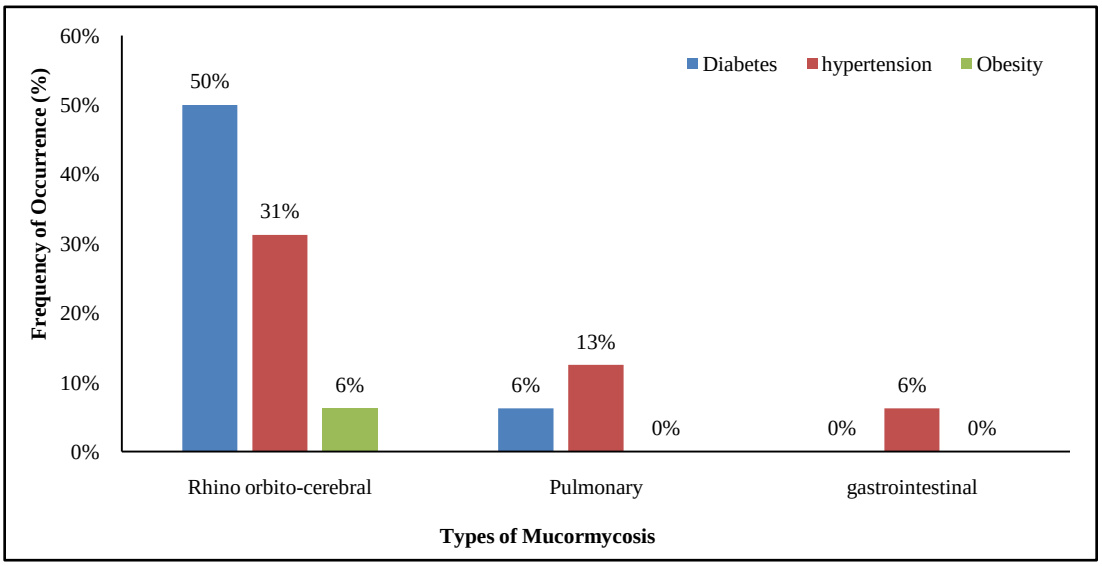

Figure 3 Comparison of types of mucormycosis in post COVID-19 patients with various underlying medical factors (Total number of case histories analysed = 16)

(Figure 2D). Prednisone, a corticosteroid widely used by immunocompromised patients, was found to make diabetic patients more vulnerable to mucormycosis infection due to its adverse effects. Similarly, chemotherapy disrupts the normal growth and development of immune cells and the renewal of epithelial cells, rendering the host more susceptible to pathogenic attacks (Teoh and Pavelka, 2016).

It was also observed that individuals with competent immune systems have been infected by mucormycosis. The apparent depressed immune response in these individuals may be due to a biphasic response to sepsis, where an initial hyperinflammatory response is followed by immune paralysis, leading to neutrophil deactivation and placing the individual at high risk of mucormycosis (Bassetti and Bouza 2017). In terms of

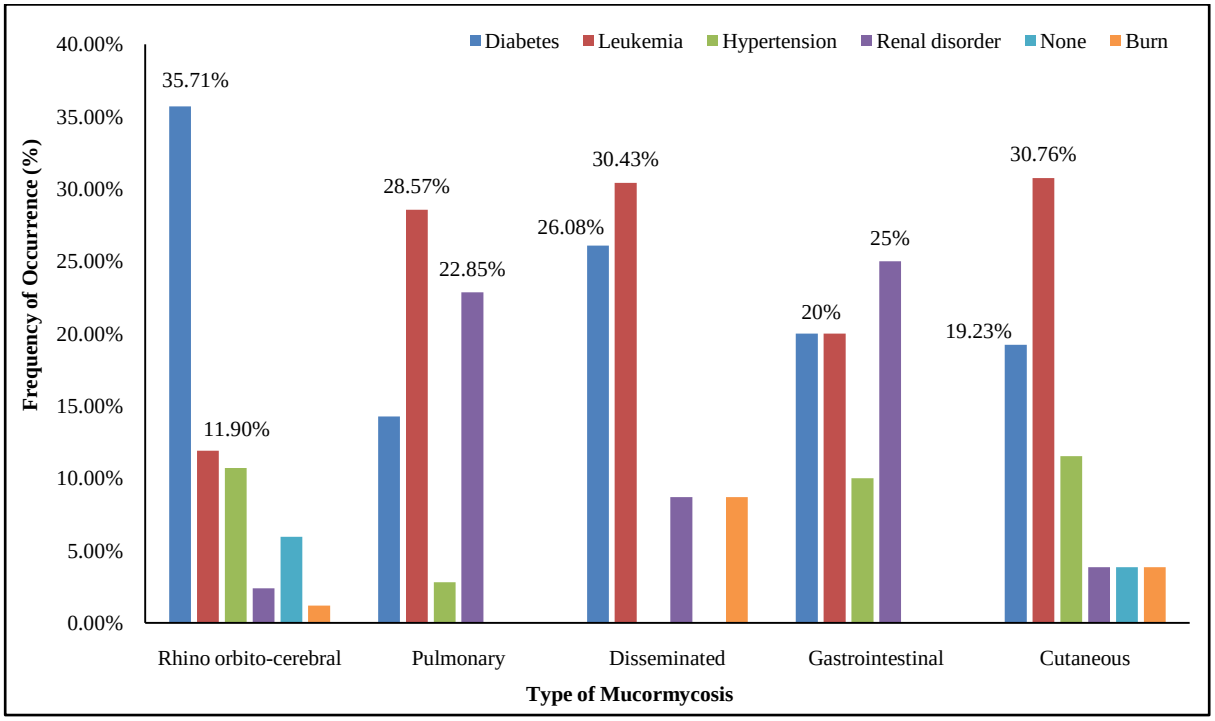

Figure 4 Comparison of types of mucormycosis with various underlying medical factors. Total number of case (histories analysed = 198)

susceptibility, the age groups most at risk of mucormycosis were patients between the ages of 40-49 years (17%), followed by those in the 50-59 years age group (16%) and the 60-69 years age group (15%) (Figure 2E). Age significantly impacts susceptibility to fungal infections due to immunological changes, administration of immunosuppressive medication, antibiotics, underlying chronic conditions, and solid organ transplantation (Kauffman 2001). Furthermore, a study on the correlation between underlying conditions and the type of mucormycosis showed that diabetes played a significant role in rhino-orbito-cerebral mucormycosis patients (35.71%). Leukemia was more prevalent in cutaneous (30.76%), disseminated (30.43%), and pulmonary mucormycosis (28.57%). Renal disorders had the highest association with gastrointestinal mucormycosis (25%) (Figure 3 & 4).

Conclusion

Mucormycosis is a rare and deadly fungal disease caused by inhaling fungal spores known as mucormycetes. The rarity of the disease makes it challenging to conduct large clinical trials. It has a very high mortality rate and is treated with antifungal agents such as isavuconazole, amphotericin B-based drugs, or posaconazole combined with surgical intervention. Several types include rhino-orbito-cerebral, pulmonary, cutaneous, gastrointestinal, and disseminated mucormycosis. Among COVID-19 patients, rhino-orbito-cerebral and pulmonary mucormycosis are most common. Certain groups, such as diabetic and immunocompromised patients, are more susceptible. The study showed that rhino-orbito-cerebral mucormycosis is most commonly diagnosed, and diabetes is the most frequent underlying condition. Age is also a determining factor. The study did not deeply explore the effect of various drugs taken for underlying conditions on the disease due to limited data, but it would be beneficial for future studies to do so.

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Conflict of Interest

Nil

Authors' contributions

Srishti Sen and Shubhangi Tiwari contributed equally to acquire data and perform the analysis. These authors performed the literature survey and wrote the entire manuscript. Sinjini Banerjee contributed to data acquisition by studying various case histories. Mihir Ghosh critically reviewed and corrected the manuscript. Boudhayan Bandyopadhyay conceived the idea of this research project and played a critical role in guiding the authors in this entire work.

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Effect of stress during exam time on immunity - A Survey based study

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ABSTRACT

Recent research indicates an escalating prevalence of stress among students during exam time. Our study aims to explore the correlation between stress induced by exams, its impact on immunity, and the varying effects of stress levels on students' health outcomes. A random online questionnaire survey involving 252 students across three educational levels, school, undergraduate, and postgraduate, have been conducted in this study. This study assessed stress levels, related symptoms experienced during exams, and stress-related health outcomes. The data were analyzed using Venn diagrams and statistically interpreted with Pearson correlation analysis and one-tailed ANOVA. The results revealed that across all three educational levels, females experience higher stress levels than males during exam periods. Additionally, females facing similar stress levels were found to be more susceptible to health issues than their male counterparts. Increased stress levels were correlated with higher incidences of weakness and digestive problems. These findings are consistent with previous research indicating that females are significantly more affected by stress than males and that stress is associated with adverse health outcomes. Our study underscores the need for further investigation into stress and immune response dynamics. Future research could explore blood biomarkers to understand these relationships better.

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1 Introduction

Stress is the body's natural response to any form of physical, mental, or emotional strain or upheaval, serving as a vital mechanism for survival in challenging situations. It comprises a sequence of events: a stressor initiates a reaction in the brain and activates the body's fight-or-flight systems (Mahassni and Eskandar 2019). When under stress, the body perceives itself to be under attack, triggering a cascade of physiological changes, including heightened heart rate, blood pressure, and release of stress hormones. These changes can impact hormones, brain function, behavior, and other bodily systems, which potentially affect immune function (Morey et al. 2015) and other health disorders like depression and anxiety (Shields and Slavich 2017).

Stress comes in various forms distinguished by its duration, i.e., chronic, acute, or episodic acute stress (Cohen et al. 2007). Acute stress is short-lived and may trigger increased levels of proinflammatory cytokines in the blood (Steptoe et al. 2007). In contrast, chronic stress persists for extended periods, ranging from days to years. Like acute stress, chronic stress is associated with elevated levels of proinflammatory cytokines but potentially differing health implications (Gouin et al. 2012). Additionally, chronic stress can activate latent viruses, indicating a loss of immunological control over these pathogens. Frequent activation of latent viruses due to chronic stress can lead to immune system strain and deterioration over time (Pawelec et al. 2005). The impact of stress can differ based on the nature of the stressors encountered, exerting varying effects on the neuroendocrine, autonomic, and central nervous systems, consequently influencing immunological function (Hossain et al. 2006). Exposure to psychological stressors can modulate the significant primary antibody response (Moraska et al. 2002). Academic exams are an example of a brief naturalistic stressor where the subject faces a short-term, real-world problem (Baum et al. 1993).

Given the variability in individual responses to stress, it becomes apparent that immune responses also differ among people. Some studies have explored these inter-individual differences in immune functioning, particularly in response to brief naturalistic stressors (Maydych et al. 2017). For example, engaging in relaxation practices has been associated with increased percentages of T helper cells and higher numbers of T and B lymphocytes during stressful periods, such as academic examinations (Glaser et al. 1986). Conversely, factors like loneliness (Kiecolt-Glaser et al. 1984), emotional instability, and high anxiety have been linked to diminished natural killer cell activity during exams (Borella et al. 1999). Moreover, psychological traits associated with resilience may protect against immune suppression or dysregulation during stressful academic situations (Segerstrom et al. 1998).

The role of cognitive factors in immunological processes, including cognitive states and beliefs, represents a relatively nascent and under-explored area in psychoneuroimmunology. However, limited studies have begun to examine how affective and cognitive factors influence immune responses during brief naturalistic stressors like academic examinations.

Stress related to academic exams has been shown to have a substantial adverse effect on students' well-being and is linked to both physical and mental health problems, including elevated anxiety, depressive symptoms, and immune system dysfunction (Cohen and Herbert 1996). The physiological stress response can become permanently activated in humans due to their capacity to create and experience psychological stresses without external stressors, which frequently have negative consequences (Dhabhar et al. 2012).

Exam stress provides a pertinent framework for investigating the impact of psychological stress on the immune system (Stowell 2003). Confronting academic examinations represents a real-world challenge that elicits varying degrees of stress among individuals. Within the field of psychoneuroimmunology, academic stress is often characterized as a brief naturalistic stressor, encompassing acute moments (immediately before and during exams) as well as prolonged periods (such as during preparation or revision) (Segerstrom and Miller 2004; Preuss et al. 2010). Academic exams thus straddle the spectrum between acute and chronic stress. These designs allow comparing students' immune status before and after exams (Katsuura et al. 2010). Typically, baseline immune status is assessed weeks before and compared with measurements taken shortly before or after exams. However, fewer studies examine immune parameters throughout the exam stress period, including the anticipation and post-exam periods. Various stressful events, including academic examinations, combat tasks, vigilance, and sleep deprivation, have been associated with diminished human immune system functioning (Loft et al. 2007). Psychological or behavioral events such as anger, anxiety, and depression can impact autonomic nervous system activity and hormonal regulation, thereby influencing immune responses. This intricate relationship underscores the brain and immune system's significant influence on each other. Academic exam stress has been shown to affect students' well-being, leading to heightened anxiety profoundly, negative mood shifts, and alterations in immune function (SHAMS et al. 2010). Understanding these dynamics provides valuable insights into the complex interplay between psychological stressors and immune function, offering potential avenues for intervention and support. This study assesses the direct relationship between exam-induced stress and its impact on immune-related outcomes. In this study, we surveyed students across three educational stages, i.e., school level (SL), undergraduate level (UG), and postgraduate level (PG), which represent diverse academic disciplines. The main objective of this

study was to analyze the stress levels experienced by students during exam periods and how it affects their health and immunity. During the study, significant variations were observed in stress levels between genders, and different stress levels were investigated to determine how they correlate with various health outcomes, revealing differences in health impacts between males and females.

2 Materials and Methods

2.1 Survey and subject selection

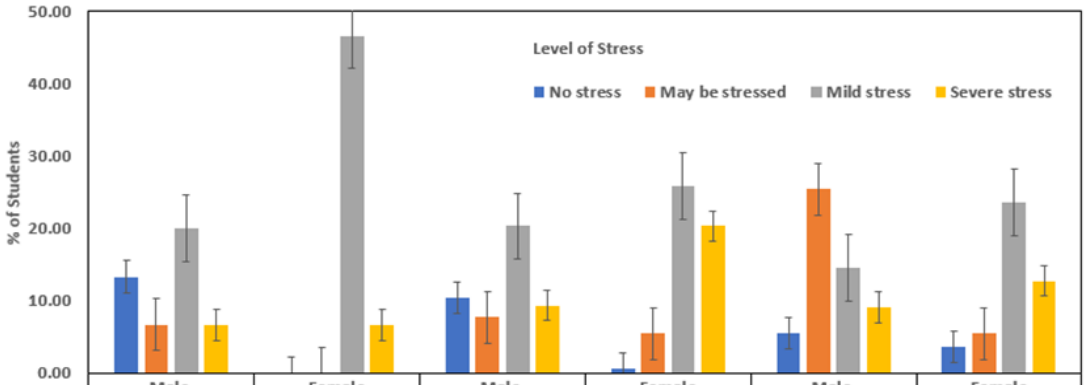
A random questionnaire study was conducted online using Google Forms during the academic year 2021-2022, following the COVID-19 pandemic in April and May 2021-2022. The questionnaire primarily aimed to assess the levels of stress and the related symptoms experienced by students during exam periods. All questionnaires were completed, although some respondents were excluded from the study as the information was inaccurate. Stress levels were categorized into four tiers: no stress, maybe stressed, mild stress, and severe stress, with symptoms also categorized according to stress severity. A total of 252 students responded to the questionnaire. Among them, 15 were SL students, 182 were from UG, and the remaining 55 belonged to PG. All the questionnaires were filled out with prior consent from the participants, including the fact that the identity of the subjects shall remain confidential.

2.2 Study Design

The questionnaire consisted of three main sections. The first part gathered personal data such as gender, age, program of study, and current class levels. In the second part, stress levels were assessed as no stress, maybe stressed, mild stress, and severe stress, along with associated symptoms like increased heart rate, stomach upset, nausea, diarrhea, headaches, lower back pain, menstrual cycle changes, feeling a "lump in the throat," sore or aching muscles, fatigue that does not improve with sleep, feeling uncoordinated and increased or decreased appetite which may be accompanied with weight loss or gain. Additionally, participants rated their level of weakness as slightly weak, moderately weak, very weak, and no weakness at all and indicated cognitive effects such as memory problems/forgetfulness, disorientation (state of mental confusion), confusion, slowness in thinking, analyzing or comprehending (understand) and loss of objectivity (ability to make decisions based on facts rather than on your personal feelings or beliefs). Finally, the third part of the questionnaire asked whether participants experienced several health outcomes during exam time, such as diseases, digestive problems, infections, or inflammation during exam periods, with response options provided as Yes, No, and Maybe.

2.3 Data Interpretation

The survey data was structured into a tabular format to organize responses regarding stress levels for males and females across different educational levels (SL, UG, PG). A graph depicting this information was generated using Excel, and the data has been provided in (Figure 1). Assessment of co-occurrence between health outcomes and stress levels among participants was done by Venn diagrams created using Meta-charts. We utilized the data from the Venn diagrams by converting it into percentage values and organizing it into a tabular format. This method has also analyzed how different health outcomes coincide with varying stress levels. The collected survey data on various health outcomes involved assigning a value of 1 to responses indicating "Yes" or "Maybe." Specifically, "Maybe" was assigned a value of 1 only



	SL		UG		PG	
	Male	Female	Male	Female	Male	Female
No stress	13.33	0.00	10.44	0.55	5.45	3.64
May be stressed	6.67	0.00	7.69	5.49	25.45	5.45
Mild stress	20.00	46.67	20.33	25.82	14.55	23.64
Severe stress	6.67	6.67	9.34	20.33	9.09	12.73

Figure 1 Stress level comparison between male and female of different educational levels SL, UG and PG with data table

Table 1 Demographic and Education level of the studied respondents

Studied Characteristics	MALE	FEMALE
EDUCATIONAL LEVELS		
School Level (SL)	7	8
Undergraduate (UG)	87	95
Post Graduate (PG)	30	25
STRESS LEVELS		
No stress	24	3
Maybe stressed	29	14
Mild stress	48	65
Severe stress	23	46
WEAKNESS LEVELS		
No weakness at all	33	12
Slightly weak	58	52
Moderately weak	26	51
Very weak	7	12
HEALTH OUTCOMES		
Digestive problems	39	58
Infection	18	23
Inflammation	24	22
Disease	36	63

when respondents exhibited at least one symptom of stress. Responses indicating "No" were assigned a value of 0 for all health outcomes except for levels of weakness.

2.4 Statistical Analysis

The data obtained from the survey study was statistically interpreted for correlation among several parameters and the significance of the data. Correlation analysis and one-tailed ANOVA were performed using the data analysis package of MS-Excel 2010 and PAST 4.03. The stress level was marked on a scale of 0 – 3 based on no, mild, moderate, or severe stress.

3 Results

3.1 Demographic details

Students under three categories were selected, i.e., SL < age 17 years; UG < 18- 22 years; and PG > 23 years. Within SL, seven subjects were male, and eight subjects were female. Within UG, 87 subjects were male, and 95 subjects were female. Within PG, 30 subjects were male, and 25 subjects were female. Among the total number of respondents, 24 males and three females responded no stress, 29 males and 14 females responded may be stressed, 48 males and 65 females responded mild stress, and 23 males and 46 females responded severe stress. The weakness has been categorized into four levels, i.e., no weakness, slightly weak, moderately weak, and very weak. Various health outcomes experienced by students during exam time, such as digestive problems, infection, inflammation, and being prone to any disease, have been considered in this study. Further, among the surveyed respondents, 39 male and 58 female subjects responded to digestive problems, 18 male and 23 female subjects responded that they faced infection during exam time, 24 male and 22 female subjects responded to inflammation, and 36 male and 63 female subjects responded that they are prone to diseases during exam time (Table 1).

3.2 Level of stress among students of various groups

The survey encompassed three educational levels, SL, UG, and PG, with notable findings across each category. For SL students (15 participants), stress responses varied between genders. Among male students, 20% reported mild stress, while 6% reported severe

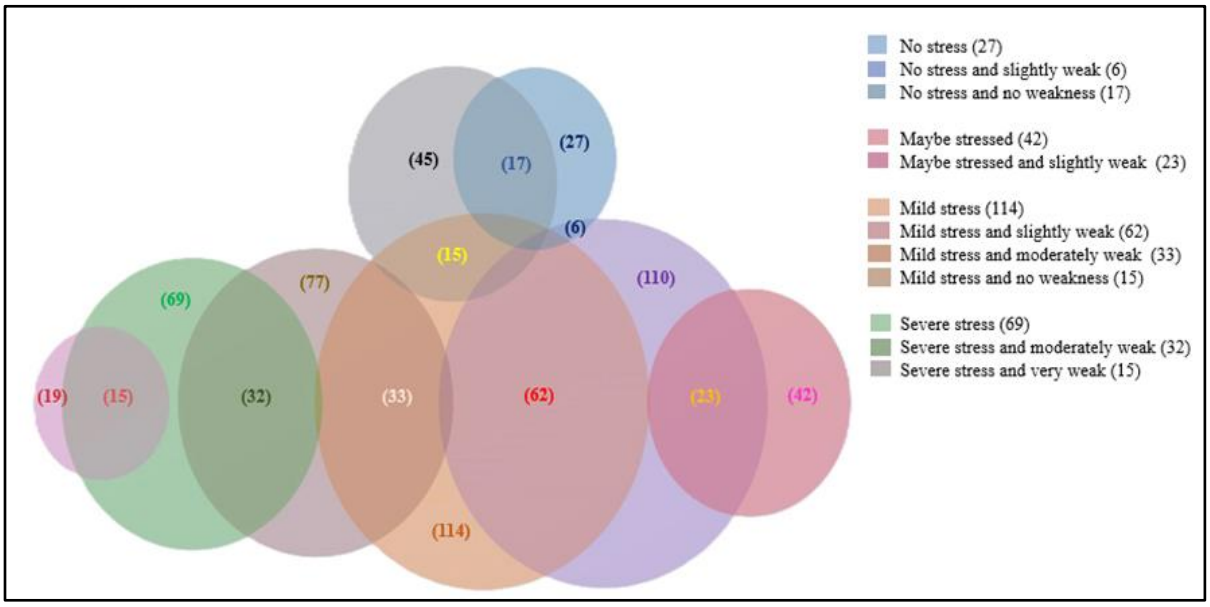


Figure 2 Venn diagrams showing co-occurrence between stress and weakness levels
(numbers withinn parentheses indicate no. of respondents)

stress. Conversely, female students exhibited different stress patterns, with no respondents indicating no stress and a significant proportion experiencing mild (46%) and severe (6%) stress. Notably, the highest incidence of stress among female students was observed during exam periods. Similarly, UG students (183 participants) displayed gender-disparate stress responses, and 20% of male students reported mild stress, while 9% reported severe stress. Conversely, only a minimum of 0.55% of female students reported no stress. However, a substantial portion of female students reported experiencing mild (25.8%) and severe (20.3%) stress levels. The data collected from PG students (55 participants) also showed similar stress trends. Among male respondents, 25.4% expressed being may be stressed, while 14.5% and 9% reported mild and severe stress, respectively. In contrast, female postgraduate students exhibited higher stress levels, with 23.64% experiencing mild stress and 12.7% reporting severe stress (Figure 1). Based on the survey results, it is evident that stress among students is a prevalent issue, particularly during exam times. Noticeably, the survey findings highlight a concerning trend of heightened stress levels among female students across all educational levels.

3.3 Co-occurrence of various health outcomes with different stress levels

3.3.1 Weakness level

The analysis of the co-occurrence between different stress levels and weakness has been observed using Venn diagrams. These diagrams demonstrate an overlapping percentage of 38% among respondents experiencing "no stress" who also report "no weakness." However, no significant overlap was observed between "no stress" and other levels of weakness. Furthermore, no co-occurrence was observed between respondents who reported being "may be stressed" and "very weak." However, there were similar percentages of overlap between "may be stressed" and "slightly weak" (21%) and between "may be stressed" and "no weakness" (22%). Among respondents experiencing "mild stress," the majority (56%) were found to be "slightly weak," while only 16% reported being "very weak." In the case of respondents reporting "severe stress," the maximum co-occurrence was observed with the "very weak" level, accounting for 79%. Conversely, there was minimal co-occurrence (7%) with the "no weakness" level (Figure 2).

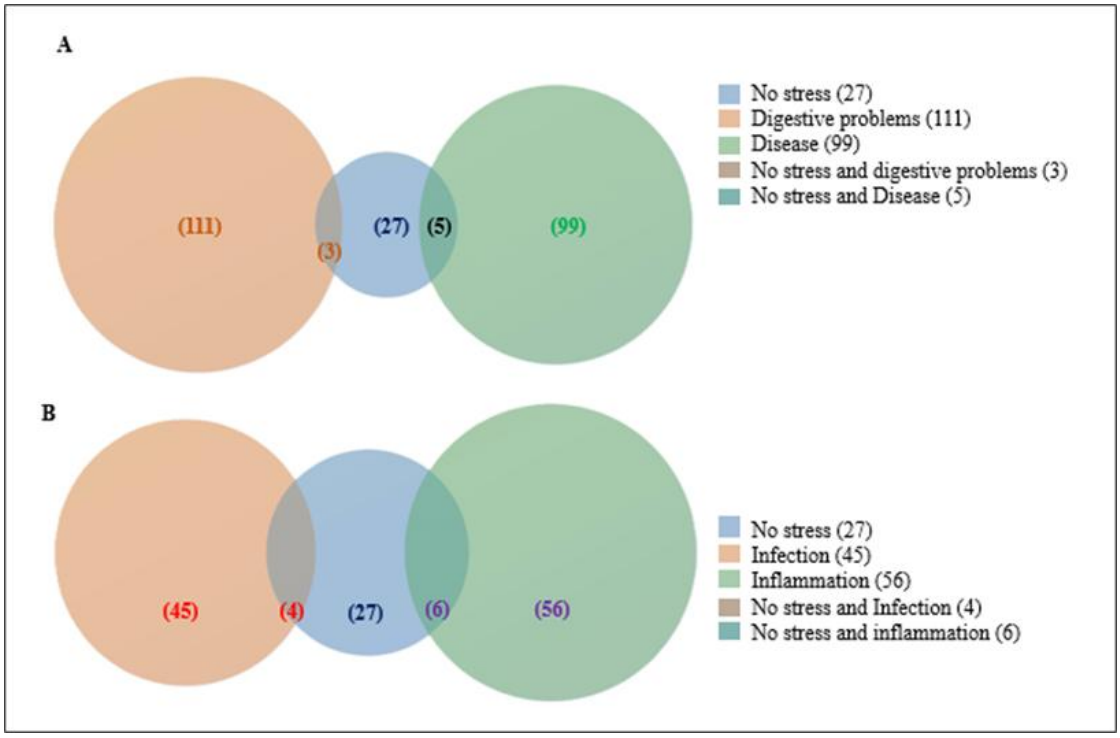
3.3.2 Digestive problems

The analysis of the co-occurrence between different stress levels and digestive problems was observed using Venn diagrams. It was noticed that 49% of respondents experiencing mild stress (Figure 3_III) and 39% of those facing severe stress encountered digestive problems during the exam period (Figure 3_IVA). In contrast, only 3% of respondents who reported no stress encountered digestive issues (Figure 3_IA).

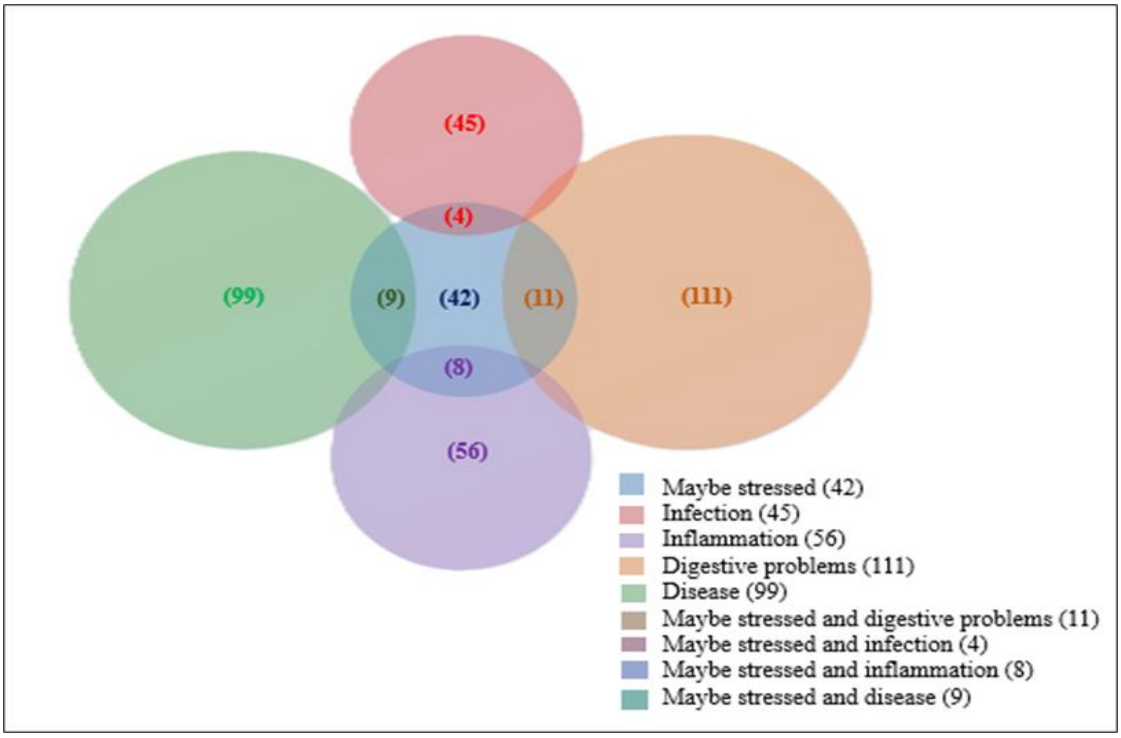
3.3.3 Infection

Venn diagrams were used to analyze the co-occurrence between stress levels and infection. The findings revealed that 49% of respondents experiencing severe stress were susceptible to infection (Figure 3_IVB). In contrast, 33% of those facing mild stress were prone to infection (Figure 3_III). While a mere 9% of those experiencing either stress or no stress were similarly affected (Figure 3_II; 3_IB).

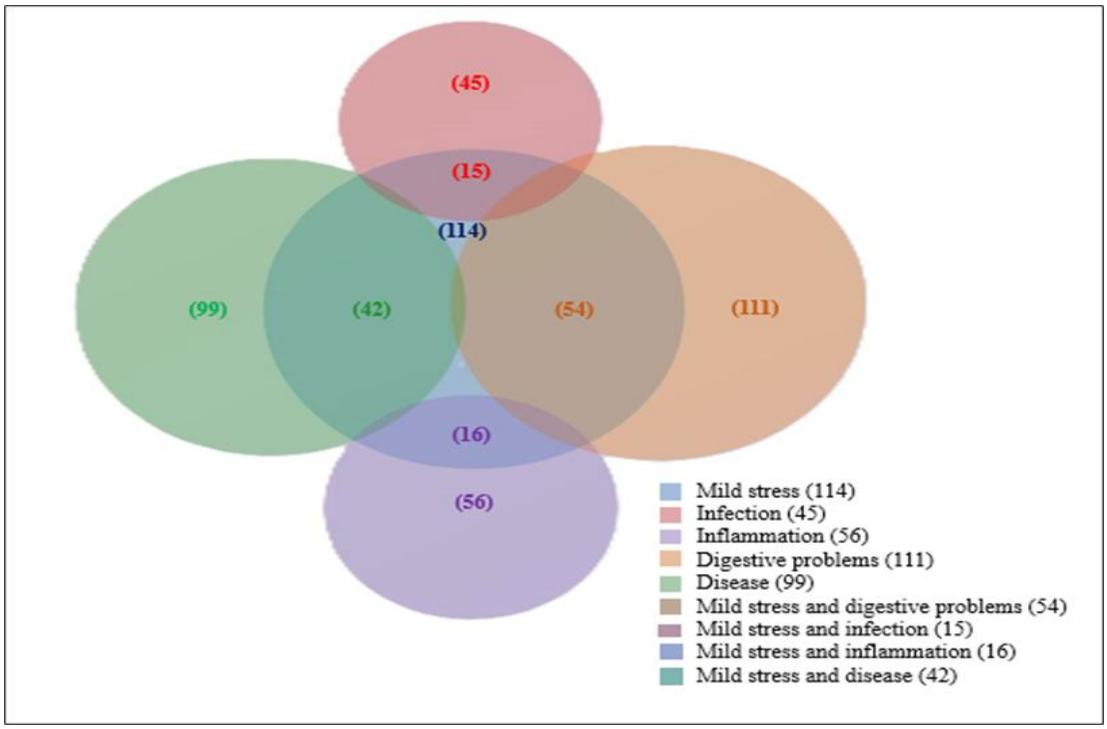
I.


A
(111)
(3)
(27)
(5)
(99)
No stress (27)
Digestive problems (111)
Disease (99)
No stress and digestive problems (3)
No stress and Disease (5)
B
(45)
(4)
(27)
(6)
(56)
No stress (27)
Infection (45)
Inflammation (56)
No stress and Infection (4)
No stress and inflammation (6)

II.


(45)
(4)
(99)
(9)
(42)
(11)
(111)
(8)
(56)
Maybe stressed (42)
Infection (45)
Inflammation (56)
Digestive problems (111)
Disease (99)
Maybe stressed and digestive problems (11)
Maybe stressed and infection (4)
Maybe stressed and inflammation (8)
Maybe stressed and disease (9)

III.



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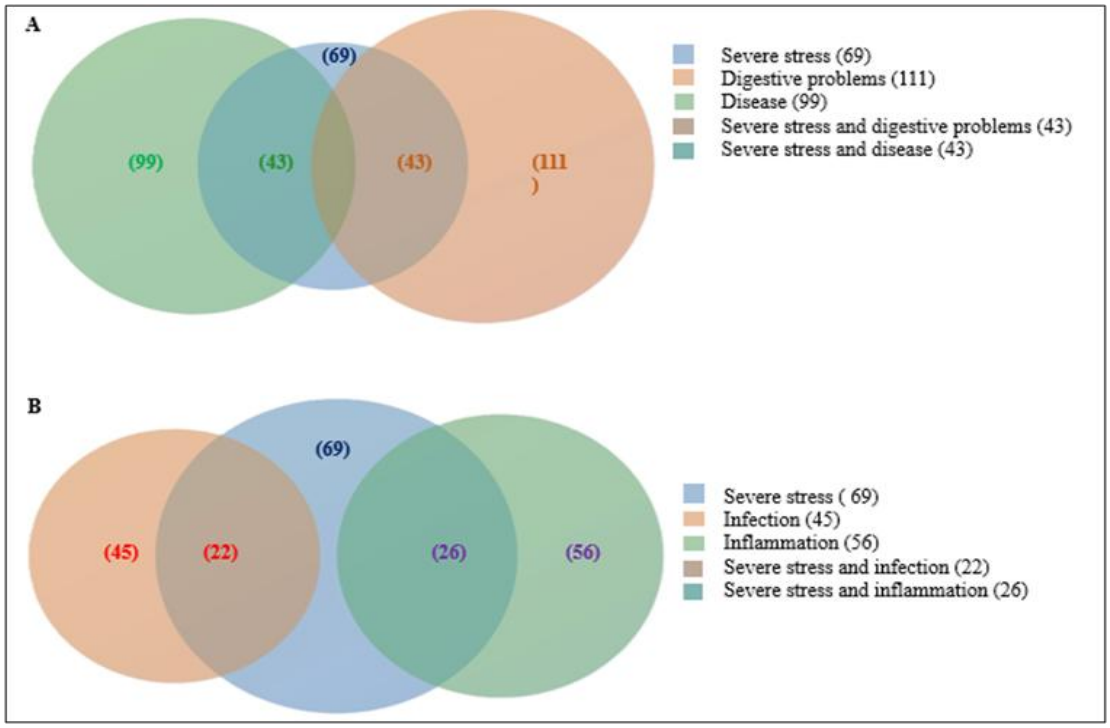


Figure 3 Venn diagrams showing co-occurrence between different stress levels i.e. (I) no stress; (II) maybe stressed; (III) mild stress; (IV) severe stress and various health outcomes (numbers within parentheses indicate no. of respondents)

3.3.4 Inflammation

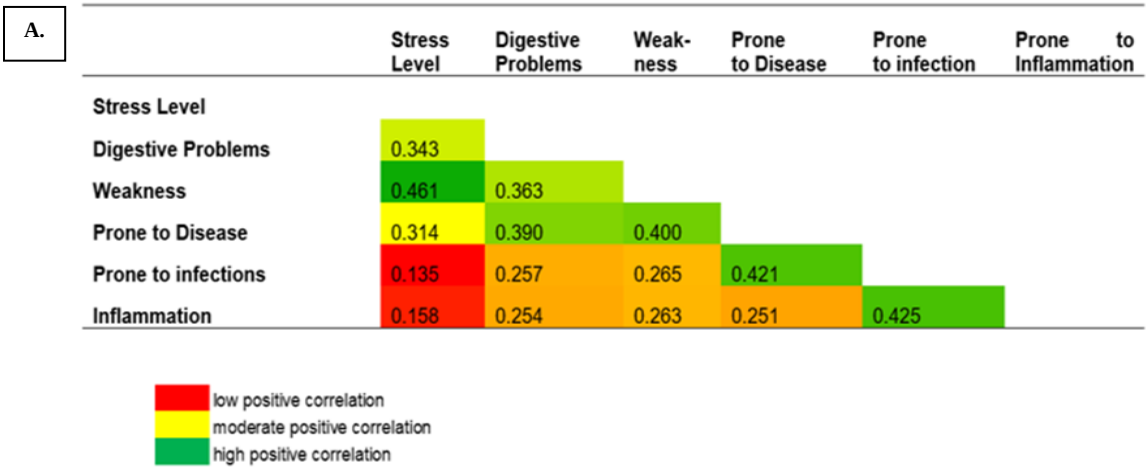
The co-occurrence between different stress levels and inflammation was examined using Venn diagrams. It was noted that 46% of respondents under severe stress were susceptible to inflammation (Figure 3_IVB), while only 11% of those experiencing no stress encountered inflammation during the exam period (Figure 3_IB).

3.3.5 Disease

Examining Venn diagrams, the co-occurrence between various stress levels and prone to diseases was noted. The diagrams revealed that respondents with mild stress (42%) and severe stress (43%) exhibited disease susceptibility (Figure 3_III; 3_IV.A). Conversely, only a tiny proportion (5%) of respondents experiencing no stress suffered from any form of disease during the exam period (Figure 3_IA).

3.4 Correlation analysis of stress levels and health outcomes

Pearson correlation study among the parameters questioned in the survey showed an overall positive correlation among the level of stress, digestive problems, weakness, prone to disease and infections, and inflammation. Stress level was found to show a maximum positive correlation with weakness (r = 0.461) followed by digestive problems (r = 0.343). A moderate correlation was found with 'prone to disease' (r = 0.314), and a very low positive correlation could be detected with 'prone to infection' and 'inflammation.' The survey data was also subjected to one-tailed ANOVA analysis to understand if the variances of means of the response of each question by 250 subjects varied significantly. The



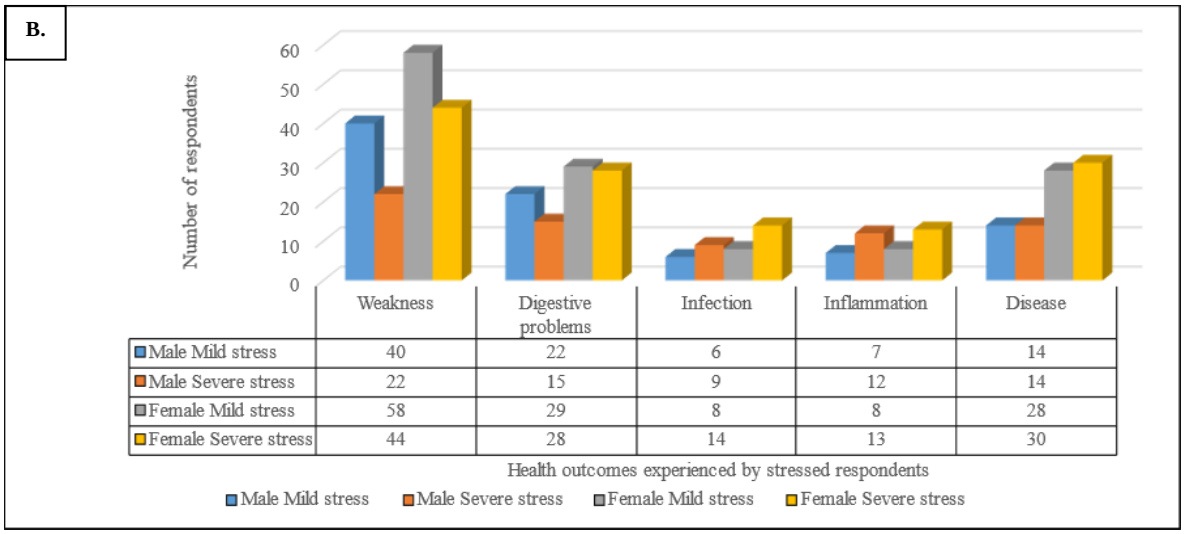


Figure 4 Correlation analysis based on survey response on stress levels and various health outcomes (A); Plot showinghealth outcomes of mild and severe stress in male and female subjects (B)

p-value for one-tailed ANOVA analysis was 0.000, much below the α of 0.05 with a very high F-value, i.e., 174.31. The analysis indicates that the variances of means of each parameter surveyed are unequal. Hence, the differences are significant (Figure 4A). A correlation between stressed male and female respondents and health outcomes has also been noted. Interestingly, both severe and mild stress appear to affect females more significantly, rendering them more prone to various health issues such as weakness, infection, inflammation, and disease compared to their male counterparts experiencing similar levels of stress. Conversely, mild and severe stress among males correlates more strongly with weakness than other health outcomes (Figure 4B).

4 Discussion

The findings of this study shed light on the significant prevalence of stress among individuals across different educational levels, especially during examination periods. Across SL, UG, and PG, the study's results indicate notable gender-dependent differences in stress responses. Among SL students, females exhibited substantially higher rates of mild and severe stress compared to males, with 46% reporting mild stress and 6% reporting severe stress. Similarly, UG and PG female students showed elevated stress levels, with significant proportions experiencing mild and severe stress compared to their male counterparts (Figure 1). These findings resonate with existing research by Shah et al. (2010) and Eman et al. (2012), which emphasize higher levels of perceived stress among female students, particularly at the PG level. This trend is consistent with previous studies conducted by Dyrbye et al. (2006) and Wadikar et al. (2017), further reinforcing the notion of gender-specific stress responses in academic settings. While Sulaiman et al. (2009) reported lower stress levels among male students compared to females, a body of literature including studies by Hall et al. (1998), Adlaf et al. (2001), Hudd et al. (2000), Kelly et al. (2006), and Kudielka and Kirschbaum (2005) consistently support the notion that female students tend to report greater levels of stress overall. These significant psychological differences between men and women may primarily arise from women's greater inclination to express their concerns and emotions openly. This contrast may be attributed to women's heightened vulnerability to a range of physical and emotional challenges, including depression and fatigue, which are more prevalent among women than men (Basudan et al. 2017; Zinurova and Dehart 2018). Remarkably, our study data revealed a distinct trend between stress levels and perception of weakness during exam time; as stress levels intensify, so does the likelihood of experiencing feelings of weakness. The Venn diagram analysis reveals intriguing patterns between stress levels and weakness. While "no stress" correlates with "no weakness" in 38% of cases, "mild stress" predominantly aligns with "slightly weak" in 56% of respondents, and "severe stress" is strongly associated with "very weak" in 79% of cases (Figure 2). The findings from our data align closely with existing literature, as evidenced by the study conducted by Murphy et al. 2010 and their investigation revealed a notable decrease in secretory immunoglobulin A (S-IgA) levels by 29% during high-stress periods, such as exam weeks, with concurrent increases in cortisol levels (Murphy et al. 2010). Cohen et al. (2001) highlight the indirect influence of stress on immune response through various behavioral changes triggered by stress, including alterations in smoking habits, sleep patterns, and dietary choices (Cohen et al. 2001), which may further exacerbate the immune system's vulnerability during stressful periods. Expanding on these insights, a wealth of literature underscores stress's profound effects on university students' immune function. For instance, research has linked exam stress to reductions in natural killer (NK) cell activity. The recent study also delves into student health, uncovering notable connections between stress levels and health outcomes. Among those experiencing severe stress during exams, a significant 39% reported digestive issues (Figure 3_IVA), while for those facing mild stress, 49% reported similar problems (Figure_ 3III). Interestingly, only 3% of respondents experiencing no stress reported digestive issues (Figure IA), underscoring the strong correlation between higher stress levels and gastrointestinal ailments; various studies also support this finding. For instance, Huerta-Franco et al. (2013) highlighted how stress can trigger gastrointestinal health problems, while Knowles et al. (2008) observed spikes in digestive troubles, cortisol levels, and alterations in gut bacteria during exam periods. The link between stress and stomach discomfort, including conditions like irritable bowel syndrome (IBS), has been extensively studied (Moloney et al. 2016). Faixová et al. (2023) found that nearly 60% of students encountered digestive issues during exams, with women being more affected. Our research corroborates this, revealing high rates of digestive problems among both severe and mild stress females (Figure 4B). Our study also found a strong link between severe stress and susceptibility to infection, with 49% of severely stressed respondents (Figure 3_IVB) affected compared to 33% of those with mild stress (Figure 3III), while only 9% of no-stress respondents are prone to infection (Figure 3_IB). These findings suggest that stress may compromise immune function, increasing infection vulnerability. These findings were also supported by several studies, where they found that stress can compromise the immune system by reducing the production of key cytokines like IFN-c and IL-2, weakening defense against viral infections, as indicated by Kang and Fox (2001). According to Assaf et al. (2017), medical and health sciences students facing heightened stress during their final exams commonly reported experiencing symptoms such as colds, flu, tonsillitis, and noticeable hair and skin health declines throughout the semester. High stress levels, particularly during exams, are associated with increased cortisol levels, shifting the Th1/Th2 cytokine balance towards Th2 dominance, potentially leading to immune dysregulation rather

than suppression (Assaf et al. 2017). This study also revealed that 46% of the respondents under severe stress experienced inflammation (Figure 3_IVB), compared to 11% with no stress (Figure 3_IB), emphasizing a significant link between stress and inflammation levels. Other research has shown similar results, suggesting that stress can activate inflammation in the body, with an increase in Th2 cytokines like IL-4, IL-5, and IL-13, which can suppress proinflammatory responses. However, this may worsen atopic diseases like asthma or atopic dermatitis. Stressful periods, such as exams, can exacerbate conditions like asthma, as seen with elevated IL-5 levels in students with mild asthma during exams (Wills-Karp 2004; Kamezaki et al. 2012).This study also observed that both mild stress of 42% (Figure 3_III) and severe stress of 43% were associated with disease susceptibility (Figure 3_IVA). In contrast, only 5% of no-stress respondents experienced any disease (Figure 3_IA) during the exam period, suggesting that stress may play a role in disease susceptibility, with higher stress levels correlating with a greater likelihood of experiencing health issues during stressful periods like exams. The findings of this research revealed a relationship between disease and stress levels, and these are reinforced by Trueba et al. (2013), who propose that prolonged stress and depression could result in a reduction in nitric oxide (NO) production, potentially impacting the progression of cardiovascular diseases. Similarly, Barth et al. (2004) and other researchers have also established an association between chronic stress and cardiovascular risks. Researchers studying medical students undergoing exam stress discovered that stress contributes to elevated cholesterol levels. Because blood pressure and serum cholesterol tend to rise during stress, the association between stress and hypertension has long been suspected (Salleh 2008).

Our Pearson correlation analysis revealed that stress exhibits its strongest correlation with weakness ($r = 0.461$) and digestive problems ($r = 0.343$), indicating that increased stress levels are closely related to increased reports of weakness and digestive issues among students (Figure 4A). These findings are consistent with research by Faixová et al. (2023), which highlighted that a majority of female students (66.6%) and nearly half of male students (46.7%) experienced higher digestive problems during exam periods. Furthermore, our observations also revealed distinct gender disparities in how stress influences health outcomes, highlighting a more significant impact on females. Specifically, the findings of this study indicate that both severe and mild stress disproportionately affect females, leading to increased susceptibility to health issues such as weakness, infection, inflammation, and disease compared to males experiencing similar levels of stress (Figure 4B). These findings align with research indicating that neuroinflammatory conditions like multiple sclerosis, Alzheimer's disease, and chronic pain are more prevalent among women (Loram et al. 2012). Oertelt-Prigione (2012) and Markle et al. (2013) propose that dysregulated immunocyte function, steroid hormone signaling, and alterations in the gut microbiome may contribute to this heightened susceptibility among females.

Additionally, research indicates that females may be more vulnerable to the effects of peripheral inflammation. In a study where healthy men and women received endotoxin, both groups had increased proinflammatory cytokines. However, only women showed cytokine-induced depressed mood and feelings of social disconnectedness (Moieni et al. 2015).

In the future, this study presents exciting opportunities for further investigation. Research endeavours could explore blood biomarkers to understand the dynamics between stress and immune response, elucidating the physiological mechanisms connecting exam-induced stress and immunity. Furthermore, analyzing the gut microbiome of individuals experiencing digestive issues before and after exams may offer valuable insights into the impact of stress on gut health and overall well-being. These efforts hold the potential to advance our comprehension of the intricate relationship between stress, immunity, and gut health.

Conclusion

The study underscores a notable correlation between stress levels and diverse health outcomes among students across various educational levels during exam periods. Female students experience more pronounced stress impacts than their male counterparts at UG and PG levels. The findings indicate that females facing equivalent stress levels are more vulnerable to health issues than males. Elevated stress levels correlate with increased occurrences of weakness and digestive problems.

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Investigating the antimicrobial activity of neem and clove extract on biofilm-producing oral microflora

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ABSTRACT

Periodontal disease, a serious gum infection, is reported to be widespread in the Indian population. A heterogeneous microbial population, predominantly consisting of gram-negative anaerobes such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Porphyromonas intermedia*, is associated with periodontal plaque formation. This condition may be worsened by the invasion of soft tissues by different species of *Candida*. Natural products like clove (*Syzygium aromaticum*) and Neem (*Azadirachta indica*) are very popular and easily available in the Indian climate and have great potential in preventing periodontitis. *Azadirachta indica* (Neem) exhibits versatile modes of action, including reported antimicrobial effects against several species associated with *periodontal* disease. Therefore, this study aims to detect the antimicrobial and antifungal effects of Neem and clove on oral biofilm both before and after biofilm formation. Results of the study revealed that both neem and clove crude extracts and their different dilution showed a significant reduction in the growth of fungal strains (*Candida sp.*) isolated from oral samples from people with poor hygiene and the biofilm produced by them.

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1 Introduction

The oral microbiota, consisting of hundreds to thousands of unique species of bacteria, viruses, and fungi, plays a significant role in the human microbiome (Arweiler and Netuschil 2016; D'Ambrosio et al. 2023). The various habitats in the oral cavity provide a complex environment for microorganisms on the teeth' hard surfaces and the oral mucosa's soft tissues. It is the second most extensive and diverse microbiota after the gastrointestinal tract (Arweiler and Netuschil 2016; Deo and Deshmukh 2019; Kitamoto et al. 2020). The oral microbiome is crucial in preventing the colonization of harmful bacteria, which could lead to oral disorders such as periodontitis, gingivitis, and caries (John et al. 2017). Additionally, many microorganisms can form biofilms resilient to mechanical stress or antibiotic treatment. Some commensal species may exhibit virulence in response to changes in the oral cavity's environment, behavioral aspects, or an individual's hygiene (Rajhans et al. 2011). There is a growing interest in understanding the role of the oral microbiome in health and disease, and recent advances in metagenomic technologies are being employed to characterize its diversity (Rajhans et al., 2011).

Periodontitis is a complex disease caused by an infection that affects the tissues supporting the teeth, known as the periodontium (Gasner and Schure 2024). It is not a single disease but rather a combination of diseases characterized by the loss and destruction of alveolar bone, deterioration of gums, periodontal ligament, jawbone, and the surfaces of the teeth covered by the gums (Könönen et al. 2019; Van Dyke et al. 2020). Periodontal disease is widespread in the Indian population, affecting over 1 billion people. It is more prevalent in urban areas (22.7%) than in rural areas, and it is also more common in males (42.2%) than in females (34.4%) (Janakiram et al. 2020; Antimicrobial Resistance Collaborators 2024).

The clinical diagnosis of the disease and its categorization involves identifying signs and symptoms in periodontal tissues that help medical professionals identify illnesses based on their origin, pathophysiology, and therapy. In addition to causing tooth loss, this disease also leads to systemic inflammation (Van Dyke et al. 2020). Severe periodontitis is rare in young individuals, while chronic periodontitis primarily affects adults (Zhu et al. 2023). Bacteria are commonly found in saliva and gingival plaque in the oral cavity, while they are present in fewer numbers in keratinized gingiva (Aas et al. 2005). Saliva affects the distribution of microorganisms in different parts of the oral cavity and lacks a stable indigenous biota due to its quick turnover and low amounts of nutrients. Bacteria shed from other oral tissues contribute to the high alpha diversity of salivary microbiota (Janakiram et al. 2020). The initiation and progression of periodontal disease are attributed to a polymicrobial synergy and dysbiosis in the microbial community, leading to the formation of a dysbiotic biofilm, plaque, and overgrowth of key pathological entities in the microbiome (Wilson et al. 2017; Lamont et al. 2018; Cugini et al. 2021). This triggers a destructive and dysregulated host immune response, leading to inflammation, destruction of host tissue, and eventually, the formation of periodontal pockets and bone loss (Hajishengallis and Chavakis 2021, Lamont et al. 2018; Caselli et al. 2020).

The pathologic condition persists in active or dormant phases until the affected tooth is extracted or the microbial biofilm is surgically removed. Environmental and host risk factors, including modifiable (e.g., quitting smoking) and non-modifiable factors (e.g., genetics) which, may determine the severity of periodontal diseases (Van Dyke and Sheilesh 2005).

Dental caries and periodontal disease are two major oral illnesses. Periodontal disease can appear in various forms, with gingivitis and periodontitis being prominent ones. Gingivitis is a common inflammatory disorder of the gingiva, the soft tissue surrounding the teeth, caused by oral microbial plaque (Rathee and Jain 2023). Periodontitis follows severe gingivitis in a smaller number of people, based on an individual's immune response, and is characterized by the destruction of the tooth's skeletal, periodontal, and connective tissue supports (Janakiram et al. 2020; Aas et al. 2005).

A diverse microbial population, predominantly of gram-negative anaerobes like *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia*, is associated with periodontal plaque formation. This may be exacerbated by the deeper invasion of soft tissues by different species of *Candida*. At a subject-specific and tissue-specific level, a microbiome comprising members like *Neisseria* spp., *C. albicans*, *P. aeruginosa*, *Hafnia alvei*, *Serratia marcescens*, *Filifactor alocis*, and *Enterobacteria* is related to periodontal inflammation and tissue destruction (Paul et al. 2021; Li et al. 2022). Conversely, healthy periodontal tissues are associated with *Lactobacillus acidophilus*, *Fusobacterium necrophorum*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.

Ghannoum et al. (2010) state that a healthy oral mycobiota comprises 74 culturable species and 11 genera of non-culturable fungi. These researchers have reported the presence of specific fungal isolates such as *Candida*, *Aspergillus*, and *Cryptococcus*, which may predispose the host to opportunistic infections and have shown significant inter-individual variance (Ghannoum et al. 2010; Saigal et al. 2011; Padminee et al. 2020). Recent research has revealed that most commensals in saliva are Malassezia species, previously characterized as pathogens of the skin and lungs (Aas et al. 2005; Ghannoum et al. 2010; Nagakubo and Kaibori 2023).

Herbal medicine is an alternative and effective therapy that can be used in conjunction with or instead of conventional chemical drugs

to treat various diseases. Many plant products have antimicrobial, antioxidant, antiviral, antiseptic, and analgesic properties, which greatly interest dentistry. Herbal properties are widely used to alleviate toothaches, gum inflammation, and canker sores (Amanpour et al. 2023). It's important to understand how these phytochemicals work and how they interact with the human body and other medications, as many of these extracts have anti-inflammatory effects and can prevent bleeding, which is vital in dental treatment (Taheri et al. 2011). Herbal products are preferred over-prescribed medicines for treating ailments due to their lower cost and reduced toxicity or side effects (Cruz Martínez et al. 2017; Mosaddad et al. 2023). Recent studies have shown the potential of herbal agents in global dental therapy, especially in the fields of periodontics and endodontics, with plant extracts such as propolis, noni fruit, burdock root, clove, neem leaf, and others (Cruz Martínez et al. 2017; Padminee et al. 2020; Milutinovici et al. 2021; Pasupuleti et al. 2023).

Neem (*A. indica*) from the Meliaceae family is significant in promoting health and is of medicinal importance due to its antibacterial, antihelminthic, anticariogenic, antioxidant, astringent, cytotoxic, and anti-inflammatory properties. Active compounds like Nimbidin, Azadirachtin, Nimbinin, Nimbin, Nimbolide, and Limonoids contribute to these versatile actions (Almas 1999; Subramaniam et al. 2005). The first polyphenolic flavonoids identified in fresh neem leaves were quercetin and ß-sitosterol, known for their antifungal and antibacterial properties (Mathur et al. 2010; Shamsudin et al. 2022). Polyphenols from neem leaf extract adhere to oral surfaces, providing long-lasting antibacterial, anti-inflammatory, and synergistic antioxidant actions, which help combat periodontal disorders (Subramaniam et al. 2005; Buakaew et al. 2021; Wylie et al. 2022). Neem has been demonstrated to have antibacterial properties against various types of *Streptococcus* sp and *C. albicans* (Wolinsky et al. 1996).

Furthermore, Clove (*S. aromaticum* or *Eugenia caryophyllata*) is an aromatic flower bud commonly used as a spice, flavoring, or scent in consumer goods (Afolabi et al. 2008; Danthu et al. 2020; Maggini et al. 2024). Significant constituents of clove oil such as eugenol, β-caryophyllene, caracole, thymol, eugenol, and cinnamaldehyde exhibit antimicrobial activity against Gram-negative (*E. coli*, *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus* sp, *Streptococcus* sp, *Listeria monocytogenes*) bacteria as well as fungal strains like *Aspergillus* sp. (Afolabi et al. 2008; Al-Ahmad et al. 2014; Marchese et al. 2017; Ben Hassine et al. 2021). Clove essential oil also possesses antibacterial, antioxidant, antifungal, and antiviral properties, along with anti-inflammatory, cytotoxic, insect repellent, and anesthetic properties (Dorman and Deans 2000). The phenolic nature of eugenol makes it highly effective against various bacteria. It can easily penetrate the cell walls of gram-positive bacteria, causing the cell wall to degrade (Al-Ahmad et al. 2014), followed by damage to the cytoplasmic membrane, impairment of the bacterial enzyme system, increased permeability, leakage of the cell's contents, and ultimately cell lysis (Prabuseenivasan et al. 2006).

Both neem and clove extracts have been reported to demonstrate antimicrobial activity against common microbes like *C. albicans* and *S. mutans* present in the oral cavity (Barua et al. 2017; Bansal et al. 2019). In countries like India, where there is a rich resource of indigenous flora with medicinal significance, and a significant proportion of the population is affected by periodontal diseases, it is important to investigate the effects of natural, easily available medicinal plants like Neem and clove on the oral biofilm of pathogenic microflora (Zhang et al. 2017). This study aims to explore the impact of plant derivatives on microbes associated with the pathogenicity of periodontitis, with the potential for use as an alternative treatment or in conjunction with existing conventional drugs.

2 Materials and Methods

2.1 Sample collection

We collected oral swab samples from five individuals with poor oral hygiene. We used a sterile swab stick to collect samples by rotating the tip on their teeth and gums before they rinsed their mouths in the morning. The swab sticks were placed in labeled test tubes (OS1-OS5) and transported to the laboratory. We recorded demographic data for each patient. We collected samples from each person thrice and then isolated microorganisms for further analysis (Table 1).

2.2 Revival of samples and culture of microorganisms

The swab samples (OS1-OS5) were placed in 5ml of sterile nutrient broth and left overnight at 37°C to allow any microorganisms in the sample to revive. The next day, 200µl of liquid culture from each sample was spread onto sterile nutrient agar plates and mannitol salt agar plates, and each plate was labeled accordingly. Gram staining was performed on the colonies obtained from OS2-OS4 to establish pure cultures and identify the bacterial morphology and gram characteristics under a light microscope at 40X magnification. To isolate fungi from the oral swab, collected oral samples were plated on potato dextrose agar

Table 1 Details of the sampling Patient's

No. of individuals	Age range (in years)	Sex	Habit
5	11-60	Male-3 Female- 2	Tobacco- 2 (males); Without tobacco- 3; (1 male, 2 females)

(PDA) media and incubated at 30°C overnight. Fungal growth on PDA media was confirmed by staining with lactophenol cotton blue and observing under a light microscope (40X). Pure cultures of colonies obtained from the five samples were prepared and maintained in PDA media and labeled as OS2, OS4A, and OS4B (two types of colonies identified from sample 4). The isolated fungal species from sample 4 were identified as Candida species, which were then cultured on selective Corn meal agar media formulated using 0.2 gm of corn infusion, 0.7ml Tween 80, 1.5 gm agar, and 100 ml distilled water, and autoclaved.

2.3 Preparation of clove and neem extract in the organic solvent

To prepare the extracts, 10 grams of clove were collected and crushed using a pre-sterilized mortar and pestle. Absolute alcohol (99.9%) was added intermittently until the volume reached 20 ml. The sample was soaked for 24 hours, stirred, and left for 24 hours. The upper layer was then separated using a pipette and placed into a sterile falcon tube for further use. Similarly, a 20ml extract of fresh neem leaves was prepared sterilely using absolute alcohol (99.9%), and the upper layer of the supernatant was separated into a sterile falcon tube for future use. The prepared culture stocks were stored at 4°C. For the working solution, 1/2, 1/4, and 1/6 dilutions of each sample were prepared using absolute alcohol as the solvent.

2.4 Paper disc preparation

Paper discs were prepared and sterilized by autoclaving. The sterile paper discs were soaked in the dilutions and crude solutions for 15 minutes, followed by aseptic air drying.

2.5 Antimicrobial activity of neem and clove extract

We used the paper disc method to evaluate the antimicrobial activity of Neem and clove extract. To summarize, we spread 300 µl of an overnight culture of pure culture from OS2, OS4A, and OS4B on a nutrient agar medium and let it absorb on the surface for 30 minutes. We then placed paper discs for the crude extract, respective dilutions (1/2, 1/4, 1/6), and a negative control on each plate. The plates were incubated overnight at 37°C, and observations were made the following day.

2.6 Effect of Neem and clove on preformed biofilm

The study examined the impact of herbal extracts on pre-existing biofilms. Notably, the most significant inhibition zone was observed for OS4B. Consequently, further experiments were conducted exclusively with the pure culture of this sample. To induce biofilm formation, OS4B was inoculated in freshly prepared nutrient broth with 1% glucose and incubated overnight at 37°C. Next, five autoclaved Eppendorf tubes were utilized, with four containing 500 µl of OS4B's overnight culture mixed with 500 µl of nutrient broth, while the fifth served as a negative control with only nutrient broth. The tubes were then placed in an incubator for 24 hours.

The following day, the four Eppendorf tubes containing the culture received 500µl of crude, 1/4, and 1/6 dilutions of clove extraction, respectively, while the fifth tube was left untreated and incubated for an additional 24 hours. The biofilm was subsequently separated from the spent media, washed with 1X PBS, stained with 1% crystal violet solution, and extracted with 33% glacial acetic acid. The absorbance was measured at 600nm using a spectrophotometer. In addition, the effect of clove extract and its dilutions on a 48-hour-old culture of OS4B was assessed through spectrophotometric analysis at 600nm. Similarly, the impact of neem extract on 24 and 48-hour-old biofilms of OS4B was also investigated using the same protocol.

2.7 Effect of neem and clove extract on biofilm formation

Five small sterile Petri plates were taken, and sterile coverslips were placed in each plate aseptically. In plate 1, 2 ml of sterile nutrient broth with 1% glucose was poured, while in the remaining four plates, 1 ml of the same media was added after adding 1 ml of 24-hour-old OS4B liquid culture on the cover slip. This was followed by adding 500 µl of 1/2, 1/4, and 1/6 dilutions of clove extraction in individual plates. The plate with only liquid culture was not treated with any extract. All the plates were then incubated at 37°C overnight. The following day, without disturbing the biofilm, the supernatant was discarded. This was followed by a PBS wash and staining with 1% crystal violet for 15 minutes. After a second PBS wash, the plates were observed under a phase contrast microscope to check for inhibition of biofilm formation. The crystal violet attached to the biofilm was extracted with 33% glacial acetic acid (1 ml) from each plate and transferred to separate Eppendorf tubes. Subsequently, absorbance was measured at 600 nm using a spectrophotometer to estimate the extent of biofilm formation in terms of binding crystal violet. Using the same procedure, the effect of crude neem extract and its dilutions was checked for their impact on the biofilm formation of OS4B.

2.8 Statistical analysis

Experimental data were presented as the mean ± standard error of triplicate measurements and were analyzed using IBM SPSS Statistics version 16. Statistical significance was determined using Student's t-test and one-way ANOVA.

3 Results

3.1 Isolation of microorganisms from oral swab sample

Oral swabs were collected aseptically from five individuals and labeled as OS1-OS4. The swabs were used to streak on sterile nutrient agar

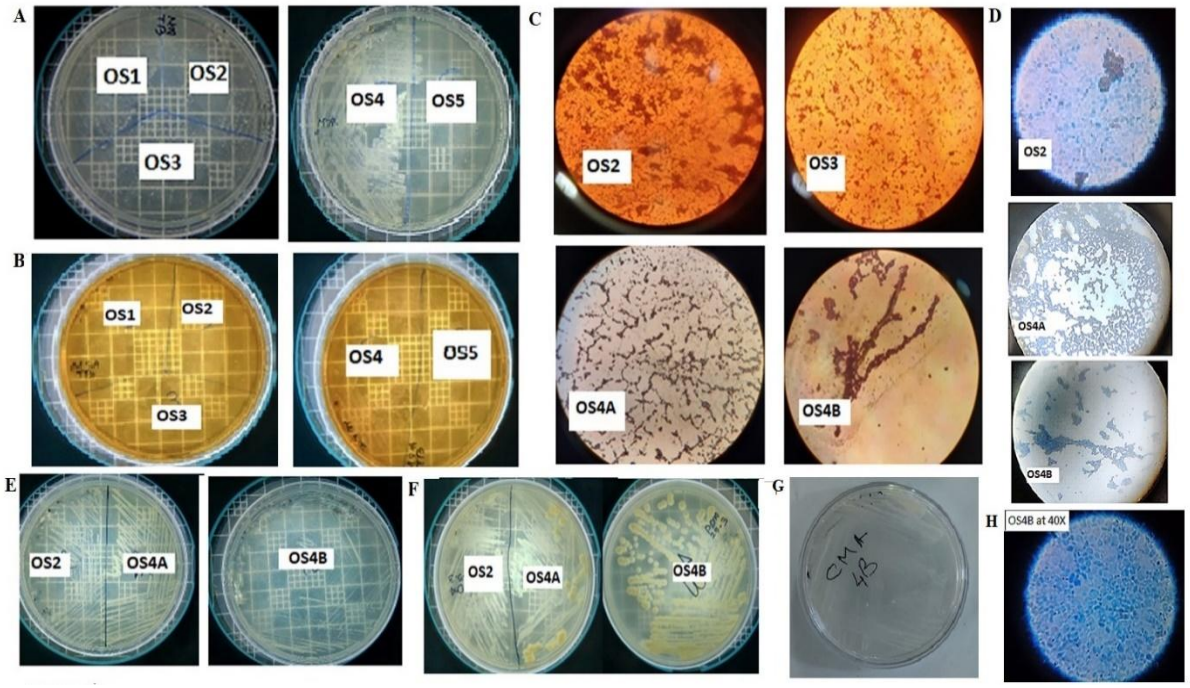

Figure 1 Pictorial representation of: 1A) The growth of samples OS1-OS5 on nutrient agar plates. After 24 hours, samples OS1 and OS5 showed no growth on nutrient agar plates, while samples OS2, OS3, and OS4 exhibited growth; 1B) None of the samples from OS1-OS5 showed growth on MSA plates; 1C) Gram staining of microbial growth from samples OS1-OS5; 1D) Fungal staining with lactophenol cotton blue indicated the presence of fungal culture in OS2 and OS4; 1E) The growth of samples OS2 and OS4 on potato dextrose agar media after 24 hours of incubation; 1F) Pure culture of OS2 and two types of colonies found from sample OS4 (OS4A and OS4B) were grown using PDA media; 1G and 1H) Growth of OS4B samples in corn meal agar media followed by staining with lactophenol cotton blue confirmed the presence of Candida sp. in OS4B sample.

plates and mannitol salt agar plates. The plates were then incubated for 24 hours at 37°C. After incubation, samples OS1 and OS5 showed no growth on the nutrient agar plates and were not included in further study. However, samples OS2, OS3, and OS4 showed growth (Figure 1A). None of the samples showed growth on mannitol salt agar plates (Figure 1B). Gram staining of microbial growth did not provide information regarding gram character and morphology (Figure 1C). Fungal staining with lactophenol cotton blue indicated the presence of fungal culture (Figure 1D). Therefore, samples OS2-OS4 were then plated on potato dextrose agar (PDA) media. After 24 hours of incubation, visible growth was observed for OS2 and OS4 on their respective PDA plates. The fungal samples presence in each was further confirmed by staining with lactophenol cotton blue and observing under a light microscope at 40X magnification (Figure 1E). Pure cultures of OS2 and two colonies found from sample OS4 (OS4A and OS4B) were prepared using PDA media (Figure 1F). Further experiments were continued with these three fungal samples only, i.e., OS2, OS4A, and OS4B. To confirm the identity of the fungal strains, they were plated on corn meal agar media, which is used as a selective media for *Candida* sp. The growth of OS4B in this media confirmed the presence of Candida sp., which was further validated by staining with lactophenol cotton blue (Figure 1G and 1H).

3.2 Examination of antimicrobial activity of Neem and clove

To test the antimicrobial effect of Neem on isolated fungal strains, we selected pure cultures of samples OS2, OS4A, and OS4B. We prepared neem crude extract at 1/4, 1/2, and 1/6 dilutions using absolute alcohol as the solvent. We then dipped sterile paper discs in the respective plant extracts to create antimicrobial discs. Each fungal sample was plated on individual plates, and the antimicrobial discs were placed to assess their antifungal activity. For sample OS2, there was no zone of inhibition for the crude extract or any of the dilutions of Neem. However, for sample OS4A, zones of inhibition were observed 0.833±0.115 cm (1/2 dilution), 1.3±0.1 cm (1/4 dilution), and 1.36±0.058 cm (1/6 dilution), with no reported zone for the crude extract or control. As for sample OS4B, the obtained zones of inhibition were 0.9±0.1 cm (1/4 dilution) and 1.1±0.17 cm (1/6 dilution), but no zone of inhibition was reported for the crude extract or the half dilution (Figure 2A and 2B).

A similar experiment was conducted with different clove extracts, and no zone of inhibition for sample OS2 was observed again. However, for the other two samples, OS4A and OS4B, zones of inhibition for the 1/2 dilution (OS4A: 1.3±0.1 cm, OS4B: 1.1±0.1

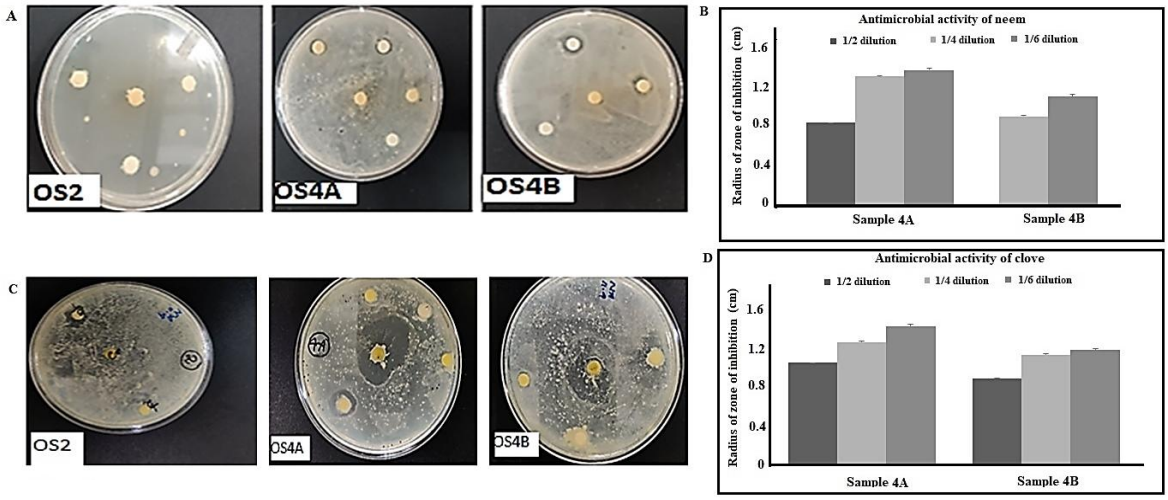

Figure 2 illustrates the zone of growth inhibition of samples OS2, OS4A, and OS4B: 1A) in the presence of neem extract and its dilutions; 1C) in the presence of clove extract and its dilutions; 1B) displaying the zone of inhibition for samples OS4A and OS4B in crude neem extract and its various dilutions (1/4, 1/2, and 1/6 dilutions); 1D) showing the zone of inhibition for samples OS4A and OS4B in crude clove extract and its various dilutions (1/4, 1/2, and 1/6 dilutions).

cm), 1/4 dilution (OS4A: 1.567±0.15 cm, OS4B: 1.4±0.1 cm), and 1/6 dilution (OS4A: 1.766±0.15 cm, OS4B: 1.4667±0.058 cm) were observed (Figure 2C and 2D).

3.3 Effect of neem and clove extract on preformed biofilm

In this experiment, biofilm formation was induced by adding 1% glucose to freshly prepared nutrient broth in five Eppendorf tubes, which were then kept at 37°C for 48 hours (Figure 3A – E). The effects of both clove and neem extracts and various dilutions of these extracts were observed on 24-hour and 48-hour-old biofilms using spectrophotometric analysis at 600nm. The study results indicated that the crude extract of clove didn't cause any change in the biofilm after 24 hours, but after 48 hours, there was a reported 19% reduction in the biofilm ($P<0.001$). Significant reduction in the formed biofilm was observed for different dilutions of clove extract, with a 20% reduction reported after 24 hours and a 41% reduction reported after 48 hours for the 1/4th dilution ($P<0.001$). In addition, the 1/6th dilution resulted in a 50% reduction after both 24 and 48 hours of exposure ($P<0.001$).

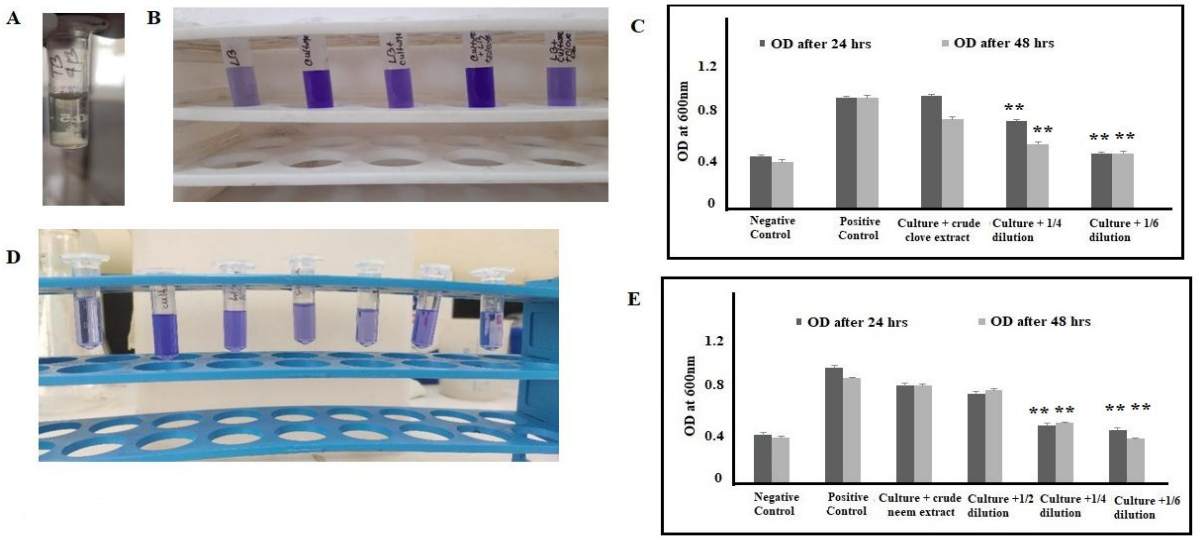

Figure 3A) Biofilm formation in Eppendorf for sample OS4B; 3B) color variation obtained after extraction of crystal violet stain from preformed biofilms of OS4B after treatment with crude extract and increasing dilutions of clove in a series of Eppendorfs; 3C) reduction in OD reading at 600nm with increasing dilution of clove for OS4B; 3D) color variation obtained after extraction of crystal violet stain from preformed biofilms of OS4B after treatment with crude neem extract and dilutions; 3E) reduction in OD reading at 600nm with increasing dilution of Neem for OS4B; ** indicates P value < 0.001

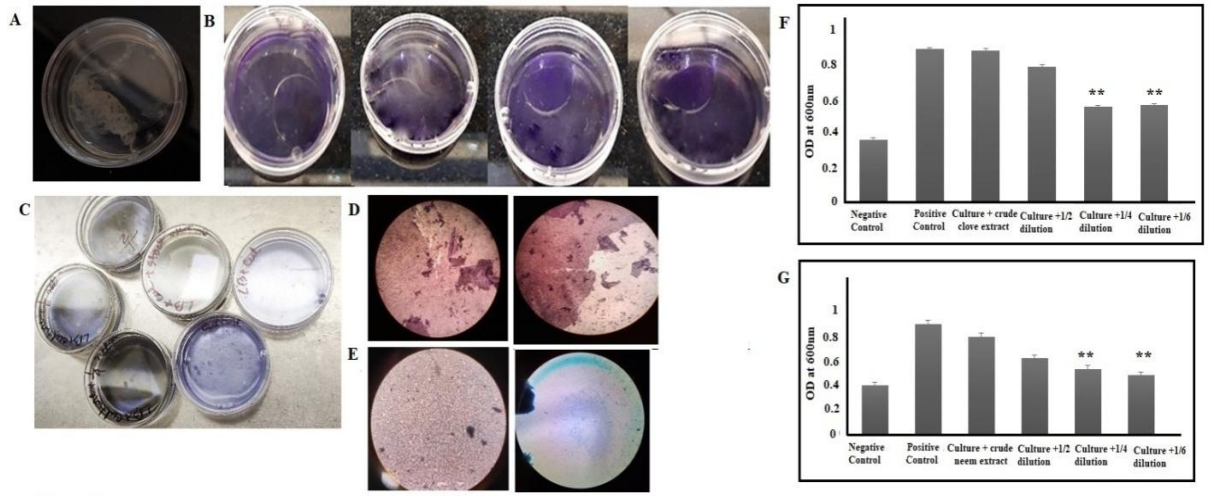

Figure 4A: Biofilm formation in a petri plate for sample OS4B; 4B & C: Variation in color obtained after extracting crystal violet stain from preformed biofilms of OS4B after treatment with crude extract and various dilutions of clove and Neem, respectively; 4D: Microscopic view of biofilm without (left panel) and with a 1/6 dilution of clove and Neem (right panel); 4E & F: A significant reduction in OD reading at 600 nm with increasing dilution of clove and neem extracts respectively; ** indicates P value<0.001

As for the effect of neem extract on the biofilm, the crude extract showed a 15% reduction after 24 and 48 hours of exposure (P<0.01). Different dilutions of neem extract also demonstrated a significant reduction in the formed biofilm, with a reported 23% and 20% reduction after 24 and 48 hours, respectively, for the 1/2th dilution (P<0.001) and a 49% and 47% reduction after 24 and 48 hours, respectively for the 1/4th dilution (P<0.001). The 1/6th dilution of neem extract showed a 54% and 56% reduction after 24 and 48 hours of exposure (P<0.001). In conclusion, both clove and neem extract at 1/6th dilutions showed the greatest capacity to disrupt biofilm formation compared to the crude extracts or the 1/2th and 1/4th dilutions of the extracts.

3.4 Effect of neem and clove extraction on the formation of biofilm

A fresh culture of OS4B was inoculated in sterile petri plates containing freshly prepared nutrient broth with 1% glucose. The growth was allowed to spread on sterile coverslips. After 48 hours, the impact of natural extracts on biofilm production was evaluated. This was done by crystal violet staining followed by observation under a phase-contrast microscope and measurement of absorbance using a spectrophotometer (Figure 4 A-E). Compared to the positive control plates with no extracts (Figure 4D, E), a significant reduction in biofilm formation was noticed in plates with 1/6 dilutions of clove and neem extracts, respectively (Figure 4D, E). Analyzing the effect of clove extract on biofilm formation, the crude extract showed no effect, while only an 11% reduction was noted for the 1/2 dilution. However, a significant reduction in biofilm formation was observed with 1/4 (37%) and 1/6 dilution (36%) (Figure 4F). Similarly, with neem extract, there was a 12% reduction with the crude extract, whereas a more significant reduction was noted with 1/2 dilution (31.5%), 1/4 dilution (41.6%), and 1/6 dilution (46.7%) (Figure 4G).

4 Discussion

Herbal remedies have been widely used in dentistry to treat tooth pain, periodontal inflammation, and oral mucosal diseases caused by sores and microbial infections. Medications of herbal origin, made up of natural ingredients, are considered safe with minimal toxicity and side effects on humans and the environment. Additionally, being indigenous, they are readily available and inexpensive, making them a useful resource for medicinal purposes. In this study, the effectiveness of two popular herbal products, neem, and clove, was evaluated against biofilm-producing pathogens isolated from the oral cavity of people with dental problems and an unhygienic lifestyle. Natural ingredients extracted from these herbal products in organic solvents were found to have antimicrobial activity against fungal strains isolated from oral swabs collected. The fungal strain identified as a species of *Candida* showed rapid biofilm production, which is characteristic of symptoms for different periodontal diseases. Both neem and clove extracts effectively disrupted preformed biofilms of *Candida* sp. and prevented biofilm formation. The efficiency was higher with more prolonged incubation for 48 hours than 24 hours. It is also worth mentioning that the effectiveness of anti-biofilm activity was enhanced significantly with higher dilutions (1/4, 1/6 dilution) compared to crude extract or 1/2 dilution. This finding is consistent with reports stating that within heterogeneous mixtures of bioactive compounds derived from plants, antimicrobial combination effects may include synergy and antagonism (Vaou et al. 2022). Similar observations were reported against both bacterial and fungal strains isolated from the oral

cavity and other sources using extracts of both neem and clove (Agarwal et al. 2010; Harjai et al. 2013; Nagarajappa et al. 2018; Barua et al. 2017; Bansal et al. 2019; Batiha et al. 2020). The antagonism between bioactive compounds in essential oils can occur during antimicrobial interactions in herbal extracts when (i) a combination of bacteriostatic and bactericidal antimicrobials exists, (ii) antimicrobials target the same site, or (iii) antimicrobial natural components interact with each other (Roller, 2003). The results of this study indicated that the antagonistic effect that masks the activity of the prime antimicrobial component may be relieved with higher dilutions. An example of such alteration of antagonism has also been reported for the antimalarial agent artemisinin. In this case, the flavone casticin exhibited antagonistic activity at a 1:3 ratio. However, the in-vitro activity of artemisinin significantly increased with higher dilutions (Liu et al. 1992; Vaou et al. 2022). However, further validation at a larger scale of experiments is needed for confirmation.

Conclusion

The use of herbal medication in dentistry has proven to be effective. Studies have shown that neem and clove extracts exhibit antifungal properties against *Candida* sp., commonly found in individuals with dental diseases. These extracts can reduce preformed biofilms and prevent biofilm formation, making them promising candidates for treating oral Candida infections. Neem and clove extracts are non-toxic and have significant antifungal activities, which could complement existing commercial medications for treating periodontitis and other biofilm-related oral disorders. However, further research with larger sample sizes and detailed investigation of the mode of action of these herbal extracts is needed before their actual application as prescribed medication.

Conflict of Interest

Nil

Authors' Contribution

Conception: Tanushree Bhattacharya, Rudra Prasad Saha, Rajib Majumder, and Sanmitra Ghosh; Literature review: Tanushree Bhattacharya and Sanmitra Ghosh; Data interpretation: Tanushree Bhattacharya, Rajib Majumder, and Sanmitra Ghosh; Manuscript drafting: Tanushree Bhattacharya and Sanmitra Ghosh; Supervision: Sanmitra Ghosh.

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