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Dear Authors,

It is great pleasure to announce that volume 5 issue 5 of Journal of Experimental Biology and Agricultural Sciences (JEBAS) is now available online for worldwide researchers. On behalf of entire JEBAS Editorial Team, I would like to extend a very warm welcome to the readership of JEBAS. I take this opportunity to thank our authors, editors and anonymous reviewers, all of whom have volunteered to contribute to the success of the journal. I am also grateful to the staff at Horizon Publisher India [HPI] for making JEBAS a reality. I extend my sincere thanks to Mr. Purn Mal Jangir, Nagaur, Rajasthan, for providing some beautiful images for cover page of this issue.

JEBAS is dedicated to the rapid dissemination of high quality research papers on advances in various aspects of Experimental Biology, Biotechnology and Agricultural sciences along with computational algorithm which can help us meet the challenges of the 21st century, and to capitalize on the promises ahead. We welcome contributions that can demonstrate near-term practical usefulness, particularly contributions that take a multidisciplinary / convergent approach because many real world problems are complex in nature. JEBAS provides an ideal forum for exchange of information on all of the topics related to Biological and Agricultural Sciences, in various formats such as full length research papers, survey papers, work-in-progress reports on promising developments, case studies and best practice articles written by industry experts.

Finally, we wish to encourage more contributions from the scientific community and industry practitioners to ensure a continued success of the journal. Authors, reviewers and guest editors are always welcome. We also welcome comments and suggestions that could improve the quality of the journal.

Thank you. We hope you will find JEBAS informative.

Dr. Kamal K Chaudhary
Managing Editor - JEBAS
October 2017
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ANTIMICROBIAL PEPTIDES IN SEMEN EXTENDERS: A VALUABLE REPLACEMENT OPTION FOR ANTIBIOTICS IN CRYOPRESERVATION - A Prospective Review

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KEYWORDS
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Antibiotics
Antibiotic resistant
Semen cryopreservation

ABSTRACT

Semen extenders or diluents are added to semen before cryopreservation for maintaining its fertilizing ability even after post thaw. Various types of semen extenders are commercially available for porcine, boar, bull, equine and humans. Bacteriospermia in human and animal samples appears to be concentration-time dependent which might affect quality, quantity and longevity of post ejaculated semen in either neat or with extended states. Antibiotics play a significant role in semen extender which ensures long shelf-life to spermatozoa. Also, it protects the female reproductive tract by hindering the transmission of invading pathogens. Although the use of antibiotics in semen extender is monitored by many authorities of respective concerned government, a lot of guidelines and experiments ensuring a proper usage in cryopreservation is in need. The use of antibiotics in semen extenders also pose a threat of antibiotic-resistant bacterial strains in artificial insemination centers (AI) as well as in assisted reproductive technology (ART) laboratories. Development of multi-drug resistant bacteria has been found to be the primary concern when mixture of a wide range of conventional antibiotics is used in semen extenders. Several potent strategies and critical point analysis are of urgent need to hinder the
Antimicrobial peptides in semen extenders for cryopreservation

1 Introduction

Cryopreservation of semen is used routinely in many areas and fields including assisted reproduction, artificial insemination, military persons, in case of pre-radiation and chemotherapy treatments and so on (Schulze et al., 2017). It is treated as fertility insurance for the males undergoing vasectomy and storage of donor semen. Testicular semen samples collected from non-obstructive azoospermia patients were cryopreserved for later usage in in vitro fertilization (IVF) treatment cycles. Cryopreservation allows a single biopsy collected semen samples from testicular sperm aspiration (TESA) and percutaneous epididymal sperm aspiration (PESA) target patients to be used for many intra-cytoplasmic sperm injection (ICSI) cycles. In order to preserve the semen samples in a better way for a long time and to ensure the feasibility of vital biological functions at subzero temperatures, semen extenders are used. Many types of semen extenders like bovine serum albumin (BSA) based, lecithin based, plant components based, animal protein free and tris based are available commercially in the market for different organisms (Paulenz et al., 2000). Various antibiotics are added to semen extenders to remove the contaminants developed by bacteria and other microbes (Martin et al., 2010). Government agencies and directives are monitoring the antibiotic usage throughout the world.

Given the fact that animal food production related industries rely on cryopreserved semen for artificial insemination (AI), a huge amount of antibiotics are currently used in semen extenders by animal breeders during AI practices. Disadvantage of using a broad range of antibiotics in semen extender is the development of antibiotic resistance over the period of time in the microbe and host organism (Tiwari et al., 2013). In fact the emerging and rising problem of antimicrobial resistant is creating public health concern worldwide (Tiwari et al., 2013; World health organization, 2014; Roca et al., 2015). To overcome this particular problem during cryopreservation, recently researchers have been trying to find an appropriate replacement for antibiotics in semen extender for cryopreservation purposes. Many researchers believed that the valuable replacement option against microbial contamination and also to prevent antibiotic-resistant issues. In order to overcome these problems, low evoking resistant antimicrobial peptides (AMPs) have been used by researchers to replace conventional antibiotics. Antimicrobial peptides are positively charged polypeptides with a minimum of 100 amino acids. They possess high structural diversity and proved to be effective against extensive array of pathogens. These AMPs not only act as pathogen defenders but also exhibit immunomodulatory actions. Naturally male and female genital tract contains many antimicrobial peptides that could play not a suitable role against invading pathogens. This review discusses the different types of antimicrobial peptides that could be used in semen extenders during cryopreservation.

2 Semen extenders and their types

Semen extenders or semen diluents constitute an aqueous solution used to increase the volume of the ejaculated semen and to maintain the functional characteristics of sperm until artificial insemination (AI) (Foote, 2002). Semen extenders have been classified by duration into three different groups like short term, long term and medium term extenders. Short term semen extenders are used in small distance transport and where the semen doses can frequently be made (Paulenz et al., 2000). Long-term semen extenders are used when the semen dose production area and insemination sites are too long. Long time semen
extenders advantages include: enables transport of semen to longer distances, can facilitate prediction of pregnancy outcome before insemination, and help in distributing the semen doses to different centers. In 1980’s, diluents were based on the glucose solutions without freezing the samples, and later the egg yolk and citrate buffers were used in semen extenders (Paulenz et al., 2000). The important innovation of semen extender research started in early 1960 with the addition of chelating agent like Ethylenediaminetetraacetic acid (EDTA) (Plisko, 1965), which are used to block the action of calcium that helps in capacitation and acrosome reaction during cryopreservation. The major components present in commercial semen extenders are glucose, sodium citrate, sodium bicarbonate, potassium chloride, EDTA, acetylcysteine, Heps, Tris, citrate, bovine serum albumin (BSA), cysteine, trehalose, polyvinyl alcohol (PVA), 3-(N-morpholino) propanesulfonic acid (MOPS) and glycerol. Semen extenders have also been classified into bovine serum albumin (BSA) based, lecithin based, soya bean based, skim milk based and tris based extenders (Westendorf et al., 1975; Gadea et al., 2003). The liquid diluents/ extenders are used in a way that should possess the following, provide nutrients and help to maintain the sperm cell metabolism, provide valid protection against the cold shock due to cryopreservation, could control the pH and osmotic pressure and the major concern is to control the development and growth of microorganism during cryopreservation (Gadea et al., 2003). Different types of semen extenders and the kind of antibiotic used by various researchers in the recent years has been tabulated in Table 1. Commercial availability of successful extenders with its antibiotic concentration for use in boar, equine, porcine and human has been tabulated in Table 2.

3 Importance of antibiotics in semen extenders

Microbial contamination occurs mainly during the collection process of the semen (Rillo et al., 1998). Antibiotics in semen extender are added to overcome the growth of contaminated bacteria which is promoted by the presence of glucose in the extenders. The temperature range of 15 to 16°C also promotes the growth of most common Gram negative bacteria in the ejaculate. The addition of antibiotics at an appropriate concentration is needed for sperm survival during cryopreservation and this enhances pregnancy outcomes (Althouse et al., 2000; Gadea et al., 2003). Penicillin and streptomycin were the first used antibiotics in semen extender for cryopreservation. Later, antibiotic cocktail including tylosin (50 μg), gentamicin (250 μg), spectinomycin (300 μg) and lincomycin (150 μg) was used first time in long term bull semen extenders. Most recently, antibiotics like ceftiofur were also used in semen extenders and are being used, but there are no conclusive and evident results on resistance development (Gadea et al., 2003; Schulze et al., 2016).

4 Why should antibiotics be replaced in semen extender preparation?

Due to lack of hygienic standards in AI centers, the antibiotic usage is not regulated in many centers. To counteract the progress of antibiotic resistance in contaminant bacteria, there is a need of highly sophisticated hygienic standard management in AI centers and proper identification of hygienic critical control points (HCCP) (Schulze et al., 2017). Many alternates are there for antibiotics such as physical removal of bacteria during semen collection and processing time. Further alternatives are using single layer centrifugation, and to carry out the process in aseptic conditions. In very recent years, the use of antimicrobial peptides (AMPs) instead of antibiotics has been found to have great importance for the researchers as well in AI centers and cryobanks for better results (Dietrich et al., 2017). The disadvantage of using antibiotics and advantage of using antimicrobial peptides are represented in figure 1. The antibiotic resistances in semen extenders were discussed by Morrell in articles (Morrell & Wallgren, 2014; Morrell et al., 2016).

5 Antimicrobial peptides (AMPs) and their functions

Evolution has gifted every single organism with a wide variety of tools for survival. Evolution and natural selection are concurrent processes which helps human to develop innate mechanisms against microbial evolution. This scenario happens in microbes as well, making them more virulent. The shorter lifespan of microbes makes them accumulate more evolutionary changes than humans, thus giving the microbes a winning edge (Jeral & Porro, 2004). AMPs are produced as first line of defence by human innate immunity system. For the first time, AMPs were initially isolated in 1980’s from varieties of insects and frogs (Zasloff, 1987). After that, a large number of AMPs were identified and are in use for various clinical and medical applications. In general, AMPs possess different functions that cover a broad array of activities. While identifying AMPs in the initial days, researchers pointed that AMPs will have only bactericidal activity, but later found that AMPs also posses antifungal (De Lucca & Walsh, 1999), antiviral (De Lucca & Walsh, 1999), antitumor (Papo & Shai, 2005), β-defensins an antimicrobial peptide is the best example for immunomodulatory peptides which possess both innate and adaptive immune
response, and they reveal direct antimicrobial activity (Dietrich et al., 2017).

6 Uses of AMPs:

The use of AMPs in semen extender was first used with the boar semen ejaculate, and antibiotic resistance was not reported in that case (Paulenz et al., 2000). The use of cyclic hexapeptides and synthetic magainin derivatives was also investigated by many researchers to be used in semen extenders for preserving boar and other species ejaculates (Coelho et al., 2017). The AMPs were found with little/no on eukaryotic cells, and this is the major prerequisite for the application of AMPs in semen extenders. Proteolytic stability, thermodynamic stability, bacterial selectivity and sensitivity, made hexapeptides as the best replacement for antibiotics in semen extenders for long time storage of semen samples especially for humans (Hall-Stoodley et al., 2012).

Many higher species including humans possess innate immune defense mechanisms, of which AMPs were found to be necessary components. The biological activity of each AMPs varies as the primary and secondary structure differences exhibited by the peptides affect their functionalities (Hall-Stoodley et al., 2012).

This is the crucial step that has to be focused while selecting AMP for the replacement of antibiotics in semen extenders. Conversely, the cationic charge and amphipathicity of AMPs were found to be conserved characteristics which explain its selective action on bacterial membranes that are highly negatively charged lipid molecules. Lysozyme isolated from human saliva (130 amino acids), by Alexander Fleming in 1922 still stands as the widely used antimicrobial protein of human origin, (Fleming, 1922).

Many peptides have been found to be rich in particular amino acids especially phenylalanine, tryptophan or arginine. For this reason the antimicrobial motifs in natural proteins, acts as potent candidates for designing selective and effective AMPs in clinical and medical applications (Chan et al., 2006). Many alternate methods were there to replace antibiotics in semen extender like doing Single Layer Centrifugation (SLC), carried out with more strict aseptic conditions may be very helpful in reducing the bacterial contamination (Morrell & Wallgren, 2014), but this technique (SLC) has limitations too. The best alternative is to replace the antibiotics with AMPs in semen extenders. Many researchers have worked with different AMPs for substituting conventional antibiotics in semen extenders. Critical studies related to AMPs in semen extenders and their implications were tabulated in table 3.
### Table 1 List of different semen extenders with its antibiotics used by researchers

<table>
<thead>
<tr>
<th>Semen extender used</th>
<th>Remarks</th>
<th>Antibiotics used in semen extender</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS Citrate, TRIS Milk</td>
<td>Short term storage of Ram semen</td>
<td>20 µM Penicillamine/ 50 µg mL⁻¹ of gentamicin</td>
<td>Acharya et al., 2017</td>
</tr>
<tr>
<td>Many different extenders were used with supplementation of lipoprotein fraction from ostrich egg yolk</td>
<td>Boar semen/ lipoprotein fraction of ostrich egg yolk</td>
<td>50 µg mL⁻¹ of gentamicin is the major antibiotic used in all the different extenders</td>
<td>Dziekonska et al., 2017</td>
</tr>
<tr>
<td>TRIS citrate, Milk, Soyabean with Ringer’s solution and Glucose solution 5%</td>
<td>Cocktail semen preservation with mentioned semen extenders</td>
<td>70 µg mL⁻¹ of gentamicin and 20 µM Penicillamine</td>
<td>Schneider et al., 2017</td>
</tr>
<tr>
<td>Boars semen extender with soyabean and lecithin</td>
<td>Boars semen to check the quality after addition of betaine in semen extender</td>
<td>Sperm parameters were maintained with betaine with addition of gentamicin antibiotics 70 µg mL⁻¹</td>
<td>Lugar et al., 2017</td>
</tr>
<tr>
<td>Human semen extenders (E4), TRIS extender with antioxidants</td>
<td>Human semen extenders supplemented with Tea Polyphenol-T. Arjuna Bark, antioxidant extender</td>
<td>Penicillin-streptomycin 50 µg mL⁻¹</td>
<td>Parameswari et al., 2017</td>
</tr>
<tr>
<td>Boar semen preservation, TRIS semen extender</td>
<td>Boar semen</td>
<td>Gentamicin varying concentration with median 220.37 mg/L</td>
<td>Schulze et al., 2017</td>
</tr>
<tr>
<td>Boar semen extenders, TRIS Semen extenders</td>
<td>Boar semen samples preservation</td>
<td>Replacement of antibiotics with cationic antimicrobial peptides</td>
<td>Schulze et al., 2016</td>
</tr>
<tr>
<td>Turkey, EK, Lake and Chicken semen extenders, TRIS semen extenders</td>
<td>Indian Red Jungle Fowl (Gallus gallus marghi)</td>
<td>Gentamicin/penicillin 50 µg mL⁻¹</td>
<td>Rakha et al., 2016</td>
</tr>
<tr>
<td>Two different egg yolk based semen extenders and soyabean lecithin based semen extender</td>
<td>Bull semen samples</td>
<td>100000 IU penicillin, 100 mg streptomycin</td>
<td>Abdussamad et al., 2016</td>
</tr>
<tr>
<td>Boar semen extenders</td>
<td>Liquid semen storage of boar semen and effect of bacteriospermia</td>
<td>Streptomycin 50 µg mL⁻¹</td>
<td>Kuster &amp; Althouse, 2016</td>
</tr>
<tr>
<td>Egg yolk and soybean extenders</td>
<td>Evolution from egg yolk to soybean-based extenders</td>
<td>Gentamicin/penicillin 50 µg mL⁻¹</td>
<td>Layek et al., 2016</td>
</tr>
<tr>
<td>egg yolk and soya milk-based extenders</td>
<td>buffalo semen sample cryopreservation</td>
<td>Streptomycin 50 µg mL⁻¹</td>
<td>Chaudhari et al., 2015</td>
</tr>
<tr>
<td>Magnetized semen extenders containing BSA</td>
<td>Boar semen sample long time preservation</td>
<td>Gentamicin/penicillin 50 µg mL⁻¹</td>
<td>Lee &amp; Park, 2015</td>
</tr>
<tr>
<td>Modified Beltsville extender</td>
<td>Rooster post-thaw semen quality</td>
<td>Streptomycin 50 µg mL⁻¹</td>
<td>Amini et al., 2015</td>
</tr>
<tr>
<td>Four different semen extenders, TRIS, soyabean, Lecithin, Milk based</td>
<td>Human semen samples preservation</td>
<td>Gentamicin/Penicillin/ Streptomycin 50 µg mL⁻¹</td>
<td>Vickram et al., 2015</td>
</tr>
<tr>
<td>Twelve different semen extenders</td>
<td>Boar semen samples</td>
<td>Gentamicin/Penicillin/ Streptomycin 50 µg mL⁻¹</td>
<td>Akandi et al., 2015</td>
</tr>
<tr>
<td>Soybean lecithin-based semen extender</td>
<td>Ram semen samples</td>
<td>Streptomycin and different antioxidants 50 µg mL⁻¹</td>
<td>Sharafi et al., 2015</td>
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<tr>
<td>Soybean-Based Extenders</td>
<td>Friesian Bull semen samples</td>
<td>Gentamicin/Penicillin/ Streptomycin 50 µg mL⁻¹</td>
<td>Rehman et al., 2014</td>
</tr>
<tr>
<td>Different types of bovine and canine commercial available semen extenders, soyabean and lecithin semen extenders</td>
<td>Brown bear semen samples</td>
<td>Gentamicin 50 µg mL⁻¹</td>
<td>Gomes-Alves et al., 2014</td>
</tr>
<tr>
<td>plant-based soybean lecithin semen extenders</td>
<td>Goat semen sample preservation</td>
<td>Streptomycin and different antioxidants 62 µg mL⁻¹</td>
<td>Salmani et al., 2014</td>
</tr>
</tbody>
</table>
### Table 2 Commercial semen extenders and the antibiotics used

<table>
<thead>
<tr>
<th>Commercial successful semen extenders</th>
<th>Model for semen cryopreservation</th>
<th>Antibiotics used</th>
</tr>
</thead>
<tbody>
<tr>
<td>AndroPRO® Plus</td>
<td>synthetic long-term porcine semen extender</td>
<td>Ampicillin, Apramycin, Enrofloxacin 60 µg mL⁻¹</td>
</tr>
<tr>
<td>AndroMed®</td>
<td>Long-term Bull semen extender</td>
<td>Antibiotic cocktail includes Tylosin 50µg, Gentamicin 250µg, Spectinomycin 300 µg and Lincomycin 150 µg</td>
</tr>
<tr>
<td>Triladyl® &amp; Biladyl®</td>
<td>Long-term Bovine semen extender</td>
<td>Tylosin, Gentamicin, Spectinomycin, Lincomycin</td>
</tr>
<tr>
<td>EquiPRO CoolGuard</td>
<td>Long term Equine semen extender</td>
<td>Amikacin &amp; Penicillin</td>
</tr>
<tr>
<td>Kobidil+ semen extender</td>
<td>Short term semen extender for Boar semen samples</td>
<td>Gentamycin sulphate 200 mg/L.</td>
</tr>
<tr>
<td>Beltsville semen extender</td>
<td>Short term semen extender</td>
<td>Gentamycin sulphate 250 mg/L.</td>
</tr>
<tr>
<td>Acromax semen extender</td>
<td>Long term Boar semen extender</td>
<td>Lincomycin 13.3 mg/L and spectinomycin 26.6 mg/L.</td>
</tr>
</tbody>
</table>

### Table 3 Important studies related to antimicrobial peptides (AMP’s) in semen extender and cryopreservation:

<table>
<thead>
<tr>
<th>Theme of the study</th>
<th>Implications</th>
<th>Conclusion</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can antimicrobial peptides be an alternative for antibiotics in semen extenders?</td>
<td>There is a possibility of multi-bacterial resistant in offspring via artificial insemination (AI). Enormous steps have been taken to employ AMPs to substitute conventional antibiotics in semen extenders</td>
<td>Magainin and cyclic hexapeptides, antimicrobial peptide derivates can be used in boar semen extenders to replace the existing conventional antibiotics</td>
<td>Schulze et al., 2016</td>
</tr>
<tr>
<td>Can cationic synthetic peptides be used in semen extender for cryopreservation?</td>
<td>The antibacterial activity of two types of cyclic synthetic peptides (c-WWW, c-WWF) and one helical peptide called magainin II against Gram-positive and Gram-negative bacteria</td>
<td>These antimicrobial peptides, not a complete alternative to conventional antibiotics and still to avoid multi-strain resistant. Cyclic and helical peptides may be the possible alternative for antibiotics in boar semen preservation</td>
<td>Speck et al., 2014</td>
</tr>
<tr>
<td>hCAP-18 (human cationic antimicrobial peptides produced from epithelium of epididymis and contributes counter immunity against female reproductive tract bacteria</td>
<td>By immunohistochemistry, hCAP-18 was able to detect in the epididymis and not in the testis. hCAP-18 was found to have antibacterial activity against broad spectrum of bacteria</td>
<td>This hCAP-18 antimicrobial peptide can probably use in many medical applications including cryopreservation</td>
<td>Malm et al., 2000</td>
</tr>
<tr>
<td>Processing of hCAP-18 to ALL-38 antimicrobial peptides in female reproductive tract</td>
<td>Seminal plasma antimicrobial peptide hCAP-18 was further processed to generate a 38 amino acid antimicrobial peptide called ALL-38 by gastricsin</td>
<td>ALL-38 was found to possess antibacterial activity against a wide range of bacteria. Further, it protects from infection after sexual intercourse</td>
<td>Sorensen et al., 2003</td>
</tr>
<tr>
<td>Is GL13K - an antimicrobial peptide are effective against Pseudomonas aeruginosa</td>
<td>GL13NH2 was not showing any bactericidal activity, later it was introduced with three lysine residues and converted into an active antimicrobial peptide called GL13K</td>
<td>GL13K - an antimicrobial peptide found to be very active against Pseudomonas aeruginosa</td>
<td>Hirt &amp; Gorr, 2013</td>
</tr>
<tr>
<td>Is Semenogelin derived antimicrobial peptides protects spermatozoa?</td>
<td>Cationic antimicrobial peptide was derived from Semenogelin protein</td>
<td>It poses number of applications including antibacterial and antiviral activity could be used in semen extenders</td>
<td>Bourgeon et al., 2004</td>
</tr>
</tbody>
</table>
7 Cationic antimicrobial peptides (CAMPs):

Cationic antimicrobial peptides exert direct antibacterial and antifungal activity. It also possesses various biological functions like inhibition of lipopolysaccharide related inflammatory, immunomodulation and immunosuppressant activity (Mulder et al., 2013). Along with the above mentioned functions, antibiotic resistance evolution in using conventional antibiotics makes cationic antimicrobial peptides a better replacement for conventional antibiotics in semen extenders for cryopreservation (Zasloff, 2016). It was found that 80-85% of the infections were with biofilm etiology in the United States (Fux et al., 2003; Davies et al., 2006). Extracellular matrix along with low growth rates associated with biofilms necessitates a 10 to 1000 fold higher concentrations of antibiotics to curtail bacterial growth (Hawkey, 2008). Antibiotic resistance among medically important microbes is on rising, and this also adds to the existing challenge (Boucher et al., 2009).

CAMPs were seen as a potential alternates to deal with the problem of antibiotic resistance development and biofilms (Schulze et al., 2014; Speck et al., 2014). In comparison to traditional antibiotics, CAMPs lower the probability of microbial resistance. Antigenic sites on bacterial membranes change as a result of antibiotic resistance evolution and less specific activity expressed by CAMPs on bacterial membranes makes development of resistance against CAMPs in bacteria impossible (Yenugu et al., 2004). This circumvents the restriction of many traditional antibiotics that require bacterial growth (Ernst & Peschel, 2007). Bradshaw discussed issues for potential clinical use of CAMPs (Bradshaw, 2003). The conception that CAMP may not possess broad-spectrum activity was found wrong. Ambicin, gramicidin S are found to be the best examples for broad spectrum activity of antimicrobial peptides. Researchers showed that 13-amino-acid peptide GL13K exhibited low toxicity and broad antibacterial activity against mammalian cells and planktonic bacteria, respectively (Abdolhosseini et al., 2012). GLK13K is effective against Pseudomonas aeruginosa biofilms formed on pegs of a Calgary device, associated with a static biofilm system (Ceri et al., 1999). Overall, Ceri results showed that a 99.9% reduction in bacterial cell numbers in a 4-h treatment with 100 µg/mL concentration of this GLK13K. These results support the usage of GLK13K in human and animal semen preservation in extenders instead of standard antibiotics. Biofilm growth can be inhibited/controlled by employing mechanical forces, which is not possible in cryopreservation process. Hence the use of CAMPs is greatly realized. More specifically, CAMPs are highly sensitive to physiological salt concentrations. In turn, this high sensitivity limits the utilization of CAMPs in biological fluids (Goldman & Widom, 1997).

Two different synthetic cationic antimicrobial peptides like c-WWW and c-WFW which belongs to cyclic hexapeptide group were investigated with boar semen extender for cryopreservation (Speck et al., 2014). Speck used a synthetic helical amide analog, magainin II (MK5E), for boar semen sample preservation invitro. This was found to be effective against gram positive and gram negative micro flora found in boar semen. In this study boar semen samples were initially maintained only with Beltsville Thawing Solution (BTS) with 250 µg/mL gentamicin which is treated as a control group. Boar semen samples were then grouped into three like BTS+c-WWW, BTS+c-WFW, and BTS+MK5E. Minimum inhibitory concentration (MIC) for all the AMPs was done using broth microdilution technique (Speck et al., 2014). The author concludes that both c-WWWW and c-WFW was showing activity against almost all bacteria in liquid preserved boar semen samples. Speck et al finds that antimicrobial peptides in semen extenders would be a better replacement for conventional antibiotics and it will lead to limit the selection of multi-resistant strains (Speck et al., 2014).

8 Human parotid secretory protein associated AMPs in semen extender:

Human parotid secretory protein (PSP) which is found to have structural similarity with bactericidal, and two potential proteins, permeability-increasing protein, and lipopolysaccharide binding protein, could be possibly used in semen extender (Abdolhosseini et al., 2012). These two peptides were found as potential antimicrobial peptides and the sequence information was identified from PSP. This could be more relevant replacement option, as it is found to have structural similarity with lipopolysaccharide binding protein, as prostasomes were rich in lipid content and played a significant role in motility (Schiessel, 2003). Helmut Hirt derived a 13-amino acid peptide called GL13K, from human PSP. Initially, the antimicrobial and various functions of this particular protein was attributed to amino acid residues 141 to 153 of PSP (GL13NH2) (Hirt & Gorr, 2013; Chen et al., 2014). This peptide was found to have the property of aggregating both Gram-negative and Gram-positive bacteria and capable of binding with LPS, but many times the peptide lacks bactericidal activity.

GL13K derived its name from the replacement of charged aminoacids in 5th and 11th position by lysine (K) residues. This replacement event confers bactericidal activity to the AMP but prevents it from acting as a bacterium agglutinating agent. Researchers found that this GL13K was also active against bacteria in biofilm communities as well as monospecies (Bingle & Gorr, 2004). GL13K was reported to significantly reduce the cell count in biofilms grown either under aerobic or anaerobic conditions. So, as per these results, GL13K could be the source
9 Male and female genital tract derived AMPs

Mammalian organs synthesized several types of antimicrobial peptides with multi-functionalities. Amongst them, the most focused areas are the epithelial layer of skin, and entire digestive, respiratory and reproductive tracts (Zhang et al., 2000; Gallo et al., 2002; Boman, 2003). Compared to any other tract, genital tract of human males and females produces enormous endogenous proteins and peptides which have anti-infectious characteristics. In humans, the most studied cationic AMP’s are β-defensins and cathelicidins (Frew & Stock, 2011). Defensins contain a series of cysteine molecules that are considered as part of immune defense system, in the genital tract. Many β-defensins are synthesized mainly in the epithelial cells of epididymis during sperm maturation, later released into the lumen, finally detected on the surface of sperm membrane (Com et al., 2003). This attachment refers to not only the function of antimicrobial activity but also in the proper function of fertilization. In a study, defensin depleted mutant spermatozoa was found with impaired motility, morphology, a destabilized microtubule structure and finally results in infertility (Zanic et al., 2003; Zhou et al., 2004).

The precursor protein hCAP18, which is considered to be the only representative for antimicrobial activity from the human male genital tract, was predominantly synthesized in the epididymis (Hammami-Hamza et al., 2001). CAP18 molecules have also been identified in head, tail and neck region of the sperm cell as well in seminal plasma. The majority of hCAP18 proteins were associated with proteasomes. The primary functions of the prostasomes were sperm cell-proteasome interaction, enhancing sperm cell rapid motility, induction of sperm cell capacitation, induction of acrosome reaction and finally the fertilization (Gombart et al., 2005). So, once the sperm cells are deposited in the female reproductive tract, though the cell may be matured to fuse with the ova, it requires many series of reactions in order to fertilize. Prostasomes fulfilled these series of steps in female reproductive tract. Prostasomes are rich in cholesterol-phospholipid ratio. Therefore it is opined that during fusion event of prostasomes with spermatozoa, the protein like hCAP18 which is present on the surface will also be transferred along with cholesterol to the sperm cell membrane and here the transport is mediated by the sperm into the uterus. Once the proteolytic processing of hCAP18 was over, the AMP LL-37 get released from the precursor protein. When it was releasing, the disruptive nature of this peptides were protected by the cholesterol enrichment in the sperm membrane (Yarbrough et al., 2014). This particular antimicrobial peptide can also be isolated from the male genital tract and can be processed to use in the semen extender for cryopreservation of sperm cells against contamination produced by many bacteria as well to maintain the integrity of sperms (Ciornei et al., 2003). Many antimicrobial peptides were synthesized by sperm cells and seminal plasma and transferred to the female reproductive tract, but female reproductive tract itself is also capable of synthesizing some antimicrobial peptides to evade the pathogens and immunomodulatory functions. Lipophilin, an antimicrobial peptide derived from female reproductive tract (FRT) showed broad spectrum activity. This type of peptide can also be used in semen extender during cryopreservation (Dcruz et al., 1995).

10 Development of AMPs resistant pathogens:

There are some bacteria which are resistant to even AMPs through many mechanisms. The major mechanism of getting resistance is through the selective production of bacterial proteases (Hashemi et al., 2017). After the penetration, peptides are very prone for transportation outside the cell through the energy-dependent mechanism and finally resided at the cytoplasmic membrane of bacteria. In addition to this, the formation of extracellular polymers as a biofilm further makes the microbes insensitive to AMP effects. A study was conducted with pexiganan and reported that resistance of AMPs was predicted when some bacterial population is regularly exposed with AMPs (Perron et al., 2006). These types of reports put many questions on researchers and on ART professionals to include AMPs in semen extender and its practices, but there is no timescale limit to predict the development of AMP resistant pathogens (Van Nierop et al., 2008).

11 Plant-derived AMPs:

AMPs can also be isolated from different plant species, within those different parts including flowers, leaves, fruits, roots, tubers, and especially seeds (Pelegrini et al., 2008; Benko-Iseppon et al., 2010; Silva et al., 2011; Astafieva et al., 2013; Singh et al., 2016). Plant-derived AMPs have many characteristic features in common that includes, low molecular mass, amphipathic properties, net positive charge at physiological pH, having more repeats of cysteine residues connected in pairs forming disulfide bridges, resulting in stable peptides (Astafieva et al., 2013). These types of AMPs have an explicit interaction with particular cellular membranes and active against certain pathogens, with these properties, AMPs from capsicum have some role in preventing the infections of various pathogens evading. Different fractions of enriched peptides have been isolated from capsicum including PEF1, PEF2, and PEF3 that exhibited a potent antifungal activity against different yeasts (Dias et al., 2013). The N-terminal sequence of these peptides was shown with highest similarity level with serine protease inhibitors (SPI) when analyzed through proper database systems. Taveira et al. identified a protein that is equal to the compound thionin from capsicum that has severe
antifungal and antibacterial activity, and this shows effective therapeutic reactions against Candida species (Taveira et al., 2014; Taveira et al., 2016). In this research, the author concluded that antimicrobial peptides isolated and purified from capsicum demonstrated in vitro inhibitory activity against a range of fungi and sometimes with bacteria of agronomic importance.

Conclusions

Antibiotics are said to be the mandatory component while preparing semen extender for cryopreservation. High level increase in the resistance rate against conventional antibiotics in semen extenders demands an alternative for antibiotics in semen extender. In this review, we suggest that the only best alternative to traditional antibiotics is antimicrobial peptides in semen extender. Especially, the antimicrobial peptides derived from male and female genital tract could be the best peptides that render the antibiotic functions in semen extender.

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Conflicts of interest:

All authors declare that there exist no commercial or financial relationships that could in any way lead to a potential conflict of interest.

References


Da Costa P, Loureiro M, Matos AJ (2013) Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans,


Gadea J (2003) semen extenders used in the artificial insemination of domestic animals and is strongly upregulated in myeloid cells by 1, 25-dihydroxyvitamin D3.


BENEFICIAL IMPACTS OF CHOLINE IN ANIMAL AND HUMAN WITH SPECIAL REFERENCE TO ITS ROLE AGAINST FATTY LIVER SYNDROME

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KEYWORDS
Choline
Micronutrient
Fatty liver syndrome
Body fat
Human
Poultry

ABSTRACT

Choline exists in feed ingredients and also synthesized in the body. It is essential for the physiological functions such as performance and lowering liver and body fat. If choline is insufficient in the diet, the liver fat content and abdominal fat content increase causing a metabolic disorder known as a fatty liver syndrome. Thus, dietary supplementation of choline as synthetic choline chloride or through natural herbs to the diets is compulsory. Besides fatty liver syndrome, choline deficiency causes loss of hepatocytes, heart diseases, bone and growth development abnormalities and impairment in kidney functions. The main indication of choline deficiency is raised up the level of liver enzymes like alanine transaminase (ALT). This ALT is usually measured clinically during diagnostic evaluation of hepatocellular injury to fix liver health. Lipotropic nutrients like choline could prevent fatty liver disorders through several mechanisms, such as increased hepatic very low-density lipoproteins (VLDL) secretion. Human and animal studies have reported protective impacts of choline for fatty liver disease (FLD) in addition to coronary heart disease (CVD) prevention from epidemiological data. Nowadays, choline supplementation is below the dietary recommendations because of a lack of understanding the importance of this vital nutrient for human and animal health. In the current review article, literature...
showed that choline can be considered as a powerful lipotropic agent that should be used as commercial feed additive to cope the metabolic disorder like fatty liver syndrome in poultry, exclusively in layers reared in cages and thus replaced synthetic medicine that being used against fatty liver syndrome.

1 Introduction

Choline is a water-soluble micronutrient. Sometimes, it is classified among vitamins like vitamin B group and defined as a natural compound (Alagaway et al., 2016). But, it is not related to vitamins and considered as an essential nutrient due to destined much nutritional value. Currently, choline has got great importance in the last years because it can prevent liver fattening, support brain development and play a crucial role in the neural conduction. Choline has an essential role in the synthesis of beta-lipoproteins and phospholipids. It enables the transportation and burning of fats, which are also associated with its preventive effect against the fatty liver disorder. In some experimental animal models, choline deficiency caused liver fattening and loss of hepatocytes heart-related diseases, bone development abnormalities and impaired kidney functions (Biswas & Giri, 2015). In poultry industry, nutrition represents about 70% of total costs, thus constitutes are a key factor in poultry production. Choline is classified as an essential vitamin for day-old chicks; it is usually added to diets for the purpose of furnishing the body with labile methyl group for formation of creatine and methionine. In addition, it also assists in the prevention of hemorrhagic kidney in different animal models and perosis in turkeys and broilers (Ross et al., 2013). The deficiency of choline is normally noticed in chicks from 1–4 weeks of age. However, the rate of choline synthesis in chickens’ increases in growing chicks over 8 weeks of age (Ross et al., 2013). Corbin & Zeisel, 2012 theorized that laying hen seems to have substantial ability to synthesize choline. The latest authors found that the addition of choline to 100 mg/kg diet did not show any effect on the aspects of egg size, egg production and relative weights of albumin and yolk. Supplementation of choline at 0.4% to broiler diet improved growth traits (Ross et al., 2013). In another study by Emmert & Baker (1997) reported that the addition of choline chloride at the rate of 2000 mg/kg to the broiler diets showed a positive effect on body weight gain of chicks. Methionine is a sulfur amino acid and it is crucial for repair, growth, and metabolism of all tissues and also for reproduction (Ross et al., 2013). The level of methionine in the animal diet is important because of the relationship with the choline needy as a methyl donor and vice versa. Choline can be supplemented in addition to methionine, but will not spare the basic methionine requirements for protein synthesis without the diet contains homo cystine (Ross et al., 2013). Pesti et al. (1981) perceived that the addition of either choline or methionine to basal diet improves growth. Some trials like Huang et al. (2015) were conducted on rats and revealed that choline insufficiency could increase the risk of getting cancer. Many kinds of literature have naked associations between choline metabolism and cancer (Kirienko et al., 2015; Marina et al., 2016). The utilization of herb and lipotropic supplements to the feed decreases the adverse metabolic consequences of the high-calorie diet in poultry farming (Khosravinia et al., 2015; Saeed et al., 2017). Therefore, L-carnitine, vitamin B12, and vitamin E are routinely added to the poultry diet in order to reduce the liver fattening syndrome (Farrokhhyan et al., 2014). The study of Jiang et al. (2014) reported that choline chloride has a lowering effect regarding cholesterol of broiler. Nowadays, the fatty liver syndrome is a metabolic disorder and it is generally encountered in the poultry industry, especially in hens kept in cage farming system. Because birds kept in cages cannot move enough to burn the calories they were taking. During egg lying, the liver, which has already become rather brittle, can easily tear. If the tears occur on large blood vessels, the bird will be susceptible to sudden death as a result of this bleeding. The present review article aimed to give more light on the structure of choline, its sources, functions, and metabolism. In addition, the current article aimed to broaden the knowledge among researchers and poultry breeder about the use of choline on a commercial level to overcome the fatty liver syndrome disorder and its repercussions that are a big threat to the poultry industry.

2 Choline chemical structure

Choline is a quaternary ammonium (also known as trimethyl, β-hydroxy ethyl ammonium) compound found in lipids and its molecular weight is 121.18 (Sheard & Zeisel, 1989) as shown in Figure 1.

![Chemical structure of choline](http://www.jebas.org)
3 Different sources and requirements of choline

In 1849, choline was firstly isolated from ox bile (Chole in Greek). Since 1930, the nutritional significance of choline has been recognized and nowadays it’s commonly dietary additive for humans and animals. Choline as chloride or sometimes as other salts like citrate is recognized as not harmful. The chief source of choline in poultry industry can be derived from the green leafy material. Moreover, the study by Song et al. (2012) reported that liver and glandular meal, fish meal and soybean meal are the richest sources of choline in poultry feedstuffs’ industry. Vitamins are essential in poultry, animal and human nutrition. The requirement of these nutrients cannot be covered by the native content in feedstuffs, so extra supply is necessary to fulfill the nutrient requirements in the poultry diet (Song et al., 2012). The standard content of choline chloride in various feedstuffs based on chemical composition of crops according to NRC (1994) is given in Table 1. Normal requirement of choline in various poultry species are given in Table 2. Emmert & Baker (1997) assessed choline bioavailability which naturally presents in peanut, soybean meal and rapeseed at rates of 83, 24 and 76 %, respectively. Rape seed meal has high levels of choline (6198 ppm) than those in soybean meal (2218 ppm) and peanut meal (1685 ppm).

4 Functions and synthesis of choline

The functions of choline are categories in four broad terms in the animal body (Zeisel & Niculescu, 2006; Garrow, 2007).

I. Choline is important for metabolism in maintaining and building cells. In addition to, it is required for the maturation of bone cartilage matrix (Figure 2).

II. It plays a key role in the lipid metabolism in the liver. Also, choline suppresses the abnormal deposition of lipids by increasing the consumption of fatty acids or by activating its transport as lecithin in the liver (Xue & Cui, 2001). Choline is considered as a “lipotropic” agent due to its function of acting on lipid metabolism (Corbin & Zeisel, 2012). In the liver of broilers, adding choline (760 mg/ kg diet) reduced fat content (Rama Rao et al., 2001).

III. It is crucial for the formation of acetylcholine that plays an important role in transferring the nerve singles from presynaptic to postsynaptic fibers of sympathetic and parasympathetic nervous systems.

IV. It is considering the vital source of methyl group. It furnishes methyl group for the creation of methionine from homocysteine and of creatine from guanido acetic acid. Zeisel (1990) confirmed that a disturbance in metabolism of methionine results in alterations in metabolism of choline and vice versa. In rats, Kim et al.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Corn</td>
<td>620</td>
<td>713</td>
<td>200</td>
</tr>
<tr>
<td>Soybean meal (48%)</td>
<td>2731</td>
<td>3140</td>
<td>3560</td>
</tr>
<tr>
<td>Fat meat meal (55%)</td>
<td>2077</td>
<td>2388</td>
<td>1570</td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>330</td>
<td>379</td>
<td>660</td>
</tr>
<tr>
<td>Wheat</td>
<td>1002</td>
<td>1152</td>
<td>440</td>
</tr>
</tbody>
</table>

1NRC values are present in choline hydroxide; they have been converted in to choline chloride (equivalent multiply by 1.15). 2IEEB: (Institute European de Environment de Bordeaux, F.3300 Bordeaux): results are established on chemical analysis.

<table>
<thead>
<tr>
<th>Avian Species</th>
<th>Feed</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler Chickens</td>
<td>Cereal grain basal diet</td>
<td>1300 mg/kg of diet</td>
</tr>
<tr>
<td>0-3 weeks</td>
<td></td>
<td></td>
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<tr>
<td>3-6 weeks</td>
<td></td>
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<tr>
<td>6-8 weeks</td>
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<tr>
<td>Laying Hen</td>
<td>Cereal grain basal diet</td>
<td>105 mg/hen/day</td>
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<tr>
<td>0-6 weeks</td>
<td></td>
<td></td>
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<tr>
<td>6-12 weeks</td>
<td></td>
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<tr>
<td>12-18 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turkey</td>
<td>Cereal grain basal diet</td>
<td></td>
</tr>
<tr>
<td>0-4 weeks</td>
<td></td>
<td>1600 mg/kg of diet</td>
</tr>
<tr>
<td>4-8 weeks</td>
<td></td>
<td>1400 mg/kg of diet</td>
</tr>
<tr>
<td>8-16 weeks</td>
<td></td>
<td>1100 mg/kg of diet</td>
</tr>
<tr>
<td>16-20 weeks</td>
<td></td>
<td>950 mg/kg of diet</td>
</tr>
<tr>
<td>20-24 weeks</td>
<td></td>
<td>800 mg/kg of diet</td>
</tr>
<tr>
<td>Breeding cycle</td>
<td>Cereal grain basal diet</td>
<td>800-1000 mg/kg of diet</td>
</tr>
</tbody>
</table>
Importance of choline in animal and human (1994) observed that the deficiency in folic acid may be caused secondary deficiency of choline in the liver.

V. Increasing the gain of interest for choline and its important role as a methyl donor is possibly the key factor that evaluates how rapidly a diet deficient in choline will bring pathology (Finkelstein et al., 1982). The liver can produce large amounts of betaine-homocysteine methyltransferase under the conditions of methionine-deficient, exclusively in the presence of excess betaine or choline (Emmert & Baker, 1997).

VI. Choline may reduce the risk of hepatic and cardiovascular diseases by being available in local markets as choline-enriched eggs that are favorable needs among consumers (Krishnan, 2010).

5 Choline metabolism pathway

Choline is a key source of methyl group through its metabolite, which takes part in the synthesis of S-adenosyl methionine. Moreover, choline and its metabolites have many biological and physiological functions such as structural integrity, acetylcholine synthesis and signaling roles for cell membranes (Cuccurullo et al., 2017). Choline is a vital nutrient that is essential for neurotransmitter (acetylcholine) synthesis, cell membrane structure, methyl-group metabolism and signaling, and lipid transport. Phosphatidylcholine is synthesized in nucleated cells by the pathway of CDP-choline; this way used choline as the preliminary substrate, and therefore it depends on dietary level of choline. The liver is a unique organ that possesses a second pathway for phosphatidylcholine synthesis; phosphatidylethanolamine N-methyltransferase (PEMT) converts phosphatidylethanolamine (PE) to phosphatidylcholine via 3 sequential methylations using S-adenosylmethionine as the methyl donor (Gibellini & Smith 2010). Figure 3 summarizes the metabolism of choline.

6 Choline digestion

Choline is generally absorbed in the jejunum and ileum parts of the intestine by sodium and an energy-dependent carrier pathway (Veth et al., 2016). After absorption, choline is transferred to the lymphatic circulation basically in lecithin form that linked to chylomicron in the phospholipids tissues (Veth et al., 2016).

**Figure 2** Synthesis and various metabolic functions of choline and related compounds.
7 Incredible impacts of choline

7.1 Choline as Antioxidant

Choline plays an important role in multiple clinical manifestations. The function of choline as methyl is a key importance in maintaining balanced cellular antioxidant defense systems subsequently checking oxidative stress and apoptosis (Table 3). Corbin & Zeisel (2012) illustrated the connection between choline deficiency and development of non-alcoholic fatty liver disease (NAFLD) which may finally progress to hepatocarcinogenesis. The previous study in human as well as in mice confirmed that a deletion of choline-related genes may alter mitochondrial membrane composition owing to choline deficiency. Levels of gut microbiome moderating the availability of choline might enhance the fatty liver disease. These findings established a new understanding that choline is a vital component of diet requirement and gave new insight ways in which many physiological conditions take place (Corbin & Zeisel, 2012).

7.2 Choline as Growth Enhancer

Choline is an essential vitamin for the prevention of perosis as well as for growth performance of poultry species. The choline requirement as demonstrated by growth that was better gained when the diet containing 3467 kcal ME/Kg diet, while the requirement for safeguard against perosis was greater to be about 1900 ppm (Fritz et al., 1967). Nesheim et al. (1971) showed that choline supplementation reduced hepatic fat content compared to control; however no improvements in weight gain for pullets fed corn-soy diet supplemented with choline during 8-20 weeks of age. Agricultural Research Council, 1975 showed that the growing poultry chicks have a requirement for choline of around 1300 mg/kg diet (ARC, 1975).

Lipstein et al. (1977) reported that the chicks that were fed choline up to 520 and 480 mg/kg in basal diets shown good responses as compared to that contained choline at the rate of 400 and 230 mg/kg diet. Pesti et al. (1979; 1980) reported large increases at the rate of 12% in body weight gain with supplementation of choline at 0.04 to 0.39% into the practical type diets for chicks and poults from day-old chicks up to 3 weeks of age.

Derilo & Balnave (1980) showed that growth of broiler was reduced with the low dietary level of choline. However, these effects were highlighted by very low nutritional total sulphur amino acid (TSAA). Increased in the number of mortality and many other pathological changes that are involving in various number of tissues that were observed in birds fed on a low choline diet on the other hand, the same later authors reported that the

Figure 3 Choline Metabolism assessed on https://en.wikipedia.org/wiki/Choline (Gibellini & Smith, 2010)
high dietary concentration of choline (1750 mg/kg feed) boost the requirement for dietary TSAA.

Neither choline nor cystine significantly affected the requirement of methionine or SAA, as estimated by body weight gain (Blachman & Waldroup, 1980). Derilo & Balnave (1980) obtained that when different dietary combinations of choline and TSAA were used with broiler chicks fed purified diet, maximum growth was obtained with a combination of high choline (>1750 mg/kg) and high TSAA (8.49/ kg diet). Anderson & Dobson (1982) showed that increasing the amount of choline supplementation in the avian diet range from 300 to 800 mg/kg feed did not increase the performance or reduce the value of the supplementary methionine. On the other hand, Miles et al. (1983) reported better growths of chicks occurred from the concurrent addition of 0.1% potassium sulfate and 0.066% choline but larger increases were attained from the addition of 0.25% methionine, representing that both choline and sulfate may be involved in sparing methionine in turkeys birds.

The choline requirements of broiler chickens were 1300, 850 and 500 ppm for the starter, grower and finisher periods, respectively (NRC, 1994). Parsons & Leeper, (1984) stated that supplementation of choline or methionine improved the productive performance of laying hens. Yeo et al., (1985) pointed out that supplementation of choline chloride in broiler diets increased body weight gain. Sonbol & Habeeb (1991) showed that broiler chicks fed on basal grower diet up to 4 weeks of age, particularly in the deficiency of supplemental methionine; also, they added that an increase was more difficult to demonstrate at other age. Tillman & Pesti (1986) found that chicks offered a feed that supplemented with L-methionine at 12% gained significantly more than those fed the basal diet, while, poultry chicks that supplemented with choline diets had gained as well as those fed L-methionine.

Andriguetto et al. (1987) studied the effect of diets based on maize meal and soybean oil meal without or with choline supplements at the rate of 200, 400, 600, 800, 1000, 1200, or 1400 mg/kg feed from hatching until 42 days old in male and female chicks. The diets were supplemented with pyridoxine 3.00; folic acid 1.00 and cyanocobalamin 0.030 mg/kg diet. The authors found that weight gain of male and female was not significantly different among groups.

Okolelova et al. (1988) studied the influence of feeding on a basal diet plus choline chloride as liquid, or as 9% mixture with lignin or microcrystalline cellulose, or as a 43% mixture with maize cobs and found that average body weight gain at 49 days old was 1641.4, 1592.0, 1749.6 and 1677.1g, respectively. Krsmanovic et al. (1990) studied the addition of choline without or with 25 or 50 g/kg on broiler diets and found average total body weight gain was 1862, 1867 and 1884g, respectively. Baranova, (1991) pointed out that supplementation of choline chloride in broiler diets increased body weight gain. Sonbol & Habeeb (1991) showed that broiler chicks fed on basal grower diet up to 4 weeks of age (22.14% CP and 2810 kcal ME Kg) and the basal finisher diet up to 7 weeks of age (18.98%CP and 2912 Kcal ME/Kg) were supplemented with 0.15% methionine +1000 mg/kg choline.
The addition of methionine and choline showed the highest significant live weight (1707g).

Vogt (1992) reported that growth of broiler chicks of 6-weeks old was improved by supplemented choline at levels of 200, 400, 600 and 800 mg/kg diet with corresponding addition DL-methionine at levels of 750, 1500, 2250 and 3000 mg/kg diet, respectively. Mohamed et al., (1994) indicated that addition of choline to corn-soybean meal diets for chicks had a significantly effect on body weight gain. Ryu et al. (1995) showed that broiler chicks were fed on a basal diet supplemented with choline 0, 500 and 1000 mg/kg diet. Body weight was significantly higher when supplemented choline was fed. In two experiments carried out to determine the effect of dietary choline on the performance of broiler chicks. Men-Kin et al. (1996) found that broiler chicks fed on a basal diet containing 23.5-24% CP less methionine and supplemented with choline at levels of 250, 500 or 750 g/ton in experiment 1 or at levels of 1000, 1250 or 1500 g/ton in experiment 2. Jokic et al. (2000) showed that the addition of choline to 950 and 850 mg/kg diet during the starting and finishing period, respectively with 0.20% and 0.15% methionine and 0.10 magnesium sulfate resulted in significantly (P<0.05) increase in body weight gain (from 6.42 to 7.41%) and body mass of chicks from 6.31 to 7.25%. Shrivastav et al. (2004) reported that choline is essential for growth and helpful to prevent leg disorder (perosis) in turkeys.

Simon et al. (1995) demonstrated that extra methionine supplementation above the recommended dose required for broilers chickens to improved their performance including body weight gain and food conversion efficiency, so choline may spare the methionine for broiler's growth (Pesti et al.,1981). Feeding a diet with choline supplementation improved body weight gain and feed efficiency in broilers either alone or in combination with methionine (Combs,1992). The study of Rama Rao et al. (2001) found that choline and methionine should be supplemented to broiler diets at higher levels to gain better results in aspects of health and production. Furthermore, Combs, (1992) postulated that choline is an absolute dietary requirement for broilers, particularly at younger ages as the chick cannot synthesize satisfactory amounts until up to 13 weeks of age. Body weight gain was improved (P<0.01) with increasing choline percent in the diet up to 2000 mg/kg diet, but increasing choline level-up to 2500 mg/kg diet caused a significant decrease in body weight (Slawinska et al., 2014).

### 7.3 Choline as Immune Booster

Choline is being considered as a member of the B-complex vitamin group and as an essential nutrient for laying hens, broilers and also for other poultry diets for the formation of the phospholipid lecithin found in egg yolk and liver (Maiorano et al., 2012). In animal nutrition, essential nutrients playing an important role on growth performance, meat quality and carcass traits (Kadam et al., 2013) and immune system development (Hhm et al., 2012). Choline showed a positive effect on immune functions by improvements in primary antibody titer of broiler chicks (Maiorano et al., 2012).

#### 7.4 Choline as a Potent Lipotropic Agent

In poultry feed industry, maize is considered the main energy source. Due to non-availability of maize in many developing countries, so nutritionists are always searching for alternative energy sources for animal and poultry feeds. The replacement of maize with pearl millet or brokenrice in the diets of broilerbreeders and layer caused an accumulation of fatin the liver and abdomen. As shown in Figure 4, choline -as a lipotropic factor- has been established to fix this problem by the donation of methyl in chicken metabolism (Zeisel et al., 2003). Similarly, Rama Rao et al., (2001) reported that birds fed a diet containing choline at the level of 760 mg/ kg diet significantly reduced the liver fat. So, choline must be a part of the human and animal diets (Sheard & Zeisel 1989; Gholami et al., 2015). Therefore, it is undertaken that the supplementation of choline in poultry feed can alter the deposition of fat and also the hatching and laying performance of broiler breeders that fed on different sources of energy.

![Figure 4 Effect of choline against fatty liver syndrome](http://www.jebas.org)

### Conclusion

From the aforementioned information, it could be concluded that choline has main three vital metabolic functions that relate to its structural integrity of cell membranes, a lipotropic mediator in fat metabolism in liver and also act as a precursor for acetylcholine synthesis that known as neurotransmitter agent for nerve impulses. So choline can be considered as a powerful lipotropic agent that would play an important role in fat metabolism by...
removing deposition of fat in the liver or ganan and consequently prevents fatty liver disorder. So, these vital properties highly recommend the use of choline as commercially feed additive to cope with the metabolic disorders and enhance bird's health and productivity.

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**Conflict of interest**

Authors have no conflict of interest.

**References**

Agricultural Research Council "ARC" (1975) Nutrient requirements of farm livestock No 1 poultry, 2nd Ed –London, Her Majesty s stationery office


IMMUNE PROFILING OF GRAIN ADAPTED I-2 (GAI-2) PELLETED FEED VACCINE IN 6 WEEKS OLD COCKERELS IN VOM NIGERIA

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ABSTRACT

In this study, a modified wet harvest of NDVI-2 virus strain with an EID₅₀ of 9.0/ml and a thermostability of 6 hours at 56°C was adsorbed to heat treated Digitalia ibura which served as the virus carrier and was used to prepare pelleted feed vaccine. In this trial, 100 (six weeks old) cockerels were divided into five groups of twenty each. They all were vaccinated against other diseases except Newcastle disease (ND). Group 1 was not given the pelleted feed vaccine and served as the vaccine control, while groups 2, 3, and 4 were feed with 10, 15, and 20 grams per birds respectively while group 5 was treated with the conventional NDVI-2 through the intra ocular route. Groups 2, 3 and 4 were given booster doses at different intervals. The detectible antibody was analysed using haemagglutination inhibition (HI) test. The geometric mean titre of protective antibody response in cockerel vaccinated with GAI-2 pelleted feed vaccine compared favourably with the conventional NDVI-2. Groups 2 to 5 were later challenged with wild virulent field virus (Kudu 113 ND strain) and the antibody response was astronomical, with group 2 recording the highest geometric mean titre of 2352.5 post challenge. Thus,

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the GA I-2 pelleted feed vaccine could be an excellent alternative to the conventional vaccination method, since the stress associated with handling of individual birds will be greatly minimized. The significance of the rapid increase in the antibody titre when challenged with wild virus strain is discussed.

1 Introduction

Newcastle disease (ND) is an acute, highly contagious, rapidly spreading viral disease affecting birds of all ages. It varies widely in type and severity of symptoms (Alexander, 2000; Abdu, 2005; Saidu et al., 2006). The global impact of ND is enormous and it is responsible for international trade barriers of poultry and poultry products (Alexander, 2003). It is a devastating disease of poultry particularly in village and backyard poultry and a major constraint to developing poultry industry (Alexander, 1988; Spradbrow, 1993), where control remains extremely difficult or even impossible (Alexander & Jones, 2001).

In Nigeria, Newcastle disease was first reported by Hill et al. (1953). Since then, it has continued to be a major setback in the development of rural poultry production. The application of biosecurity measures as a control option is possible however; its application in an endemic region is very difficult. Thus, vaccination remains the only possible alternative. Though vaccines are available for the control of this disease, they are mostly geared toward the control of ND in commercial flocks. In developing countries, rural backyard poultry are rarely vaccinated against ND.

Current conventional vaccination programs for ND virus control include the use of either low-virulent live-virus vaccines or inactivated vaccines to induce protective immunity while producing minimal adverse effects in birds (Zoth et al., 2008). Birds may also be inoculated with eye drops containing vaccines based attenuated viruses. Vaccination using non virulent ND strains protects susceptible birds against ND disease, and antibodies are produced either locally or systemically or both (Zoth et al., 2008).

The commercial potential of rural poultry is enormous, but an occasional annual loss resulting from ND outbreaks discourages huge investment in rural backyard poultry production. Vaccination against Newcastle disease has been reported as the major safe guard against outbreaks especially in countries with endemic very virulent Newcastle disease (vND) and where highly efficient biosecurity measures are impossible to apply (Usman, 2002), especially in rural situation in developing countries due to the nomadic and free range rearing of village poultry.

NDVI-2 vaccine was originally developed for the control of ND in rural flocks where cold chain storage is almost absent. The development of GAI-2 feed vaccine is believed to be a better vaccination alternative to conventional NDVI-2 in terms of heat resistant and immunogenicity. Therefore, present study has been carried out to access the immune profiling of grain adapted I-2 (GAI-2) pelleted feed vaccine in 6 weeks old cockerels in Vom Nigeria

2 Materials and Methods

2.1 Proximate and antinutritional analysis

Proximate and antinutritional tests were carried out on Digitalia iburua using Association of Official Analytical Chemists (AOAC) 1990 method. The proximate analysis of the Digitalia iburua sample for protein, crude fibre, total ash, moisture were carried out in triplicate using the method described by Association of Official Analytical Chemists (AOAC, 1990).

2.2 Moisture content

The moisture content determination was carried out gravimetrically after oven drying the Digitalia iburua sample at 105°C to a constant weight.

2.3 Crude protein

Nitrogen content of the Digitalia iburua sample was determined using the Kjeldhal method and the titrated value of the nitrogen multiplied by 6.25 as a conversion factor.

2.4 Lipid content

The determination of crude fat (Lipid) content of the Digitalia iburua sample was carried out by extraction with petroleum ether in a soxhlet apparatus and the fat content determined gravimetrically.

2.5 Ash content

Ash content was determined by heating 2 grams of Digitalia iburua sample in a muffle furnace at 550°C to a constant weight.

2.6 Crude fibre

The determination of crude fibre of the Digitalia iburua sample was carried out using an acid–alkaline gravimetric method in line with the principles of AOAC (1990).
2.7 Nitrogen free extract

The nitrogen free extract (NFE) of *Digitalia iburua* was obtained by difference i.e. the sum of crude protein (CP), crude fat (CF), and Ash subtracted from one hundred.

2.8 Determination of antinutritional factors

2.8.1 Determination of oxalate

Oxalate was extracted from powdered sample of *Digitalia iburua* and precipitated as calcium salt. The precipitate was dissolved in 25% sulphuric acid and the concentration of oxalate determined by titration with 0.01N potassium permanganate (AOAC, 1990).

2.8.2 Determination of Tannins

This was carried out spectrophotometrically by the method of Joselyn (1970). The sample of the *Digitalia iburua* was extracted by refluxing with 50% ethanol in a soxhlet apparatus for 18 hours. The extract was then dissolved in 25ml water treated according to standard procedure and absorbance read at 760nm

2.8.3 Determination of Phytic acid

Phytic acid (Inositol hexaphosphate) content of the *Digitalia iburua* was determined by the modified method of AOAC(1990). For this, two grams (2g) of *Digitalia iburua* sample was extracted according to standard procedure and absorbance read at 476nm or 640nm.

2.9.1 Modification of thermostable NDVI-2 virus

NDVI-2 parent virus with a thermostability of 3 hour at 56°C was subjected to series of heat treatment in water bath. The heating duration was standardized after a modification of the heating method of Ibu et al. (2010). 1ml of NDVI-2 parent stock with a thermostability of 3 hours at 56°C was placed into each 8 bijou bottles and allowed to stand in a water bath at 56°C for 8 hours. After every hour one bijou bottle is removed from the water bath and placed in the refrigerator at +4°C until the 8th hour when the last bijou bottle was removed from the water bath and stored at +4°C. At the end of the heat exposure the aliquots were inoculated into 10day old embryonated chicken eggs at 0.1ml per egg and incubated at 37°C for 96 hours, with daily candling, to assess its infectivity. On the death of any inoculated embryonated eggs following candling, the allantoic fluid was harvested and checked for haemagglutination activity.

The embryonated eggs inoculated with virus from the aliquot with the highest heat treatment duration showing haemagglutination was harvested and subsequently used for further heat treatment in water bath at 56°C for 8 hours. After repeated three passages in embryonated chicken eggs and three heat treatments at 56°C, a thermostable heat resistant virus with a thermostability of 6 hours at 56°C was produced and harvested, and used for the preparation of GAI-2 pelleted feed vaccine.

2.9.2 Preparation of virus carrier for GAI-2 pelleted feed vaccine

2.5kg of *Digitalia iburua* was soaked in 10 liters of clean sterile water overnight. After which it was sieved and boiled in equal volume of water at 100°C and the boiling mixture stirred for ten minutes. Thereafter the cooked *Digitalia iburua* was dried and roasted in an oven for 4 hours at 100°C. The complete dried *Digitalia iburua* was then allowed to cool before it was blended into fine particles, using a manual blender.

2.9.3 Virus mixture

2.5 litres of modified NDVI-2 virus harvested from infective allantoic fluid of embryonated chicken eggs after three passage with a thermostability of 6 hours at 56°C and a titre of 9.0 log._50_ EID per ml was added to 100ml of peptone. 100ml of antibiotic and antimycotic suspension made up of (penicillin 1000 000 i.u, streptomycin 5g streptomycin base, gentamycin 280mg/ml, and amphotericine B250μg/ml). This mixture was added to 2.5kg of the blended *Digitalia iburua* and mixed. The mixture was allowed to stand for 10 minutes for complete adsorption. The adsorbed virus mixture was pelleted using an electric powered pelleting machine. The generated pellets were stored at -80°C. The EID50 of the pelleted vaccine was determined using the Karber method (1931).

2.10 Experimental Trial

The facility for the trial was provided by National Veterinary Research experimental station, at Vom, Plateau state Nigeria. The pen and cages were fumigated with formaldehyde and potassium permanganate, and disinfected with potassium peroxomonosulphate 50% m/m. The facility was rested for two days before stock fencing the birds. Movement into the pens were restricted and provision for foot dip was provided in each of the experimental house and application of the “all in all out rule” in the facility during the study period was strictly adhered to during sample collection.

The birds were divided into 5 groups of 20 cockerels each viz: Group 1- unvaccinated (control) Group 2 -vaccinated with 10gm of pelleted feed vaccine per bird and booster dose administered at day 14 Group 3 - vaccinated with 15gm of pelleted feed vaccine and booster dose administered at day 14 Group 4 -vaccinated with 20gm of pelleted feed vaccine per bird, Group 5 - vaccinated with NDVI-2 conventional vaccine.
Antinutritional screening equally showed low phytic acid, oxalate and tannins content all of which are natural viral inhibitors in grain. Therefore, based on these results Digitaliaiburua was subsequently subjected to heat treatment for complete inactivation of these natural inhibitors. Afterwards, the heat treated Digitaliaiburua was adsorbed to modified virus harvest for the preparation of grain adapted feed vaccine, and the produced pelleted feed vaccine EID50 compared with the EID50 of standard NDVI-2 vaccine in an in-vitro assay prior to on-station trial in 6 weeks old cockerels.

Viral content assay of the pelleted feed vaccine produced using heat treated Digitaliaiburua are shown in (tables 3). The obtained EID50 was comparable with EID50 of standard NDVI-2 vaccine. The resultant titre EID50 of log10 EID50 8.5 per dose obtained after pelleted feed vaccine production showed that the heat treated Digitaliaiburua was 3 logarithms above minimal acceptable dose range for NDVI-2 standard vaccine of log10 EID50 5.5 per dose. The result showed that heat treated Digitaliaiburua when adsorbs with the modified NDVI-2-6 virus derivative, an EID50 that was comparable with standard NDVI-2 vaccine recommended dose for field vaccine was obtained (Table 3).

3 Result

Proximate and antinutritional factors analysis showed that Digitaliaiburua has low moisture, fat, and calcium content. It equally showed low content in terms of phytic acid, oxalate and tannins content (table 1 and 2)

The result of the proximate analysis of Digitaliaiburua revealed low; moisture content, crude proteins, crude fat, ash, nitrogen free extract calcium and phosphorus.

### Table 1: Proximate analysis: [weight (g) per 100 grams Digitaliaiburua sample]

<table>
<thead>
<tr>
<th>Sample</th>
<th>MOISTURE</th>
<th>Crude PROTEIN</th>
<th>Crude FIBRE</th>
<th>Crude FAT</th>
<th>ASH</th>
<th>NFE</th>
<th>CA</th>
<th>Phosphorous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digitalia</td>
<td>4.60</td>
<td>10.49</td>
<td>7.23</td>
<td>2.88</td>
<td>14.15</td>
<td>65.25</td>
<td>0.14</td>
<td>0.08</td>
</tr>
</tbody>
</table>

### Table 2: Anti-nutritional screening weight (mg) per 100 grams digitaliaiburua sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>PHYTIC ACID</th>
<th>OXALATE</th>
<th>TANNINS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Acha</td>
<td>49.34</td>
<td>20.00</td>
<td>0.827</td>
</tr>
</tbody>
</table>

The birds were housed in separate cages of 10 birds per cage and each group in duplicate. Blood samples were collected from all the birds for determination of presence of ND antibody titre, prior to the administration of the GAI-2 pelleted feed vaccine. Following vaccination, birds were bled 2 weeks, 3 weeks, 4 weeks and 6 weeks post vaccination (Table 4)
This was attributed to the systemic response of the vaccinated birds to wild ND viral challenge.

3.1 Effect of Vaccination on Birds

The birds were divided into twenty in each group and were administered the GAI-2 adapted pelleted feed vaccine, and the standard NDVI-2 vaccine.

Results of the on-station trial of grain adapted feed vaccine in chicken (GMT) is as presented on table 5. There were significant differences (p < 0.05) in the mean result in all the quantity of feed vaccine administration (groups) across the period. The highest mean is obtained in the 2 weeks post challenge in each of the groups.

Table 6 showed the result of the statistical analysis during the on-station trial of grain adapted feed vaccine in chicken (GMT) in relation to pre-vaccination, two weeks post-vaccination, and two weeks post challenge. There was significant difference of (p < 0.05) from each other. The highest mean value was found in the 2 weeks post challenge this sequel to astronomical rise in the antibody production due challenge activity with the Kudu 113 strain.
4 Discussion

Researchers at University of Queensland in Australia carried out extensive research on ND virus and as a result, an avirulent thermostable virus was developed to control ND in rural poultry (Spadbrow & Sabine, 1995; Bensink & Spradbrow 1999). The NDVI-2 vaccine strain which requires less cold chain handling has been extensively tested to protect rural poultry against ND in some Asian and African countries (Tu et al., 1998; Wambura et al., 2000; Wambura et al., 2007).

Since ND affect chickens of all ages and due to free range nature of village poultry production system where chickens of all ages scavenge for food together, it becomes difficult to administer ND vaccine through water or intraocularly. The search for a suitable food material as vaccine virus carrier that will simplify vaccination in rural poultry therefore becomes imperative, and several attempts have been made by researchers to use food as vaccine virus carrier for thermostable ND vaccine. In Asia Africa and Australia paddy rice, cooked white rice and cooked parboiled rice has been used as suitable alternatives (Samuel & Spradbrow, 1989; Spradbrow, 1992; Ibrahim et al., 1992; Wambura et al., 2007), with a thermostability of 3 hour at 56°C.

In this study, Newcastle disease virus vaccine (NDVI-2) was subjected to heat treatment and underwent three different passages in embryonated chicken eggs. A resultant harvest with a thermostability of 6 hours at 56°C was achieved. The modified virus harvest in the presence of antibiotics, antimycotic and stabilizer was adsorbed to heat treated Digitalia iburua (locally known as Acha) without deterioration. This was pelleted to produce the grain adapted (GAI-2) pelleted feed vaccine which is more durable under field condition.

The result of the antibody response after vaccination with GAI-2 showed a steady increase in the geometric mean titre by the second and third week post vaccination. However, a sudden drop in geometric mean titre was observed on the fourth week, this could probably be attributed to unascertained natural event or stress associated with handling during bleeding for blood sample collection. Despite the drop in the mean antibody titre, 10 birds from each group were challenged on the fifth week with wild virulent field strain of Newcastle virus (kudu 113 strain) with a log10 EID50 8.7 at a dose of 0.1ml per bird intramuscular. The unvaccinated group showed 100% mortality within 96 hours while the vaccine groups i.e. those vaccinated with both conventional and the modify GAI-2 pelleted feed vaccine showed an astronomical rise in their geometric antibody titres and mortality was not recorded in these groups.

The result from table 4 revealed that birds vaccinated with 10 grams per bird and boosted two weeks later gave a highest antibody response (2352.5) compared to groups 3 (315.2), group 4 (1351.2) and group 5 (548.73). This result is similar to observations of Abdi et al., (2016) where they observed that repeated vaccine administration induces progressively higher HI antibody titer that could correspond to high levels of protection Although all the groups that were challenged with virulent field strain had an astronomical rise in the geometric mean titre, the results of birds in group 3 showed that they did get enough of the pelleted feed, thus the low mean antibody titre. From the above, it could be recommended that 10 grams per bird be administered and a booster dose given at 14 days after the initial vaccination.

This result showed that vaccination using the newly developed vaccine GAI-2 will confer protection to birds against wild virulent forms of field ND virus, especially in regions where ND outbreak is a challenge to scavenging poultry farming. Therefore, almost sixty two years sequel to the first recorded outbreak in Nigeria, ND has persistently been a setback to rural scavenging poultry development. Thus, to mitigate against consequences of occasional out breaks in rural unvaccinated flock, it is imperative that a compulsory strategic vaccination be initiated, and this must be geared towards ND eradication especially in endemic regions where bio-security measures which includes bio-exclusion and bio-containment methods of control are impossible control options.

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Conflict of interest

All the authors declare that there is no conflict of interest.

References


Abdu PA (2005) Evolution and the pathogenicity of Newcastle Disease virus and its implication for diagnosis and control. Proceedings of the workshop on improved Disease Diagnosis, Health, Nutrition and Risk management Practice in Poultry, held...
on 29th November to 1st December 2005 at Ahmadu Bello University Zaria, Nigeria.


ASSESSMENT OF HUMORAL IMMUNE RESPONSE IN VACCINATED DOMESTIC DOGS AND CATS INTENDED FOR PET-TRAVEL FROM INDIA BY RAPID FLORESCENT FOCUS INHIBITION TEST (RFFIT)

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ABSTRACT

The present study evaluates humoral response in vaccinated domestic dogs and cats intended for pet-travel from India by Rapid Fluorescent Focus Inhibition Test (RFFIT). In present study, 184 serum samples from dogs (n=149) and cats (n=35), vaccinated within the period of one year against rabies were tested by RFFIT using PV-3462 strain of rabies virus and BHK 21 cells. Out of total studied 149 dogs samples (male-96, female-53), 122 showed titre ≥0.5 IU/ml and 27 below <0.5 IU/ml. Interestingly, all the 35 samples from cats showed titre ≥0.5 IU/ml. The protection observed in vaccinated dogs was 81.87 percent and in vaccinated cats it was 100 percent. The analysis showed serum sampling between 20-50 days will have higher percentage of vaccinates with neutralizing antibody titre >0.5 IU/ml than those collected < 20 days and > 50 days. Association of the age factor of the vaccinated dogs and getting varied neutralizing antibody titre was evident. Whereas gender and breed based on size did not reveal any statistically significant effect on antibody titre. Furthermore, Out of eight vaccine brands, only one (V8) yielded 100 percent (9/9) protection, whereas the remaining...
Assessment of humoral immune response in vaccinated domestic dogs and cats intended for pet-travel from India by Rapid Florescent Focus Inhibition Test

1 Introduction

Rabies is one of the terrifying infectious disease that has affected mankind since antiquity (Jackson, 2016). Yearly 60,000 human deaths due to rabies are reported worldwide (Hampson et al., 2015), of which 20,000 deaths are from India alone which accounts for one third of total deaths by rabies (Sudarshan et al., 2007; Sudarshan, 2017). Rabies is caused by virus belonging to family Rhabdoviridae and genus Lyssavirus causes acute progressive viral encephalitis (Rupprecht et al., 2002). The virus is made of five structural proteins among which glycoprotein is important major structural protein involved in induction of production and protection against rabies virus by anti-rabies neutralizing antibodies after vaccination (Perrin et al., 1985; Etessami et al., 2000). International travel and trade have increased dramatically during the past two decades. Global travel and trade present risks for rapid, long distance movement of a variety of infectious diseases including rabies virus (IMNA, 2010). Due to this, in the past few years, serological testing of dogs and cats has increased because many rabies free countries have amended their quarantine measures and adopted a scheme requiring rabies vaccination followed by a serological test (Mansfield et al., 2004). This scheme has been promoted by the WHO, OIE and the European Commission and allows the free movement of pets from countries without rabies, or where rabies is under control, to rabies-free countries. Pre-exposure vaccination is considered successful by WHO and OIE when the neutralizing antibody titre is at least 0.5 IU/ml in serum from vaccinated humans and animal (WHO, 2005; OIE, 2008). Rapid fluorescent focus inhibition test (RFFIT) is a gold standard test which is recommended for serological testing of pets intended for international trade (European Commission, 2003).

Any pet animal moving from India to most other countries is vaccinated and neutralizing antibodies checked for protective neutralizing antibody titre. In India, KVAFSU- CVA- Crucell Rabies diagnostic Laboratory, (a laboratory twinned under OIE programme with APHA, UK, and CDC, Atlanta) Veterinary college, Bangalore is regularly processing serum samples for RFFIT from different states. The present study was conducted to estimate the neutralizing antibodies to anti-rabies vaccination in domestic dogs and cats from different states of India which were primarily intended to travel abroad.

2 Materials and Methods

2.1 Collection of Serum samples

Serum samples (n=184) of vaccinated dogs and cats from 11 different states of India were submitted to KVAFSU-CVA-Crucell Rabies Diagnostic Laboratory, Veterinary college, Bengaluru along with details of age, gender, breed, history of vaccination, date of serum collection and Microchip number. Of these 184 serum samples, 149 were from dogs (Males -96, Females - 53) and 35 from cats (Males – 18, Female - 17). These serum samples were stored at -20°C until the test was performed.

2.2 Rapid Fluorescent Focus Inhibition Test (RFFIT)

The RFFIT which is gold standard test was employed to assess the neutralizing antibodies against rabies according to Smith et al., 1996 and Neelufer et al., 2015. Initially, the test serum samples were diluted two fold and heat inactivated at 56°C for 30 min followed by further two fold serial dilution viz, 1:2, 1:4, 1:8, 1:16 in the 96 micro titre plate was carried out in duplicate and mixed

Figure 1 Results of anti-rabies neutralizing antibodies in dogs and cats
with 100 TCID\(_{50}\) constant amount of PV-3462 (Dr. Larghi’s strain) of rabies virus obtained from Pasteur Institute, Coonoor, Tamil Nadu. This was incubated for 90 minutes at 37°C for virus neutralization. Then 50 µl of BHK-21 cells (25,000-30,000) suspended in 10% growth medium was added and incubated for 48hrs at 37°C in 5% CO\(_2\). The contents were then removed and 70% chilled acetone added to the wells and fixed for 30 minutes at -20°C. Then 50µl of Rabies anti-nucleocapsid based conjugate (Light diagnostic rabies DFA III cat # 6500) was diluted 1:100 with phosphate buffer saline (PBS) along with Evans blue as a counter stain at concentration of 0.001% and incubated at 37°C for one hour. The microtitre plate was then washed with 1x PBS two times, observed under fluorescent microscope at 20X objective.

The neutralising antibody titre of test serum sample was determined by dividing the reciprocal of highest dilution of serum sample by reciprocal of highest dilution of WHO reference serum at which complete neutralization observed, then multiplied by unitage of WHO reference serum. The neutralising antibody titre was expressed in International Unit (IU) / millilitre of test serum. A antibody titre of equal or above 0.5 IU/ml was considered as protective.

### 2.3 Statistical analysis

A linear model was fitted with various factors like age, gender, breed and brand of vaccine with neutralizing antibody titre. GraphPad Prism-5 software was utilized for calculating mean, standard deviation, standard error. Chi-square (\(\chi^2\)) test was employed to detect effect of breed, gender, vaccine brand and association between time intervals for blood sampling and antibody titre. A \(\chi^2\) test was used to analyze difference among the variables (Version 5; Graphpad Software Inc., La Jolla, CA, USA). The p value less than 0.05 concluded as statistically significant where as vice versa as statistically non-significant.

### 3 Results

Out of 149 vaccinated dogs, 122(81.87%) dogs possessed anti-rabies neutralising antibody titre of \(\geq 0.5\) IU/ml. Interestingly, all 35(100%) vaccinated cats had neutralising antibody titre \(\geq 0.5\) IU/ml.

#### 3.1 Persistence of anti-rabies neutralizing antibodies and best window period for serum collection

The animals were divided into 3 sampling time groups viz., short, normal and long. This analysis showed that the 20-50 days sampling period had a higher percentage of dogs with neutralizing antibody titre \(\geq 0.5\) IU/ml than the other two groups in dogs (Table 1). This is the best window period for sampling which is having least failure rates (14.55 per cent) with in dogs and higher mean antibody titre (8.654 IU/ml) in cats.

Data were assessed in terms of protective or non-protective percentage of neutralizing antibody and mean neutralising antibody titre (Table 2). In the present study, the data was tabulated and analysed for association of various factors with anti-neutralising antibody titre. The statistical analysis with chi square test suggests that there is association of age of vaccinated dog and protective neutralizing antibody titre is not significant because p values for this is less than 0.05. The adult (1 year - 5 years) and older ( >5 years) dogs revealed a higher percentage of dogs with neutralizing antibody titre \(\geq 0.5\) IU / ml at 76.38 and 94.47 respectively compared to young dogs with 65

<table>
<thead>
<tr>
<th></th>
<th>No. of animals with (\geq 0.5) IU/ml</th>
<th>No. of animals with &lt;0.5 IU/ml</th>
<th>Total</th>
<th>Mean Titre</th>
<th>Std error</th>
<th>Per cent</th>
<th>Chi square Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Below 20 days (Short)</strong></td>
<td>Dogs 05</td>
<td>02</td>
<td>07</td>
<td>1.500</td>
<td>0.6892</td>
<td>71.42</td>
<td>Dogs 0.5742ns</td>
</tr>
<tr>
<td></td>
<td>Cats 02</td>
<td>00</td>
<td>02</td>
<td>2.250</td>
<td>1.750</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>20-50 days (Normal)</strong></td>
<td>Dogs 47</td>
<td>08</td>
<td>55</td>
<td>2.330</td>
<td>0.4739</td>
<td>85.45</td>
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</tr>
<tr>
<td></td>
<td>Cats 13</td>
<td>00</td>
<td>13</td>
<td>8.654</td>
<td>3.075</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>&gt;50 days (Long)</strong></td>
<td>Dogs 70</td>
<td>17</td>
<td>87</td>
<td>2.477</td>
<td>0.2940</td>
<td>80.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cats 20</td>
<td>00</td>
<td>20</td>
<td>2.625</td>
<td>0.8069</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>157</td>
<td>27</td>
<td>184</td>
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</tbody>
</table>
per cent. Whereas, gender, brand of vaccine, breed, had no statistical effect \((p > 0.05)\).

### 3.2. Effect of age, gender, brand of vaccine, and breed of animals on neutralizing antibody titre in vaccinated cats

Interestingly, all the 35 cats were having the protective titre of neutralising antibody against rabies (Table 3). The mean titre of adult cats was high compared to young and old. Both the genders showed similar protective neutralizing antibody titre and vaccine brands \(\text{V}2, \text{V}3, \text{V}1,\) and \(\text{V}4\) resulted in higher mean antibody titre in decreasing order. In breed wise analysis, non-descript cats revealed good mean antibody titre than the Persian and domestic short hair.

### 3.3 State wise - neutralizing antibody titres in dogs

The maximum number of serum samples were received from Maharashtra (59) and Karnataka (54) with protective neutralizing antibody titre showing (51/59) 86.44, (44/54) 81.48 percent respectively. From other 8 states the serum samples received varied from 01 to 10. It indicates more number of pets from Maharashtra and Karnataka are being transported out of India compared to other states (Table 4).

### 4 Discussion

The study was conducted to estimate the neutralizing antibodies to anti rabies vaccination in dogs and cats intended to travel abroad from India. Interestingly, all the cats (n=35) were showing titre above or equal to 0.5 IU/ml which is required and recommended by OIE and WHO for protection \((\text{WHO, 2005}; \text{OIE, 2008})\). In dogs \((n=149), 81.87\) per cent revealed protective titre of neutralizing antibodies. This confirms that cats respond better than dogs as observed by Cliquet et al. (2003). Similar study was conducted in United Kingdom by Mansfield et al. (2004) and obtained 95.88, 94.83 percent of vaccinated dogs and 97.17, 97.33 percent of vaccinated cats showing protective antibody titre tested.
by two different laboratories by Fluorescent Antibody Virus Neutralization (FAVN) test. Shyamsundar et al. (2014) observed 84 percent vaccinated dogs showing protective neutralizing antibody titre by RFFIT. In contrast to this study, Neelufer et al. (2015) and Savaliya et al. (2015) reported low, viz., 58 and 62.36 percent of vaccinated dogs with protective neutralizing antibody titre by RFFIT, respectively.

The interval between vaccination and blood sampling is one of the main significant factors in determining host response. Rabies vaccination produces a typical antibody curve with response going down over the period. In this study, the best window period for sample collection was between 20 to 50 days of post vaccination which showed better protective neutralizing antibody titre percent, i.e., 85.45 and 100 per cent in dogs and cats respectively.
The variation in production of neutralizing antibody response to length of interval of sampling following vaccination relates to response kinetics from primary vaccination. After primary vaccination, isotype shift from an Ig-M response to an Ig-G as immune response develops and optimum measurement in an appropriate window of time should measure this effect. Measurement at too early a stage captures only an Ig-M response, but will not confirm whether class switching to Ig-G response progresses. Measurement at later stage point of time may show lower antibody levels, but this may not relate to lack of immune protection. As the immunoglobulin measure may be proportionally more accounted for by Ig G (Kennedy et al., 2007).

The Chi square test in the present study, indicated the association between age and production of neutralizing antibodies against rabies where significant difference was observed between different age groups (p<0.05) in dogs. Ability of younger (<1year) dogs and cats to develop protective neutralizing antibody titre is less compared to adult and older dogs. This could be attributed to immune system being less efficient (Kennedy et al., 2007). Mansfield et al. (2004) showed that animals less than one year old have an increased risk of having poor FAVN titres. Previous researchers like Aghomo et al. (1990), Kennedy et al. (2007) confirmed that young dogs can produce rabies antibodies from four weeks of age, when the titre of maternal antibody waned. This interference by maternal antibodies and less matured immune system explains the poorer immune response in young animals. Furthermore, in dogs it was observed that older dogs (>5 years) revealed higher protective neutralizing antibody titre than adults (1-5years) and young (less than 1 year) dogs. This observation in contrast to Mansfield et al. (2004) and Kennedy et al. (2007), where they observed poor protective neutralizing antibody titre in older dogs than adults and opined that this may be due to reduction in immune regulation thought to occur.

HogenEsch et al. (2004) studied the effect of age on the immune response. However, they did not find any difference in IgM and IgG levels among the adult and old dogs. They confirmed pre-vaccination titre was higher in older dogs than in adults. Furthermore, they did not find any difference in post vaccination titre against rabies among adult and old dogs, this supports the findings of present study. The other possible explanation for higher protective neutralizing antibody titre in older dogs (94 percent) may be due to more number of booster vaccinations than the adults (76 percent) and young dogs (65 percent).

Influence of gender on neutralizing antibody titre in case of both dogs and cats was analysed by using chi square test which indicates that there is no significant difference (p>0.05) between the titre of different genders in both species of animals. This is in agreement with the study conducted by Mansfield et al. (2004), Jakel et al. (2008), Shyamsundar et al. (2014), Savaliya et al. (2015) and Neelufer et al. (2015) in dogs. But contrasting results were observed by Mansfield et al. (2004) in case of vaccinated cats, where male cats showed lesser percentage of protective neutralizing antibody titre compared to females. This was attributed to suppression of cytokine production by gonadal steroid hormone (Schuurs & Verheul., 1990; Rife et al.,1990; Verthelyi & Klinman., 2000).

Various vaccine brands such as V1, V2, V3, V4, V5, V6, V7, V8 and V1, V2, V3, V4 were used in dogs and cats respectively. Of these, only brand V8 provided 100 per cent protection (99%) and all the other vaccine brands except V1 (61.53 percent) and V7 (70 per cent) provided satisfactory seroconversion ranging from 75 percent (12/16 in V5) to 90.90 (10/11 in V6). In cats, higher seroconversion was obtained by all vaccine brands. The performance of each vaccine brand vary as they are produced by different manufacturers having different formulation, concentration, integrity of antigen content, adjuvant and maintenance of cold chain until its use, as reported by Kennedy et al. (2007). Although there is apparent, relative variation in the performance of vaccine brands, the statistical analysis of vaccine brands and antibody titres by chi square test did not showed significant difference (P>0.05). Similar observations was recorded by Shyamsundar et al. (2014) and Savaliya et al. (2015). The contrasting reports on influence of vaccine brands on antibody titre were also reported by Mansfield et al. (2004) and Neelufer et al. (2015).

In the present study, small breeds (50 / 60, 83.33 percent) compared to larger (7/9, 77.77 percent) and medium (56/69, 81.15 per cent) sized breeds showed higher mean protective neutralizing antibody titres (1.810 IU/ml). However, there was no significant difference (P>0.05) in the titre of neutralizing antibodies among different breeds (based on size) when analysed by chi square test. Similar observations were recorded by Shyamsundar et al. (2014) and Savaliya et al. (2015). In contrast, to these results, Kennedy et al. (2007) observed five percent difference in log titre between breeds based on body size. There is clear existence of general relationship between the animal size and level of antibodies responses (Mansfield et al., 2004). The larger dogs are more likely to have deeper subcutaneous fat for injection, deposition and sequestration of rabies antigen known to reduce the level of immune response as compared to smaller breeds (Ellis, 1993; Keating & Noble, 2003). In the present study, unequal sample size of breeds based on size may be the reason for not observing the difference among them.
Failure to protect 27 dogs even after vaccination is alarming and it may be attributed to single or multiple factors acting synergistically, like age, where 6/27 were found below one year of age, genetic profile of individual dog is different, as haplotype of specific breeds of dogs is a factor which leads to a difference in immune response to vaccination (Kennedy et al., 1999), reproductive status (M=17/19, 89.5 percent were intact, Female=7/8, 87.5 were intact), brand of vaccine used and the inappropriate window period of serum sample collection in 74 percent (20/27) of dogs that failed the test (<20 days=3/27, 11 percent ; >50days=17/27, 63 percent).

Conclusion

In the present study immune response of vaccinated domestic dogs and cats intended for pet-travel from India was studied by Rapid Fluorescent Focus Inhibition Test. The observed protective anti-rabies neutralizing antibody titre in dogs and cats were 81.87 and 100 per cent. Study showed that cats are better responders than dogs. The best window period for serum sampling is between 20 to 50 days post vaccination. Statistically, the age of the dog showed association for higher neutralizing antibody titre whereas gender, vaccine brands did not reveal any statistically significant association in conferring protective neutralizing antibody titre.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interest that could possibly arise.

References


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EVALUATING POSTEMERGENCE HERBICIDES FOR BROADLEAF WEED CONTROL IN IRRIGATED BREAD WHEAT (*Triticum aestivum* L.)

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**ABSTRACT**

The objective of this research was to study the effect of broadleaf herbicide treatments and grass + broadleaf herbicides on annual broadleaf weeds and bread wheat yield. Eight on-farm weed control trials were conducted in bread wheat from 2014-15 to 2016-17 in Tadla and Doukkala irrigated perimeters, Morocco: 3 trials using broadleaf herbicides and 5 trials using premix or tank mix of grass and broadleaf herbicides. In 3 trials, 9 broadleaf herbicides (metsulfuron-methyl 6 g ha⁻¹, tribenuron-methyl 9.375 g ha⁻¹, amidosulfuron 15 g ha⁻¹ + iodosulfuron-methyl-sodium 3.75 g ha⁻¹, aminopyralid 9.9 g ha⁻¹ + florasulam 4.95 g ha⁻¹, dicamba 120 g ha⁻¹ + 2,4-D 344 g ha⁻¹, florasulam 3.75 g ha⁻¹ + 2,4-D 180 g ha⁻¹, flumetsulam 5 g ha⁻¹ + florasulam 3.75 g ha⁻¹, triasulfuron 6.15 g ha⁻¹ + dicamba 98.85 g ha⁻¹, and tribenuron-methyl 5 g ha⁻¹ + thifensulfuron-methyl 10 g ha⁻¹) caused no injury to bread wheat, reduced 80 to 100% density and shoot biomass of broadleaf weeds, and provided wheat grain yields up to 8.0 Tons ha⁻¹ compared to the untreated plots. In 5 trials, 12 premix or tank mix of grass and broadleaf herbicides reduced shoot biomass of broadleaf weeds up to 100% and provided wheat grain yields up to 9.3 Tons ha⁻¹ compared to the untreated plots. Herbicide treatments containing pyroxsulam or mesosulfuron-methyl + iodosulfuron-methyl-sodium caused crop injury, but wheat plants recovered and grain yield was not affected.

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Evaluating postemergence herbicides for broadleaf weed control in irrigated bread wheat (*Triticum aestivum* L.)

Mesosulfuron-methyl + iodosulfuron-methyl-sodium and mesosulfuron-methyl + iodosulfuron-methyl-sodium + difufenican were less effective on milk thistle (*Silybum marianum* (L.) Gaertn). Further research involving grass and broadleaf herbicides is needed to explore ways, including the use of adjuvants, to avoid wheat injury and optimize herbicide efficacy on noxious weeds such as milk thistle.

1 Introduction

Bread wheat (*Triticum aestivum* L.) is a major crop that is grown on 2 million hectares in Morocco (Rerhrhaye & Ait El Mekki, 2017). Out of this, about 200,000 hectares are cultivated under irrigation. Irrigated bread wheat is usually planted in November in rotation with several crops including sugar beet (*Beta vulgaris* L.), corn (*Zea mays* L.), Egyptian clover (*Trifolium alexandrinum* L.) or alfalfa (*Medicago sativa* L.). In 2015-16, grain yield of irrigated bread wheat was estimated to 4 Tons ha$^{-1}$ (Rerhrhaye & Ait El Mekki, 2017), but area and yield increases over the past decades have been minimal. However, Jlibene & Chafai Elalaoui (2002) found that some bread wheat cultivars, when cultivated appropriately under irrigation, produced up to 7.8 Tons ha$^{-1}$.

More than 300 weed species have been identified in wheat fields (Taleb et al., 2000). Among them, major annual grass weeds are rigid brome (*Anisantha rigida* (Roth) Hyl.), sterile oat (*Avena sterilis* L.), canarygrass (*Phalaris brachystachys* Link, *P. minor* Retz., *P. paradoxa* L.), and rigid ryegrass (*Lolium rigidum* Gaud.) (Tanji, 2005). Major annual broadleaf weeds are common poppy (*Papaver rheas* L.), milk thistle (*Silybum marianum* (L.) Gaertn.), wild mustard (*Sinapis arvensis* L.), wild chicory (*Cichorium intybus* L.), spiny emex (*Emex spinosa* (L.) Campd.), and crown daisy (*Glebionis coronaria* (L.) Spach) (Tanji, 2005). All these weeds are fall and/or winter annuals, and the weed emergence pattern is a consequence of soil moisture (from rainfall and irrigation) and germination temperature requirements. In 23 weed control trials, Tanji (2002) found that weed densities in the untreated plots were up to 500 plants m$^{-2}$, and grain yield losses of irrigated bread wheat due to weed competition were up to 80%.

Weed control with herbicides is the most common practice in irrigated wheat (Tanji, 2000). Furthermore, Tanji (2003) reported that tribenuron-methyl (9.375 g ha$^{-1}$), trasulfuron + terbutryne (10 + 150 g ha$^{-1}$), and 2,4-D + MCPA (330 + 341 g ha$^{-1}$) provided up to 96% control of annual broadleaf weeds. In general, the use of selective postemergence herbicides resulted in excellent weed control and increased bread wheat yield (Benahinia, 1985; Rafrafi, 1988; Errohi, 1995; Aitounejjar & Tanji, 1997; Benslimane, 2000; Tanji, 2002; Tanji, 2003; El Antri & Madkouri, 2005).

Since 2000, several postemergence herbicides (amidosulfuron, aminopyralid, flucarbazone, iodosulfuron-methyl-sodium, mesosulfuron-methyl, metsulfuron-methyl, pyroxasulam, thifensulfuron-methyl, and many others) have been registered in Morocco for weed control in wheat. However, limited research has been conducted on weed control with these recently registered herbicides in irrigated wheat. Information on herbicide choice, effective herbicide application rates, and application timing is needed to provide producers with precise decision tools for managing weeds in wheat production systems. The objective of this study was to evaluate the response of annual broadleaf weeds and wheat production to 9 broadleaf herbicide treatments in 3 trials and 12 premix or tank mix of grass and broadleaf herbicide treatments in irrigated bread wheat in 5 trials.

2 Materials and Methods

2.1 Experiment site and design

A total of 8 bread wheat trials were conducted in farmers’ fields from 2014-15 to 2016-17, in Doukkala and Tadla perimeters, Morocco: 3 trials using postemergence broadleaf herbicides (Tables 1 & 2) and 5 trials using premix or tank mix of postemergence grass and broadleaf herbicides (Tables 1 & 3). The preceding crops were alfalfa, faba bean, sugar beet or bread wheat. Fields were plowed once with a deep plow and twice with a tandem disc. Each field measured 1 hectare that was drilled with “Amal” bread wheat at a seed rate of 200 kg ha$^{-1}$ in November 2014, 2015, and 2016. The N-P-K fertilizer used at planting was either diammonium phosphate (18-46-0) at the rate of 200 kg ha$^{-1}$ or 15-15-15 at the rate of 300 kg ha$^{-1}$. It was manually broadcast and incorporated with a tandem disc before planting. After crop emergence, ammonium nitrate 33% was broadcast 2 to 3 times during the growing season. Trials had access to irrigation to supplement rainfall (Table 1).

Trials were arranged as randomized complete blocks with 3 replications and individual plot size measured 10 m by 2 to 3 m. One nontreated control per replication was included for treatment comparisons. At the early wheat tillering stage, 9 broadleaf herbicide treatments (Table 2) and 12 premix or tank mix of grass and broadleaf herbicide treatments were applied (Table 3). The herbicides and their rates were

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Table 1 Characteristics of the 8 on-farm trials conducted from 2014-15 to 2016-17 in the Tadla and Doukkala irrigated perimeters, Morocco

<table>
<thead>
<tr>
<th>Management practices</th>
<th>3 trials with broadleaf herbicides</th>
<th>5 trials with premix or tank mix of grass and broadleaf herbicides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tadla</td>
<td>Tadla</td>
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<tr>
<td>Preceding crop</td>
<td>sugarbeet</td>
<td>alfalfa</td>
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<tr>
<td>Tillage</td>
<td>1 deep plowing</td>
<td>1 deep plowing</td>
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<tr>
<td></td>
<td>+ 2 tandem disc</td>
<td>+ 2 tandem disc</td>
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<tr>
<td></td>
<td>+ planting with a drill</td>
<td>+ planting with a drill</td>
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<tr>
<td>Date of crop</td>
<td>200 kg ha(^{-1}) DAP 18-46-0</td>
<td>200 kg ha(^{-1}) DAP 18-46-0</td>
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<tr>
<td>emergence</td>
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<tr>
<td>Fertilizer at</td>
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<td>100 kg ha(^{-1}) ammonium nitrate</td>
</tr>
<tr>
<td>planting</td>
<td>33% at the tillering stage, 100 kg ha(^{-1}) ammonium nitrate 33% at the jointing stage</td>
<td>33% at the tillering stage, 100 kg ha(^{-1}) ammonium nitrate 33% at the jointing stage</td>
</tr>
<tr>
<td>Foliar fungicide at</td>
<td>spiroxamine 200 g ha(^{-1}) + tebuconazole 133.6 g ha(^{-1}) + triadimenol 34.4 g ha(^{-1})</td>
<td>spiroxamine 200 g ha(^{-1}) + tebuconazole 133.6 g ha(^{-1}) + triadimenol 34.4 g ha(^{-1})</td>
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<td>the booting stage</td>
<td>Flood irrigation</td>
<td>Flood irrigation</td>
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<td>and repeated at</td>
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<td>Dec 30, 2014 at the tillering stage</td>
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<td>heading</td>
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<td>Jan 7, 2016 at the tillering stage</td>
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<td>Irrigation system</td>
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<td>Date of spraying</td>
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<td>Dec 30, 2014 at the tillering stage</td>
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<td>weed control trials</td>
<td></td>
<td>Jan 2, 2016 at the tillering stage</td>
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</table>
Table 2 Effect of broadleaf herbicides on the density and shoot dry weight (DW) of broadleaf weeds 2 months after treatment (2 MAT), and bread wheat grain yield in 3 on-farm weed control trials in 2014-15 and 2015-16 in the Tadla and Doukkala irrigated perimeters, Morocco

| Herbicide (rate ha$^{-1}$) | Tedla Trial 1 2014-15 | | Tadla Trial 2 2015-16 | | Doukkala Trial 3 2015-16 | | Weed density | Weed DW | Wheat yield | Weed density | Weed DW | Wheat yield | Weed density | Weed DW | Wheat yield |
|---------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| Amidosulfuron (15 g) + iodosulfuron-methyl- sodium (3.75 g) | SEKATOR (150 ml) | 4 | 7 | 7.8 | 2 | 7 | 6.8 | 3 | 13 | 5.1 |
| Aminopyralide (9.9 g) + florasulam (4.95 g) | LANCELOT (33 g) | 4 | 9 | 7.8 | 4 | 7 | 6.7 | 1 | 7 | 5.3 |
| Dicamba (120 g) + 2,4-D (344 g) | DIALEN SUPER (1 L) | 1 | 3 | 7.9 | 2 | 3 | 6.8 | 3 | 13 | 5.1 |
| Flumetsulam (5 g) + florasulam (3.75 g) | DERBY (50 ml) | 2 | 5 | 7.8 | 2 | 6 | 6.9 | 1 | 13 | 5.2 |
| Florasulam (3.75 g) + 2,4-D (180 g) | MUSTANG (600 ml) | 4 | 9 | 7.8 | 3 | 7 | 6.6 | 0 | 0 | 5.6 |
| Metsulfuron-methyl (6 g) | STARKEM (10 g) | 3 | 7 | 8.0 | 1 | 4 | 6.8 | 1 | 7 | 5.7 |
| Triasulfuron (6.15 g) + dicamba (98.85 g) | LINTUR (150 g) | 3 | 7 | 7.8 | 3 | 7 | 6.7 | 12 | 20 | 5.8 |
| Tribenuron-methyl (9.375 g) | GRANSTAR (12.5 g) | 3 | 5 | 8.0 | 2 | 5 | 6.7 | 5 | 13 | 5.1 |
| Tribenuron-methyl (5 g) + thifensulfuron-methyl (10 g) | HARMONY EXTRA (20 g) | 2 | 4 | 8.0 | 2 | 3 | 6.6 | 3 | 13 | 5.3 |
| Untreated check | | 29 | 75 | 7.2 | 26 | 60 | 6.1 | 57 | 1613 | 3.4 |
| LSD (0.05) | | 5 | 5 | NS | 2 | 3 | 0.2 | 11 | 197 | 1.1 |
Table 3 Effect of premix or tank mix of grass and broadleaf herbicides on the density and shoot dry weight (DW) of broadleaf weeds 2 months after treatment (2 MAT) and wheat grain yield in 5 on-farm weed control trials from 2014-15 to 2016-17 in the Tadla and Doukkala irrigated perimeters, Morocco

<table>
<thead>
<tr>
<th>Herbicide (rate ha⁻¹)</th>
<th>Trial 1 2014-15</th>
<th>Trial 2 2015-16</th>
<th>Tadla</th>
<th>Trial 3 2016-17</th>
<th>Trial 4 2016-17</th>
<th>Trial 5 2015-16</th>
<th>Doukkala</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weed DW (g m⁻²)</td>
<td>Wheat yield (T ha⁻¹)</td>
<td>Weed DW (g m⁻²)</td>
<td>Wheat yield (T ha⁻¹)</td>
<td>Weed DW (g m⁻²)</td>
<td>Wheat yield (T ha⁻¹)</td>
<td>Weed DW (g m⁻²)</td>
</tr>
</tbody>
</table>
| Mesosulfuron-methyl (15 g) + iodosulfuron-methyl- sodium (3 g) | ATLANTIS (500 ml) | 0 | 6.8 | 4 | 6.3 | 15 | 6.1 | 188 | 5.0 | 27 | 5.7
| Mesosulfuron-methyl (7.5 g) + iodosulfuron-methyl-sodium (7.5 g) | COSSACK (1 L) | 0 | 6.6 | 2 | 6.5 | 8 | 6.0 | 125 | 5.2 | 27 | 5.9
| Flucarbazone (61.4 g) + amidosulfuron (15 g) + iodosulfuron-methyl-sodium (3.75 g) | EVEREST (43 g) + SEKATOR (150 ml) | 1 | 6.7 | 3 | 6.5 | 3 | 6.3 | 4 | 8.8 | 13 | 4.4
| Iodosulfuron-methyl-sodium (8 g) + fenoxaprop- p-ethyl (64 g) | HUSSAR (1 L) | 3 | 6.6 | 5 | 6.4 | 10 | 6.2 | 15 | 7.8 | 0 | 4.5
| Mesosulfuron-methyl (7.2 g) + iodosulfuron-methyl- sodium (6 g) + diflufenican (96 g) | KALENKOEA (800 ml) | 3 | 6.7 | 5 | 6.5 | 4 | 6.1 | 99 | 6.1 | 13 | 4.5
| Pinoxaden (45 g) + florasulam (5 g) | NAVIGATOR (1 L) | 2 | 6.8 | 5 | 6.5 | 3 | 6.1 | 160 | 5.1 | 27 | 4.8
| Mesosulfuron-methyl (7.5 g) + iodosulfuron-methyl sodium (2.5 g) + diflufenican (50 g) | OTELLO (1 L) | 3 | 6.8 | 5 | 6.4 | 0 | 6.4 | 25 | 8.8 | 13 | 5.6
| Pinoxaden (30 g) + clodinafop (30 g) + florasulam (7.5 g) | SWIPE (1 L) | 3 | 6.7 | 4 | 6.4 | 0 | 6.1 | 236 | 8.2 | 27 | 5.5
| Pinoxaden (45 g) + amidosulfuron (15 g) + iodosulfuron-methyl-sodium (3.75 g) | AXIAL (1 L) + SEKATOR (150 ml) | 3 | 6.5 | 4 | 6.4 | 0 | 6.5 | 5 | 9.0 | 13 | 5.4
| Pyroxasulam (22.656 g) + florasulam (4,260 g) | FLORAMIX (320 g) | 0 | 6.6 | 0 | 6.4 | 7 | 6.1 | 16 | 8.6 | 0 | 5.5
| Clodinafop (60 g) + amidosulfuron (15 g) + iodosulfuron-methyl-sodium (3.75 g) | TOPIK (750 ml) + SEKATOR (150 ml) | 3 | 6.5 | 4 | 6.2 | 0 | 6.2 | 0 | 9.2 | 13 | 5.1
| Pinoxaden (22.5 g) + clodinafop (22.5 g) + amidosulfuron (15 g) + iodosulfuron-methyl- sodium (3.75 g) | TRAXOS (1 L) + SEKATOR (150 ml) | 4 | 6.6 | 5 | 6.1 | 0 | 6.5 | 6 | 9.3 | 220 | 5.3
| Untreated check | | | | | | | | | | | 67 | 6.1 | 67 | 5.2 | 120 | 4.1 | 302 | 4.6 | 780 | 4.1
| LSD (0.05) | | | | | | | | | | | 5 | NS | 3 | 0.2 | 16 | 1.4 | 38 | 1.2 | 248 | 1.6
recommended field rates for weed control in wheat (Tables 2 and 3). In the 3 trials with broadleaf herbicides, major weed species infesting wheat included spiny emex (E. spinosa), common poppy (Papaver rhoes L.), nettleleaf goosefoot (Chenopodium murale), wild mustard (S. arvensis), wild chervil (C. intybus), common sowthistle (Sonchus oleraceus), and henbit (Lamium amplexicaule). In the 5 trials with grass and broadleaf herbicides, major weed species infesting wheat were milk thistle (S. marianum), field marigold (Calendula arvensis), wild mustard (S. arvensis), wild chervil (C. intybus), and blue pimpernel (Lysimachia arvensis).

Herbicide application was done at the seedling or vegetative stage of broadleaf weeds. Foliar fungicides were sprayed at the boot stage and repeated at heading. Herbicide and fungicide treatments were applied with a backpack sprayer fitted with a spray boom with 4 flat fan 8002 nozzles calibrated to deliver 200 L ha⁻¹.

2.2 Measurements

Two months after treatments (2 MAT), weeds were collected from one 0.50 m x 0.50 m quadrat in each plot. Removed weeds were counted for density, and shoots were oven dried at 60°C to a constant weight and weighed. At full maturity, wheat was harvested at ground level in an area of 1 m² per plot. Wheat was threshed by a small combine and grain was cleaned and weighed. Wheat yield loss due to weed competition and yield increase due to herbicide use were calculated as follows:

\[
\% \text{ yield loss} = \left( \frac{\text{Grain yield in weed - free plots}}{\text{Grain yield in weed - free plots}} \right) \times 100
\]

\[
\% \text{ yield increase} = \left( \frac{\text{Grain yield in weed - free plots}}{\text{Grain yield in weedy plots}} \right) \times 100
\]

2.3 Statistical analyses

Measured variables (weed density, weed shoot dry weight, and wheat grain yield) varied across trials and cropping seasons, primarily due to differences in environmental conditions (irrigation, rainfall, soil type, crop rotation). Therefore, data analysis was performed for each trial to determine the significance of the herbicide treatments. Density and biomass of weeds and wheat grain yields were subjected to the ANOVA using SAS (SAS Institute, Cary, NC, USA). Means were compared using the Fisher’s protected LSD at P = 0.05.

3 Results and Discussion

3.1 Weed control with broadleaf herbicides (3 trials)

Wheat densities in untreated plots varied from 26 to 57 plants m⁻² (Table 2). Shoot dry weights varied between 60 and 1613 g m⁻². Five herbicide treatments viz. (i) metsulfuron-methyl 6 g ha⁻¹, (ii) tribenuron-methyl 9.375 g ha⁻¹, (iii) dicamba 120 g ha⁻¹ + 2,4-D 344 g ha⁻¹, (iv) flumetsulam 5 g ha⁻¹ + florasulam 3.75 g ha⁻¹, and (v) tribenuron-methyl 5 g ha⁻¹ + thifensulfuron-methyl 10 g ha⁻¹ provided consistently excellent weed control by reducing weed density and/or weed biomass by ≥90%. While the other herbicide treatments i.e. (i) amidosulfuron 15 g ha⁻¹ + iodosulfuron-methyl-sodium 3.75 g ha⁻¹, (ii) amipyrarid 9.9 g ha⁻¹ + florasulam 4.95 g ha⁻¹, (iii) florasulam 3.75 g ha⁻¹ + 2,4-D 180 g ha⁻¹, and (iv) triasulfuron 6.15 g ha⁻¹ + dicamba 98.85 g ha⁻¹ gave good to excellent control by reducing weed density and/or biomass by more than 80% (Table 2).

Such acceptable levels of weed control could be attributed to the herbicides reaching seedlings and small weed plants at the early wheat tillering stage, to the susceptibility of most annual broadleaf weeds to herbicides, and to better growth conditions of weeds under irrigation. Previous studies revealed that many annual broadleaf weeds were controlled by broadleaf herbicides in wheat (Benahnia, 1985; Rafi, 1988; Errohi, 1995; Diab, 1996; Aitounejjar & Tanji, 1997; Benslimane, 2000; Tanji, 2002; Tanji 2003; El Antri & Madkouri, 2005). Further, Wu et al. (2010) reported in Australia that the efficacy of early postemergent applications of metsulfuron-methyl at 4.2 g ha⁻¹ on flaxleaf fleebane (Erigeron bonariensis L.) and it was effective up to 98%.

In Egypt, Saad et al. (2011) reported that tribenuron-methyl (9.375 g ha⁻¹) and flumetsulam (5 g ha⁻¹) + florasulam (3.75 g ha⁻¹) reduced nettleleaf goosefoot (Chenopodium murale L.) density up to 91% 3 weeks after treatment.

Due to foliar or root absorption and possibly the activation by irrigation, broadleaf herbicide treatments containing residual herbicides such as amipyrarid, iodosulfuron-methyl-sodium, and triasulfuron have been effective in controlling initial weed infestations as well as multiple flushes of weeds, and provided season-long control of weeds. But, use of residual herbicides might restrict the choice of crops planted after wheat. In North Dakota, USA, Mikkelson & Lym (2011) found that residues of amipyrarid applied at 120 g ha⁻¹ injured alfalfa, soybean, and sunflower planted 8 and 11 months after treatments (MAT). They found that corn was not affected by amipyrarid when seeded 8 or 11 MAT and appeared to be the best cropping option for land recently treated with amipyrarid.
3.2 Weed control with grass + broadleaf herbicides

Shoot weights of broadleaf weeds in untreated plots varied from 67 to 780 g m$^{-2}$ (Table 3). Major weed species found in the trials were milk thistle (S. marianum), field marigold (C. arvensis), wild mustard (S. arvensis), wild chicory (C. intybus), and blue pimpernel (L. arvensis).

Six herbicide treatments consistently provided excellent weed control by reducing weed biomass by 92-100% (Table 3). The other 6 treatments, including mesosulfuron-methyl + iodosulfuron-methyl-sodium, were excellent on many annual broadleaf weed species, but less effective against milk thistle (S. marianum), as indicated by higher weed biomass (Table 3). In Portugal, Barros et al. (2009) found that premix of mesosulfuron-methyl at 12 g ha$^{-1}$ + iodosulfuron-methyl-sodium at 2.4 g ha$^{-1}$ gave satisfactory control of rigid ryegrass (Lolium rigidum), but had limited ability to control some broadleaf weeds such as night catchfly (Silene nocturna), Moroccan chamomile (Cladanthus mixtus), prostrate knotweed (Polygonum aviculare), blue pimpernel (Lysichichia arvensis), glandular plantain (Plantago afra), and Queen Anne’s lace (Daucus carota).

3.3 Grain yield after using broadleaf herbicides (3 trials)

None of the broadleaf herbicide treatments visibly injured wheat at any location. Injury was not expected since the herbicide treatments were applied under appropriate growth conditions and at the registered rates. Grain yields in sprayed plots ranged from 7.8 to 8.0 T ha$^{-1}$ in trial 1, 6.6 to 6.9 T ha$^{-1}$ in trial 2, and 5.1 to 5.8 T ha$^{-1}$ in trial 3 (Table 2). In these trials, no significant differences were observed between yields obtained in various treated plots. Yield variations between trials and years were probably due to different preceding crops and irregular durations between irrigations. Yield increases due to weed control were 11, 13, and 71% compared to the yields observed in nontreated plots in trials 1, 2, and 3, respectively. Increased grain yields recorded in all herbicide-treated plots in comparison with untreated plots could be attributed to the excellent weed control by all herbicide treatments used in this study. Weed control improved grain yields through better utilization of available resources like water, fertilizer, sunlight, and space. Other appropriate practices that could have increased grain yield were November planting, certified seeds, disease control with fungicides, and irrigation (Hamid, 1995; Ezzahiri et al., 1999; Bouhache et al., 2000; Mosseddaq et al., 2000; Zbair et al., 2000). In on-farm trials, bread wheat grain yields varied with cultivars and were up to 7.8 T ha$^{-1}$ when the best production practices were used (Jilbene & Chafai Elalaoui, 2002).

In untreated plots of all trials, grain yields were low compared to treated plots (Table 2). Yield losses due to weed competition throughout the growing season were 10, 12, and 41% compared to the highest yields observed in treated plots in trials 1, 2, and 3, respectively. Broadleaf weeds left in the nontreated plots appeared to cause significant crop production losses; hence, herbicide selection should be based on knowledge of weed species present within the weed flora. This is consistent with previous research that has emphasized the potential for bread wheat yield loss due to weed competition (Tanji, 2002). Curran et al. (2015) found that bread wheat yields declined 0.55 to 1.2 kg ha$^{-1}$ per 1 kg ha$^{-1}$ increase in hairy vetch (Vicia villosa Roth) biomass.

3.4 Grain yield after using grass + broadleaf herbicides (5 trials)

Grain yields in sprayed plots ranged from 6.5 to 6.8 T ha$^{-1}$, 6.1 to 6.5 T ha$^{-1}$, 6.0 to 6.5 T ha$^{-1}$, 5.0 to 9.3 T ha$^{-1}$, and 4.4 to 5.9 T ha$^{-1}$ in trials 1 to 5, respectively (Table 3). The increased grain yields recorded in the herbicide treated plots in comparison with those of the untreated plots could be attributed to weed control, which resulted in reduced wheat competition from weeds. Using the highest yields obtained in treated plots, grain yield increases due to weed control were 11, 25, 59, 102, and 44% compared to the yields observed in nontreated plots in trials 1 to 5, respectively. Weed control with herbicides, as well as other appropriate practices, increased wheat grain yields (Ezzahiri et al., 1999; Bouhache et al., 2000; Mosseddaq et al., 2000; Zbair et al., 2000).

Grain yields were low in nontreated plots, as well as in treated plots where herbicide treatments failed to control milk thistle (trials 4 and 5, Table 3). Yield losses due to weed competition throughout the growing season were 10, 20, 37, 51, and 31% compared to the highest yields observed in treated plots in trials 1 to 5, respectively. High yield loss (51%) was due to milk thistle competition which drastically lowered grain yield in the untreated plots. Tanji (2002) reported that yield losses due to weed competition from the mixed populations of weeds were up to 80%.

Herbicide treatments containing (i) pyroxsulam, (ii) mesosulfuron-methyl + iodosulfuron-methyl-sodium and (iii) mesosulfuron-methyl + iodosulfuron-methyl-sodium + diflufenican, caused severe injury symptoms on wheat during almost 2 months after treatments (data not shown). They caused an overall stunting and crop height reduction. However, the crop recovered later in the season with no yield reduction. It is highly possible that wheat injury was due to both mesosulfuron and pyroxsulam. Pyroxsulam at 18 g ha$^{-1}$ caused 5 to 10% wheat injury 5 to 14 days after treatment, but wheat recovered completely (Geier et al., 2011). Mesosulfuron-methyl at 15 g ai ha$^{-1}$ with the crop safener at 30 g ai ha$^{-1}$ injured wheat 11 to 32%, but tiller number and height of treated wheat were, by 9 weeks after treatment, similar to those of nontreated wheat (Bailey et al.,...
Evaluating postemergence herbicides for broadleaf weed control in irrigated bread wheat (*Triticum aestivum* L.)

2004). Other studies revealed that wheat injury was often observed after application of mesosulfuron-methyl + iodosulfuron-methyl-sodium + safener, but injury did not reduce grain yield (Kirkland et al., 2001; Wiersma et al., 2003; Crooks et al., 2004a; Crooks et al., 2004b).

**Conclusion**

The results of this study indicate that very good control of most annual broadleaf weeds and high wheat yield can be achieved by the application of herbicide treatments containing 1 to 4 active ingredients at the early wheat tillering stage. Premix of grass and broadleaf herbicides were less effective on milk thistle (*S. marianum*). Herbicide treatments containing pyroxsulam or mesosulfuron-methyl + iodosulfuron-methyl-sodium caused crop injury, but wheat plants recovered and grain yield was not affected. Further research involving grass and broadleaf herbicides is needed to explore ways, including the use of adjuvants, to avoid wheat injury and optimize herbicide efficacy on noxious weeds such as milk thistle.

**Acknowledgments**

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**Conflict of interest**

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

**References**


Crooks HL, York AC, Jordan DL (2004a) Tolerance of six soft red winter wheat cultivars to AE F130060 00 plus AE F115008 00. Weed Technology 18:252-257.

Crooks HL, York AC, Jordan DL (2004b) Wheat tolerance to AE F130060 00 plus AE F115008 00 as affected by time of application and rate of the safener AE F107892. Weed Technology 18:841-845.


MICROSPOROGENESIS MANIPULATION BY CHA’S TO ELIMINATE EMASCULATION IN HYBRID SEED PRODUCTION OF OKRA

(Albemoschus esculentum L.)

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ABSTRACT

Present study was conducted to identify effective microsporogenesis and flower bud stage for application of male gametocides to induce pollen sterility in okra. Results of study revealed the association of floral bud size and stages of microsporogenesis in female parental lines. Formation of microspore from PMC is between 3 mm to 5 mm bud size. Further, it was reported that Meiosis I completed when bud is under 5 mm and after this microgametogenesis start appearing when the bud size between 10 mm to 40 mm buds. However, pollen remains nonviable till bud reaches 45mm size and attains maturity just before flower anthesis between 45mm to 50 mm bud size. Days taken in formation of microspore to viable pollen grain are 18-20 days. Male gametocides used in the study were effective in inducing pollen sterility and vary with different stages of microsporogenesis and bud growth. Among the different treatment combinations, 45 mm bud size treated with malic hydrazide at 450 ppm induced higher pollen sterility under in vitro pollen germination test (93.40 %) and this was followed by the acetocarmine (90.77 %) and ethrel @ 3000 ppm (80.68) at 45 mm bud size.

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1 Introduction

Hybrid seed production of okra (Abelmoschus esculentum L.) has an impact on economic traits of seed production. Due to the availability of skilled labour and climatic conditions, about 29.3% of the total okra hybrid seeds of India are produced in Karnataka (Davaluri, 2015). Presently hybrid seeds are produced using hand emasculation and pollination technique, which considered as tedious, cumbersome, expensive, laborious and time consuming process. The floral biology and structure of Okra demands continuous labour for 25 to 40 days during flowering period for emasculation followed by pollination. The process of emasculation is painful as buds are thorny and sticky due to this Child labours are majorly engaged (Davaluri, 2015). This has made a way to find an alternative technique to skip emasculation process and directly go for pollination. Hand emasculation process can be skipped if female parent is a male sterile line. Apart from elimination of tedious hand emasculation technique, male sterile line will provide more flexibility to breeding programme, facilitating quick incorporation of diverse genes for stress resistance and also reducing cost of F1 hybrid seeds.

Male gametocides also called as CHA’s are known to induce male sterility in okra (Dubey & Singh, 1967; Deepak et al., 2007) and male gametocides will selectively kills only male gametes, spores or organs and render the treated plants as male sterile. However, no single male gametocide has reported to induce 100 per cent male sterility in okra. The effectiveness of these male gametocides depends on the stages of buds too. According to Rehm (1952) male sterility was effectively induced when plants are sprayed with 2, 4-D a week before the first flower. High levels of pollen sterility were also found in lettuce plants when immature flower buds were sprayed with ethrel (Han & Lee, 1972). Van der Meer & Van Bennekom (1973) also reported that foliar application of Maleic Hydrazide (MH) @ 500 ppm at bolting stage gave complete pollen sterility in cole crops. Salgare (1995) found 50 per cent male sterility by the application of MH and 2, 4- D at 100 ppm concentrations in ornamental chilli. Prakash et al. (2001) also reported that one week before flowering, spray of ethrel (400 ppm) and 2, 4- D (40 ppm) induced maximum sterility. Similarly, when Deepak et al. (2007) sprayed MH @ 200 ppm at 20+30+40 Days after sowing resulted in higher sterility (84.33 %) and this sterility percentage was followed by ethrel (1250 ppm) (82.10 %). The proper timing of the activity of enzymes during pollen development is critical for normal development of pollen. Any disturbance in the activity of callase enzyme will result in pollen sterility (Sharma & Sharma, 2005). The effectiveness of male gametocides on pre-meiotic and post meiotic stages to ensure cent per cent artificial male sterility need to be study in Okra. Hence, the present study was formulated to identify the association of bud size with microsporogenesis growth stages to identify effective bud growth stage (bud size) for application of male gametocide to induce maximum pollen sterility in okra for skipping emasculation in commercial hybrid seed production.

2 Materials and Methods

The experiment was conducted at Seed Testing Laboratory, Seed Unit, Main campus, University of Horticultural Sciences, Bagalkot, Karnataka (16.1635° N, 75.6172° E) during 2015-16. Flower buds of two different female parental lines of two commercial hybrids were used. Flower buds ranging from 3 mm (at the time of bud initiation) to 50 mm size (at the time anthesis) were collected and labelled as B1, B2, B3, B4, B5, B6, B7, B8, B9 and B10.

2.1 Fixation of buds and preparation of slides:

Meiotic studies of flower buds were studied after fixing the developmental stages. The buds of predetermined size were collected and fixed during early morning (5.00 – 5.30 am) by dipping buds in corney’s fixing solution (Yoneyama et al., 2013). Buds were soaked in/dipped in fixative solution for 24 hours, this was followed by the storing these buds in 70 per cent alcohol for dissection of anthers. From each fixed flower buds ten anthers were studied separately, developing PMC’s were extracted by squeezing anther on glass slides.

For slide preparation, Acetocarmine (1%) was added to PMC’s and desirable spread of the chromosomes was obtained by application of pressure on the cover slip over several folds of blotting paper. Later the slides were warmed and are temporarily sealed with paraffin wax and observe under microscope. Camera lucida drawings and photomicrographs were made from temporary slide preparations. All the observed developmental phases were classified into different stages of meiosis/cell division based on the cytological observations.

Further, the effect of Chemical hybridising Agents (CHA’s) on different flower buds sizes was studied in-vitro (Figure 2). Flower buds of different sizes ranging from 40 mm, 45 mm and 50 mm were collected from two female parental lines. Buds size was selected based on the results of the bud size association with pollen developmental stages. The buds collected were fixed using fixative after treating with CHA’s at different concentration viz., male hydrazide (400, 450 and 500 ppm), ethrel (1000, 2000 and 3000 ppm) and 2, 4- D (10, 20 and 30 ppm) were used to soak flower buds for 12 hours. Pollen viability was assessed using acetcarmine test (Gaaliche et al., 2013) and results were confirmed in in-vitro pollen germination test on pollen germination media (Dafni & Firmage, 2000). Anthers dissected out from treated buds by using forceps and needle to place on glass slides and a drop of acetcarmine stain was added and
tapped gently to release the pollen grains followed by stirring with the help of needle thoroughly and then cover slip was covered on the pollen. The stained slides were heated gently and uniform pressure was applied on the edge of cover slip to flatten the anther uniformly. Edges of cover slip were sealed with wax and observed under microscope by taking minimum of five microscopic fields. In each field, number of pollen grains with red stained and unstained pollens were counted and recorded. The red stained scored as fertile and unstained pollen grains as sterile. The percent pollen sterility was calculated using the formula

\[
\text{Pollen sterility (\%) = \frac{\text{Number of unstained pollen grains}}{\text{Total number of pollen grains}} \times 100}
\]

Pollen germination medium was prepared using KNO₃ (0.01%), CaNO₃ (0.03%), sucrose (20%), Boric acid (0.01%) and MgSO₄ (0.02%). A drop of freshly prepared medium was placed on clean glass slide in circular form using camel hair brush. Pollen grains extracted from anther were smeared onto the sitting droplets of the medium and spread with brush so as to assure complete saturation of pollen grains in the media. A separate glass petri plates were taken and lined with moistened filter paper. Additional 2 to 3 droplets of water was added to maintain optimum relative humidity. The pollen grains inoculated slides were then carefully kept on moistened filter paper in petri plates covered half a way using upper petri plates and these petri plates were incubated at 30°C ± 2°C temperature. After 3 hours of incubation, the slides were observed under microscope for germination of pollen grains. A drop of acetocarmine was added to germinating pollen grain to document photographs. If the pollen grain germinates by producing the pollen tube was counted as fertile pollen and pollens without pollen tubes were considered as sterile pollen. The Factorial CRD observations were analyzed statistically and critical difference (CD) values were calculated at 1 percent (p = 0.01) for laboratory experiments using OPISTAT software where experiment found significant. In case of non-significant effects, value of standard error of means alone was presented and tabulated.

3 Results and Discussion

The results of study revealed that different development stages of microsporogenesis were found to occur in 3 -5 mm bud size and complete before bud reaching 10 mm size. Meiotic cell division I was completed in 3 mm size bud while Meiotic II of triad and tetrad were recorded (Figure 1 and table 1) in 5 mm and 10 mm size bud, respectively. Microspore undergone cell division to produced gamete was observed from 10 mm bud size to 45 mm size bud. The formation of outer layer callose deposition in pollen grain occurs from bud size of 10 mm to 45 mm. The developed microspore remained nonviable till the bud size reaches 40 mm and did not show any red staining in acetocarmine test, this confirmed that pollen are formed but not matured. Completely

<table>
<thead>
<tr>
<th>Bud Size (mm)</th>
<th>Developmental Stages in Microsporogenesis and microgametogenesis</th>
<th>Microsporogenesis</th>
<th>Microgametogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₁</td>
<td>3 Meiosis I</td>
<td>PMC to Microsporocyte</td>
<td>Microspore to microgamete</td>
</tr>
<tr>
<td>B₂</td>
<td>5 Meiosis II (Dyads)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₃</td>
<td>10 Meiosis II (Tetrads)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₄</td>
<td>15 Microspore formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₅</td>
<td>20 Increased size of vacuole in microspore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₆</td>
<td>25 Initiation of mitosis I and deposition of callose on cell wall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₇</td>
<td>30 Occurrence of mitosis I and deposition of callose on cell wall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₈</td>
<td>35 Formation of vegetative nucleus (Bicellular pollen) and deposition of callose on cell wall</td>
<td></td>
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</tr>
<tr>
<td>B₉</td>
<td>40 Initiation on Mitosis II of nucleus and deposition of callose on cell wall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₁₀</td>
<td>45 Formation of vegetative nucleus (Tricellular pollen) and deposition of callose on cell wall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₁₁</td>
<td>50 Formation of vegetative nucleus (Tricellular pollen) and deposition of callose on cell wall</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1 Triad and tetrad stage of microspore development in 3 mm and 5 mm bud size, respectively in okra

Figure 2 Transverse section of stamina column as observed in different bud size of okra
developed pollen became viable when bud size increases more than 40 mm. Interestingly, maximum viability is observed only one hour before anthesis (50 mm bud size).

The meiotic phase was seen in buds of 3 to 5 mm and post meiotic phases and division of nucleus to form two or three nucleus was from 10 mm to 40 mm bud size. However, the fertility of the pollen was gained in 40 mm bud size and it was maximum at 50 mm (at the time anthesis).

The viability of the pollen extracted from buds less than 40 mm could not be assessed in acetocarmine test as pollens were non-viable. Hence, the study on CHA’s for inducing pollen sterility was implemented from 40 to 50 mm size buds. The results obtained were tabulated and presented in table 2 and 3. The results revealed that among different CHA’s, Maleic hydrazide has induced significantly higher pollen sterility (87.99 %) and this was followed by 2, 4-D (67.58 %) and Ethrel (66.68 %). Lower pollen sterility was recorded in untreated control (13.29 %) at it was not statistically different that the distilled water (13.38 %). The pollen sterility has increased along with the increase in concentration in all CHA’s. Flower buds treated with 500 ppm of MH have shown higher pollen sterility (89.76 %), which is at par the 450 ppm of MH (89.64 %). Among the different flower bud size, higher pollen sterility (64.62 %) was reported from the 45 mm bud and it was at par the 50 mm bud size (64.58 %). In three

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Female Parent 1</th>
<th>Female Parent 2</th>
<th>T x B</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B1</td>
<td>B2</td>
<td>B3</td>
<td>Mean</td>
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<tr>
<td>MH @ 400 ppm</td>
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<td>56.96</td>
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<tr>
<td>Ethrel @ 3000 ppm</td>
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<tr>
<td>2, 4-D @ 20 ppm</td>
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<tr>
<td>2, 4-D @ 30 ppm</td>
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<td>80.68</td>
<td>78.77</td>
<td>76.38</td>
</tr>
<tr>
<td>Mean B</td>
<td>60.46</td>
<td>65.07</td>
<td>64.21</td>
<td>63.25</td>
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</table>

Factors | S.E.m± | C.D (1%) | C.V |
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<tr>
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<tr>
<td>P x B</td>
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<tr>
<td>B x T</td>
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<tr>
<td>P x B x T</td>
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<td>7.54</td>
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</tbody>
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Table 2 Effect of male gametocides on inducing pollen sterility (%) studied under Acetocarmine test in okra (A. esculentus)
Table 3 Effect of gametocides on inducing pollen sterility (%) studied under *In vitro* pollen germination test in okra (*A. esculentus L.*)

<table>
<thead>
<tr>
<th>Treatment</th>
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<th>T x B</th>
<th>Mean</th>
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</thead>
<tbody>
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<td></td>
<td>B1</td>
<td>B2</td>
<td>B3</td>
<td>Mean</td>
</tr>
<tr>
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<td>66.94</td>
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<tr>
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<td>67.47</td>
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<tr>
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<td>78.27</td>
</tr>
<tr>
<td>2, 4-D @ 10 ppm</td>
<td>52.96</td>
<td>54.17</td>
<td>54.74</td>
<td>53.96</td>
</tr>
<tr>
<td>2, 4-D @ 20 ppm</td>
<td>64.64</td>
<td>75.56</td>
<td>70.37</td>
<td>70.19</td>
</tr>
<tr>
<td>2, 4-D @ 30 ppm</td>
<td>68.39</td>
<td>80.57</td>
<td>79.53</td>
<td>76.16</td>
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<tr>
<td>Distilled water</td>
<td>14.73</td>
<td>9.27</td>
<td>6.46</td>
<td>10.15</td>
</tr>
</tbody>
</table>
|Mean B              | 59.54| 64.41| 64.53| 62.83| 58.05| 64.22| 62.28| 61.52| 58.80| 64.31| 63.40 |-

Factors           | S.Em± | C.D (1%) | C.V  |
<table>
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<tr>
<td>Treatments (T)</td>
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<td>Bud size (B)</td>
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<td>1.39</td>
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<td>P x T</td>
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<tr>
<td>B x T</td>
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<tr>
<td>P x B x T</td>
<td>2.42</td>
<td>7.87</td>
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</tbody>
</table>

Legend of this table is similar to the table 2

way treatment combinations, buds of 45 mm size treated with MH @ 500 ppm recorded higher pollen sterility (91.18 %), which is not showing any significant different with 450 ppm (90.77 %) followed by ethrel @ 3000 ppm (78.33 %) and least was recorded in untreated control (6.61 %).

The results were confirmed from *in vitro* pollen germination test. Maximum pollen sterility was obtained in MH treatment (89.31 %) and this was followed by ethrel (69.05 %) and 2, 4-D (65.44 %). Lower pollen sterility was recorded in untreated control (10.19 %) and this was at par with distilled water (10.15 %).

Result of study revealed that increasing the concentration of gametocides increases the pollen sterility. Findings of this study are in agreement with the findings of Dubey & Singh (1967) and Deepak et al. (2007) who reported higher pollen sterility at higher concentration of MH.

The flower buds treated with MH (450 ppm) has shown higher pollen sterility (91.59 %) but this was not statistically different from the MH (500 ppm) (90.27 %). Further, among the different flower bud size, 45 mm recorded higher pollen sterility (64.31 %) which is at par with 50 mm bud size (63.40 %). In two way treatment combinations, buds of 45 mm size treated with MH @ 450 ppm recorded higher pollen sterility (93.40 %), followed by ethrel @ 3000 ppm (82.22 %) and least was recorded in distilled water spray (6.46 %).
Artificial induction of pollen sterility with the application of MH may cause abnormalities like shrivelling of microspores or premature disintegration of tapetum starved microspore which leads to death of the pollen (Deepak et al., 2007). Shrinkage of microspores perhaps associated with the shrinkage of cytoplasm when anther treated with MH in Capsicum annum, which might lead to pollen sterility (Chauhan, 1980). Malic hydrazide also induced maximum pollen sterility in several crops like okra (Dubey & Singh, 1967; Verma & Singh 1978), brinjal (Echlin, 1971; Sreenivasa, 2001), chilli (Chauhan, 1980) and egg plant (Swarnalatha, 2005). Being a pyridazine compound, MH is known as a clastogenic agent in plants and is known to inhibit the synthesis of nucleic acids and proteins. The application of MH in Vicia faba was reported to induce chromosome aberrations and sister chromatid exchanges in the root meristems (Rizal et al., 2015). Ethrel also induced higher level of pollen sterility when treated at bolting stage in lettuce (Han & Lee, 1972) and egg plant (Dikii & Anikeenko, 1975; Helal & El-Saied Zaki, 1981). In conclusion, it was evident that, MH (450 ppm) sprayed on 45 mm bud size stage found to be most ideal for inducing maximum pollen sterility in okra to skip emasculation process during hybrid seed production.

Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

References


Swarnalatha V (2005) Induction of male sterility and histological studies on induced male sterility in niger (Guizotia abyssinica

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http://www.jebas.org
Cass). M.Sc.(Agri.) Thesis submitted to the University of Agricultural Sciences, Dharwad (India).


ASSESSING THE SUITABILITY OF TURMERIC SEED RHIZOME SIZES ON BIOMETRIC AND QUALITATIVE TRAITS UNDER MID HILL CONDITIONS

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ABSTRACT

A field experiment was carried out with the objectives to evaluate and assess the suitability of Turmeric seed rhizome sizes under mid hill conditions of Arunachal Pradesh. The experiment consisted seven treatments of different grades of Turmeric rhizome var. Megha Turmeric-1. The results of study revealed that heavier the mother rhizome, gave better plant growth characteristics. Further it was reported that use of 50-60 g mother rhizome as a planting material resulted in maximum in case of all biometric characteristics with a plant height (121.33 cm), leaf size of length (62.79 cm), breadth (18.05 cm), number of leaves per plant (7.33), number of tillers per plant (4.24), and stem girth (2.20 cm) which were significantly higher as compared to the treatments with smaller rhizomes. As far as fresh and dried rhizome yields are concerned, mother rhizomes (50-60 g) yielded maximum fresh rhizome (24.58 t/ha) and dried rhizome yield (4.79 t/ha) which reduced considerably with small seed size. Curcumin and oleoresin, an important biochemical property in turmeric were also exhibited maximum in terms of percent and yield when mother rhizome was 50-60 g used as planting material and this amount is followed by mother rhizome (30-40 g).

KEYWORDS
Turmeric
Mother rhizome
Finger rhizome
Growth, Yield
Quality
Curcumin
Oleoresin

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1 Introduction

Turmeric (Curcuma longa L.) belonging to the family Zingiberaceae and cultivated extensively not only in India but also in other Southeast Asian countries. India is the largest producer, consumer and exporter (approximately 90%) of turmeric in the world (Anandaraj et al., 2014). It is one of the extensively used spices and colouring agents especially in the Indian subcontinent (Sarker & Nahar, 2007). Turmeric is prized and valued for its ability to impart brilliant yellow-gold colour to food due to the presence of secondary metabolite (yellow pigment) i.e. curcumin (Rakhunde et al., 1998) and is considered as an important factor in sensory and consumer acceptance of products (Wang et al., 2009). Besides being a spice crop, it has worldwide demand in cosmetic as well as in medicinal industry (Hossain et al., 2005). Its role as an antimalarial (Nandakumar et al., 2006), anti-inflammatory and antitumor (Gupta et al., 2012) has been well appreciated worldwide and it has also been known to modulate lipid metabolism, which has been implicated in obesity (Alappat & Awad, 2010). Normally turmeric is propagated through a small portion of rhizomes known as seed rhizome which gives the economic yield (Ravinathan et al., 2005). So it is obvious that the selection of right size planting material (length, weight and number of growing buds per seed) is an important factor for turmeric cultivation. Although the standard size of turmeric rhizome for planting is 20-30 g as per the scientific package of practices yet many researchers reported that planting larger turmeric seed rhizomes resulted in higher yield compared to smaller seed rhizomes (Randhawa & Mishra, 1974; Borget, 1993; Hossain et al., 2005). Further, Awasthy & Jessykutty (2017) also reported that a turmeric rhizome bit of approximate weight i.e. 7 g with 3 node recorded the highest sprouting percentage with good morphological characteristics. Therefore, in order to assess the suitability of the variety Megha Turmeric-1 seed rhizome sizes based on its important biometric characteristics and quality attributes, a preliminary investigation was carried out with the objectives to study the growth, yield and quality as influenced by different grades of turmeric seed rhizomes so as to identify the best rhizome size that could be used as planting material under mid hill condition of Arunachal Pradesh.

2 Materials and Methods

The present investigation was carried out at ICAR Research Farm, Gori, Basar, Arunachal Pradesh, India situated in the mid hill zone at the latitude of 27°59.537’ N and longitude of 94°41.269’ E with an altitude of 650 m above sea level during March 2015 to assess the suitable seed rhizome sizes with respect to its growth, yield and quality traits. The variety grown for the study was Megha Turmeric-1 which was developed from ICAR (Research Complex) for NEH Region, Umiam, Meghalaya through clonal selection having tolerant to leaf blotch and leaf spot with crop duration of 300-315 days. Cultivation practice was followed as per the recommended scientific package of practices. The field experiment was laid out in RBD (Randomised Block Design) with three replications having seven treatments viz., Mother rhizome (50-60 g), Mother rhizome (30-40 g), Mother rhizome (10-20 g), Finger rhizome (50-60 g), Finger rhizome (30-40 g), Finger rhizome (10-20 g), Finger rhizome (<10 g). In each plot, selected plants were tagged to record biometric observations on growth and yield attributes. For curcumin content measurement, finely ground turmeric powder samples (1 g) were extracted by refluxing over a water-cooled condenser with 100 ml of distilled alcohol (methanol) for 2 ½ hours. The extract was transferred to a 100 ml volumetric flask and volume was made to 100 ml with alcohol (methanol). It was then filtered and an aliquot of 2 ml was transferred to a 25 ml volumetric flask and made to 25 ml volume, mixed well and the absorbance of this solution was measured at 425 nm wavelength against blank made of alcohol (Manjunath et al., 1991). The analysis of curcumin was replicated thrice and the mean was taken for data analysis. Per cent oleoresin was also estimated as outlined by Sadasivam & Manickam, 2004 where ten grams of powder sample was taken in chromatographic column which was then eluted with 50 ml of acetone. The slurry was collected in a pre-weighed beaker and solvent was allowed to evaporate. The collected slurry was cooled and weighed. Difference in weight was calculated and then converted into per cent. Data recorded on different aspects of crop were tabulated and subjected to statistical analysis as outlined by Gomez & Gomez (1984). Significance difference between treatment means was tested through ‘F’ test and the critical difference (CD) was worked out wherever ‘F’ value was found to be significant for treatment effect. The results are presented at 5% level of significance (P=0.05).

3 Results and Discussion

3.1 Effect of different seed rhizome sizes on biometric characteristics of turmeric

In present study, uniform seedling emergence was observed irrespective of the size of seed rhizomes. The biometric characteristics of turmeric as influenced by different rhizome size are presented in (Table 1). The vegetative growth characteristics viz. plant height, leaf size, number of tillers, number of leaves and stem girth were recorded. Plants arising from 50-60 g mother rhizome were found healthier because larger rhizomes had larger buds and diameter. Among the different grades of rhizome used, the mother rhizome with 50-60 g recorded the highest plant height (121.33 cm), the reason being larger seed rhizomes contains larger amount of reserves that enhanced seedling growth, which ultimately resulted in a taller plant (Padmadevi et al., 2012).
Highest number of tillers (4.24) and leaves (7.33) per plant were also exhibited from the mother rhizome (50-60 g). The number of tillers and leaves increased as the seed size increased, because the plants from the larger seeds were longer and had a larger number of tillers. The shoot with a higher leaf number and larger leaf size received a higher solar energy for photosynthesis, which ultimately resulted in a larger shoot biomass. This result is in agreement with the report of Sarker et al. (2001). Meanwhile, the maximum stem girth (2.20 cm) and leaf area of length (62.79 cm) and breadth (18.05 cm) was also exhibited in mother rhizome of 50-60 g followed by mother rhizome of 30-40 g.

### 3.2 Effect of different seed rhizome sizes on yield of turmeric

The weight of mother rhizomes exhibited positive and significant correlation with rhizome yield. Plants from larger seeds of mother rhizome (50-60 g) had bigger shoot base and produced a higher number of daughter rhizomes, which ultimately increased the yield of turmeric. In this experiment, 50-60 g mother rhizome recorded the best performance in fresh and dried rhizome yield (Table 2) because of sufficient food reserves which probably encouraged vigorous plant growth that should have eventually transformed into yield (Padmadevi et al., 2012). Similar results

<table>
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<tr>
<th>Treatments</th>
<th>Plant height (cm)</th>
<th>Leaf length (cm)</th>
<th>Leaf breadth (cm)</th>
<th>Number of leaves</th>
<th>Number of tillers</th>
<th>Stem girth (cm)</th>
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<td>Mother rhizome (50-60 g)</td>
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<tr>
<td>Mother rhizome (30-40 g)</td>
<td>119.50</td>
<td>58.53</td>
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<tr>
<th>Treatments</th>
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<th>Curcumin content (%)</th>
<th>Oleoresin content (%)</th>
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<tr>
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<td>2.60</td>
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</table>
Suitability of turmeric seed rhizome under mid hill conditions were also reported by Kumar & Gill, (2010) who reported that increased rhizome yield in mother rhizome planting material might be attributed to better crop growth in terms of higher plant height, more number of leaves and tillers per plant which enabled more photosynthesis causing higher yields. Variation in sizes of rhizomes affects its performance as a sink (source-sink relationship). As far as the sink is concerned, mother rhizomes are better than fingers. Mother rhizomes are rich in nutrients due to better mobilization of the same. Thus, the mother rhizomes are considered as better planting material than finger rhizomes producing more vigorous plants ultimately increasing the yield. Similar findings were also recorded by Singh et al. (2000) and Alam et al. (2003).

3.3 Effect of different seed rhizome sizes on quality attributes of turmeric

The results presented in table 2 revealed that 50-60 g mother rhizome have highest curcumin (6.29 %) and oleoresin content (13.53 %) and this was followed by mother rhizome with 30-40 g and showed curcumin and oleoresin yield of 650.14 kg/ha and 297.16 kg/ha respectively as shown in Figure 1 which might be attributed to the bigger rhizome size which has higher reserved food which enhanced better vegetative growth leading to accumulation of more photosynthates in rhizomes and production of secondary metabolites like curcumin and oleoresin. The results obtained in the present study are in conformity with the findings of Kumar et al. (1992) who reported better quality attributes using mother rhizomes of turmeric in cultivar Duggirala.

Conclusion

From this experiment, it is apparent that heavier the turmeric rhizome, better the plant growth characteristics quantitatively and qualitatively. Therefore, it can be concluded that use of mother rhizome (50-60 g) of variety Megha Turmeric-1 as seed material gave the best performance in all the growth, yield and quality attributes making it a suitable rhizome size that could be used as planting material under mid hill condition of Arunachal Pradesh. However, besides the study conducted, there is need in future and scope to examine the effect of larger seed rhizome sizes (> 60 g) which might result in a different response both in terms of quantity and quality.

Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

References


ASSESSMENT OF GENETIC PURITY OF INTER-SPECIFIC F₁ HYBRIDS INVOLVING VIGNA RADIATA AND VIGNA UMBELLATA

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ABSTRACT

Genetic purity test of true hybrids from controlled crosses before further generations of selfing or crossing and selection is essential for Mungbean improvement. The present study was conducted to transfer mungbean yellow mosaic (MYM) disease resistance in mungbean from ricebean and to assess the genetic purity of developed inter-specific F₁ hybrids using morphological features and microsatellite markers. Inter-specific crosses were made involving two genotypes of mungbean viz., K 851 and TM 96-2 and one genotype of ricebean (RBL 1) where the crossability results revealed significant differences. Crossability was recorded 8.2% (TM 96-2 × RBL 1) and 4.6% (K 851 × RBL 1). Pollen fertility was recorded 1.6% and 3.4% in TM 96-2 × RBL 1 and K 851 × RBL 1, respectively. Morphological features such as epicotyl colour, hypocotyl length, petiole length, germination habit, etc., were used as indicators of true hybridity. Further, the microsatellite markers were used to confirm the genetic purity of the developed inter-specific hybrid. These hybrids exhibited resistance against mungbean yellow mosaic disease under natural epiphytotic field conditions. The present study will be useful in developing high yielding varieties or lines of mungbean coupled with stable MYMV resistance.

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Keywords
Crossability
Hybrid
Inter-specific
Ricebean
Mungbean
Yellow mosaic disease

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1 Introduction

Mungbean (Vigna radiata (L.) Wilczek) (2n=22), third in the series of important pulse crop, is an excellent source of easily digestible proteins. It has low flatulence which complements the staple rice and wheat diet in Asia (Selvi et al., 2006). Mungbean which is also known as green gram is one of the most important pulse crops of India grown in area of 3.83 m ha with 1.60 MT production and productivity 418 kg ha⁻¹ (Directorate of Economics and Statistics, 2016). Mungbean is a self-pollinated grain legume and occupies an important position as it possess high seed protein content (22-24%) as well as has the ability to restore the soil fertility by fixing atmospheric nitrogen through symbiotic relationship with Rhizobium in the root nodule of the crop (Malik, 1994; Bhanu et al., 2016). The reasons for low productivity of mungbean are biotic (Mungbean yellow mosaic virus, Cercospora leaf spot, powdery mildew) and abiotic (heat, drought and pre-harvest sprouting) stresses (Sahoo et al., 2002). Among the biotic stresses, the most prevalent problem contributing much to this yield reduction up to 85% is yellow mosaic disease (YMD) caused by Mungbean yellow mosaic virus (MYMV), a member of family Geminiviridae, has emerged into a great threat because of its severity and ability to cause high yield loss (Haq et al., 2010).

Limiting variability prevailing among the existing germplasm coupled with low harvest index also restricts in improving the productivity of mungbean. Extensive screening of the germplasm collections of mungbean has not yielded stable source of resistance to YMD. Use of in-vivo and in-vitro techniques to induce mutagenesis for the induction of resistance has also been not so effective (Javed et al., 2016). The resistance source for mungbean yellow mosaic India virus (MYMIV) has been reported in urdbean but the significance of resistance is indistinct (Anjum et al., 2010). However, in the recent times, new sources of resistance and molecular markers associated with MYMIV have been identified (Chen et al., 2012). Karthikeyan et al. (2011) proclaimed that even though urdbean can be used effectively as resistant source for introgressing MYMV resistance into mungbean, the resistance breaks down very often due to rapid evolution of new pathotype (Kumar, 2010). Thus, to diversify and broaden the genetic base of cultivated mungbean genotypes, there is a need to look for alien gene transfer from other Vigna species. Introgression of alien genes from cultivated /wild species would not only minimize the risks of biotic and abiotic stresses but will also make discernible yield advances and quality in the crop (Stalker, 1980; Kumar et al., 2011). Therefore, expeditious consideration on identification of sources resistance to biotic and abiotic stresses coupled with favourable agronomic traits is indispensable (Sehrawat et al., 2014).

Ricebean [Vigna umbellata (Thunb.) Ohwi & Ohashi], an underutilized crop possess many useful characteristics such as disease resistance, particularly to MYMV, Cercospora Leaf Spot, bacterial leaf spot and bruchids along with the highest potential grain yield among the Ceratotropis spp. (Sonta et al., 2006; Sehrawat & Yadav, 2014). However, ricebean holding immense potential is still considered as a scientifically underutilized crop which has not been subjected to systematic breeding. The plant breeding techniques such as inter-specific hybridization can be effectively used to transfer the useful characters of ricebean in other susceptible crops, consequently facilitating in developing improved varieties of food legumes for biotic stress-prone areas (Singh et al., 2013; Sehrawat & Yadav, 2014).

Genetic purity of parental variety and hybrids is of crucial importance, as one percent reduction in purity of hybrid seed, results in a reduction of about 100 kg ha⁻¹ in yield of commercial crop. In the last few years, several molecular markers have been recognized that can scrutinize complex traits into individual components. Marker assisted breeding is one of the significant molecular approaches which accelerates and aids conventional breeding (Ashraf & Foolad, 2013). Application of SSRs in V. radiata and V. umbellata includes determining the identification of hybrids, phylogeny and gene mapping establishing marker-trait association using segregating populations (Michelmore et al., 1991). Keeping above mentioned points into consideration, the present investigation was undertaken to introgress YMD resistance into mungbean from ricebean using inter-specific hybridization and to evaluate the genetic purity of developed inter-specific F₁ hybrids employing morphological features and microsatellite markers.

2 Materials and Methods

2.1 Plant material and hybridization of genotypes

Seeds of ricebean genotype namely, RBL 1 and two varieties of mungbean viz., K 851 and TM 96-2 were procured from Department of Genetics & Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. Seeds of parental lines were planted in crossing block in cemented pots during the Kharif, 2015. Hybridization was done using the method given by Boling et al. (1961). The stigma of the mungbean remains highly receptive during the early hours of the day. Consequently, emasculation was done between 04:00-06:00 pm and pollination in the subsequent morning between 06:00 and 08:00 am. Large number of flowers were pollinated for each cross, in order to obtain higher number of hybrid (crossed) pods. The pure seeds of two varieties of mungbean namely K 851 and TM 96-2 and one of ricebean i.e. RBL 1 and crossed seed (F₀) of two inter specific hybrids viz., K851 × RBL 1 and TM 96-2 × RBL 1 were raised, during Kharif, 2016-2017.
2.2 Morphological characterization

Observations were recorded on crossability, growth habit, plant type, leaf shape, leaf colour, length/ breadth ratio of primary leaf, petiole length (cm), flower colour, pod character, upper epicotyl colour, hypocotyl length, raceme, number of tubercles per inflorescence, keel tip colour, germination. 100-seed weight besides pollen fertility (%). Viability of the fresh pollen samples was determined on the basis of observations on stainability of fresh pollen grains by acetocarmine technique as described by Roberts (1977). Two hundred pollen grains were counted per slide with five slides each. Percent pollen fertility was calculated as (total number of stained pollen/total number of pollen) x 100. Normal deeply stained pollen grains were counted as viable, while weakly stained were recorded as non-viable (Pearson & Harney 1984).

2.3 Field evaluation for YMD resistance

Field trials for screening of YMD were done under natural conditions. One row of infector line CO-5 variety of urdbean was raised after every two test entries to evaluate MYMV symptoms. Plants were randomly selected and their leaves showing clear symptoms (veinal yellowing and scattered bright yellow spots) and total leaves were counted and percent disease incidence was calculated (Wheeler, 1969). Genotypes were scored on 0-5 arbitrary scale as Highly Resistant (HR), Resistant (R), Moderately Resistant (MR), Susceptible (S) and Highly Susceptible (HS) based on disease severity (Bashir et al., 2005; Akhtar et al., 2009).

2.4 Microsatellite Markers (SSRs) used in the study

For the molecular confirmation of hybrid purity the hybrids obtained in the present study, the adzukibean-specific simple sequence repeat markers (SSRs) CEDG035 and G 1034 showing polymorphism between the parental lines were utilized.

2.5 Genomic DNA extraction and PCR Amplification

Young leaves from 15-20 days old seedlings of all the F1 plants and their respective parents were collected. Total genomic DNA was extracted using the CTAB DNA extraction method (Doyle & Doyle, 1987). The quantification of the DNA was assayed on 0.8% agarose gel electrophoresis in 1X TAE. PCR reactions with SSR markers were performed in a 15 μl volume of PCR mix which consisted of 50 ng of template DNA and 10X Taq Buffer (with MgCl2) in a final concentration of 1X 1.0 μM of each forward and reverse primer (Sigma), 10 mM of dNTPs, and 0.03 units of Taq DNA polymerase (5U/μL). The PCR reaction was run on a thermocycler (Masterecycler eppendorf, USA). PCR amplification was done following the protocol consisting of initial denaturation at 94 °C for 30 s followed by 35 cycles 94 °C for 30s (denaturation), annealing at 55-60 °C for 1 min, and 72 °C for 30 s (elongation), followed by the final extension at 72 °C for 5 min and cooling at 4 °C for 10 min. The amplified products from SSR markers were resolved with 2.5% agarose gel electrophoresis. Ethidium bromide was used for staining the gels, which were subsequently visualized and photographed on a Gel Documentation System (GEL DOC TM XR*, BIORAD USA). Consequently, the DNA banding patterns of the PCR-amplified products of the derived inter-specific hybrid plants were compared with their respective male and female parents to confirm their true hybrid nature.

3 Results and Discussion

3.1 Crossability and morphological verification of hybrid purity

In present investigation, interspecific hybridization involving two genotypes of mungbean and one of ricebean have been effected to study the germination, as well as fertility behavior and attempt has been also made to assess the usefulness of testing the purity of the newly developed interspecific hybrids using morphological features and microsatellite markers.

The 30 crossed seed of each of the F1 and 10 seeds of each of the parents were sown in the pots (05 seed per pots) during Kharif, 2016. Out of 30 seeds only 15 F1 plants could survive in each of the two cross.

Considerable variation was found in interspecific crosses. The number of seeds per pod in F1 hybrid varied from 1 to 3. The crossability was recorded 8.2% and 4.6% in the interspecific cross, TM 96-2 × RBL 1 and K 851 × RBL 1 respectively. Varying degree of success in interspecific hybridization was reported in previous studies also (Chen et al., 1977; Ahn & Hartmann, 1977; Ahn & Hartmann, 1978) owing to reproductive obstructions between the species (Adinarayananmurti et al., 1993). Nevertheless, the present study revealed that all the selected male and female parents were cross-compatible with each other. Interspecific hybrids showed poor germination as only 50% crossed seeds germinated. These findings showed that V. radiata is crossable with V. umbellata and corroborate with earlier findings on hybridity among different Vigna species (Pandiyan et al., 2010; Singh et al., 2013; Sehrawat & Yadav, 2014).

The hybrid seedlings initially showed poor growth. However, after seedling stage vigorous growth of the hybrid plants was observed. The inviability or weakness of the F1 seedlings could be due to disharmonies between genomes of the parental species; between genomes of one species and cytoplasm of the other or between genotypes of F1 zygote and genotype of endosperm or maternal tissue (Cooper & Brink, 1940; Stebbins 1958; Gill & Waines, 1978, Monika et al., 2001).
Assessment of genetic purity in *Vigna radiata* and *Vigna umbellata*

The morphological characterization of F₁ hybrids and the parental genotype are depicted in Table 1. The germination habit of F₁ seedlings was intermediate between the parental species. The position of cotyledon of the hybrid seedlings were at the soil surface, and the average length of hypocotyl was 2.5 cm. The indeterminate growth character of *V. umbellata* was dominant in the F₁ plants. Change in growth habit was exhibited in the hybrids. The hybrids showed indeterminate vegetative growth which was in contrast to the determinate growth of the female parent. The growth habit of hybrid plant was intermediate, semi erect and compact type and characterized by a thicker stem with good branching (Figure 1).

Pollen fertility was observed 1.6% and 3.4% in TM 96-2 × RBL 1 and K 851 × RBL 1, respectively. The unstained pollen grains were variable in size, while the stained pollen grains were usually larger than the parental pollen. The pollen fertility was lower in F₁ as compared to parents (Figure 2). The reduced fertility has been ascribed due to pairing abnormality and the formation of the anaphase bridges and laggards leading to the unequal distribution of the chromosome by various workers (De & Krishnan, 1967; Biswas & Dana, 1975; Anandabaskaran & Rangasamy, 1996; Kaur & Satija, 1998; Gupta et al., 2002).

Pseudo-pod formation took place which later degenerated. This may be due to embryo disharmony. However, wherever fertile pods formed, a small increase in seed size was observed over the female parent. Large seed size may provide yield advantage and other yield-related attributes.

Evaluation of the morphological features of hybrid plants from germination to maturity assists in testing the genetic purity of hybrids (Dongre et al., 2010). Consequently, inheritance of biparental morphological characteristics by the inter-specific hybrids confirmed their genetic purity.

3.2 Molecular confirmation of hybrid purity

The SSR marker G 1034 primer pair produced a specific but reproducible band of 100 bp in the male parent and 200 bp in both parents. Likewise, the adzukibean-specific SSR CEDG 035 amplified a reproducible band of one male (165 bp) and female (146 bp) specific repeats. Consequently, all the F₁ inter-specific hybrids produced both bands specific to their respective parents (Figure 3 and 4). The complementary banding pattern of the male and female parents makes a way to identify the hybrid (Sudharani et al., 2014).

### Table 1 Comparison among *V. radiata*, *V. umbellata* and their hybrid

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Character</th>
<th><em>V. radiata</em></th>
<th><em>V. umbellata</em></th>
<th>Hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Growth habit</td>
<td>Determinate</td>
<td>Indeterminate</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>2</td>
<td>Plant type</td>
<td>Erect and compact</td>
<td>Semi-erect and semi-compact</td>
<td>Semi-erect and semi-compact</td>
</tr>
<tr>
<td>3</td>
<td>Leaf shape</td>
<td>Ovate</td>
<td>Lanceolate</td>
<td>Lanceolate</td>
</tr>
<tr>
<td>4</td>
<td>Leaf colour</td>
<td>Dark green</td>
<td>Light green</td>
<td>Light green</td>
</tr>
<tr>
<td>5</td>
<td>Length / breadth ratio of primary leaf (cm)</td>
<td>6.15/5.8 = 1.06</td>
<td>5.4/1.4 = 3.85</td>
<td>3.16/1.11 = 2.84</td>
</tr>
<tr>
<td>6</td>
<td>Length of petiole (cm)</td>
<td>12.2</td>
<td>6.5</td>
<td>9.6</td>
</tr>
<tr>
<td>7</td>
<td>Flower colour</td>
<td>Light yellow</td>
<td>Dark yellow</td>
<td>Dark yellow</td>
</tr>
<tr>
<td>8</td>
<td>Pod</td>
<td>Hairy</td>
<td>Non-hairy</td>
<td>Hairy</td>
</tr>
<tr>
<td>9</td>
<td>Pollen fertility</td>
<td>&gt;95%</td>
<td>&gt;95%</td>
<td>1.6-3.4%</td>
</tr>
<tr>
<td>10</td>
<td>Upper epicotyl colour</td>
<td>Green</td>
<td>Purple</td>
<td>Light Purple</td>
</tr>
<tr>
<td>11</td>
<td>Hypocotyl length (cm)</td>
<td>6.5</td>
<td>0.0</td>
<td>2.5</td>
</tr>
<tr>
<td>12</td>
<td>Raceme</td>
<td>Often compound</td>
<td>Simple</td>
<td>Simple</td>
</tr>
<tr>
<td>13</td>
<td>Number of tubercles per inflorescence</td>
<td>5-7</td>
<td>12-16</td>
<td>8-10</td>
</tr>
<tr>
<td>14</td>
<td>Keel tip colour</td>
<td>Grayish</td>
<td>Bright yellow</td>
<td>Light yellow</td>
</tr>
<tr>
<td>15</td>
<td>Germination</td>
<td>Epigeal</td>
<td>Hypogeal</td>
<td>Intermediate</td>
</tr>
<tr>
<td>16</td>
<td>100-seed weight</td>
<td>3.5 g</td>
<td>5.6 g</td>
<td>4.3 g</td>
</tr>
</tbody>
</table>

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Figure 1 Comparison of F₁ hybrid with parents

Figure 2 Comparison of pollen of F₁ hybrid with parents

Figure 4 Confirmation of the purity of the hybrids using adzukibeanspecific microsatellite G 1034 marker
Assessment of genetic purity in *Vigna radiata* and *Vigna umbellata*

The higher degree of resemblance between the male parent and offspring as well as their male specific band marker imparts a clear indication that the offspring is successful crop and true hybrid of *V. radiata × V. umbellata*. Consequently, these SSR markers were highly expedient for the identification of the true hybrid during the study and can be used unambiguously as referral markers for the identification of hybrids. The amplification of microsatellite marker across related legumes will upsurge their efficacy in breeding program (Dikshit et al., 2012).

3.3 Field evaluation of hybrids for YMD resistance

In the present investigation, screening of genotypes of mungbean and ricebean showed that ricebean genotype, RBL 1 was highly resistant. However, the mungbean genotype viz., TM 96-2 and K 851 were moderately resistant and highly susceptible respectively. During Kharif 2016, three interspecific crosses along with the parents were screened for MYM disease symptoms under natural epiphytotic field conditions. The results showed that all the hybrids and the ricebean genotype (RBL 1) were highly resistant to MYM disease, while the mungbean genotype viz., K851 was highly susceptible. Present study corroborates earlier findings of Pandiyam et al. (2010) and Sehrawat & Yadav (2014). Further evaluation of segregating generations of these hybrids will help in determining the inheritance of resistance and lead to development of stable and improved variety of mungbean.

Conclusions

Exploitation of novel genes and alleles from exotic germplasm is needed which can be a major source of robust donors for both biotic and abiotic resistance. The present study concludes that ricebean genotypes exhibits a high level of resistance against YMD which can be utilized as donor parent in inter-specific hybridization programme to develop resistant varieties in other vulnerable *Vigna* species coupled with high yield potential. The morphological characterization and the SSR-PCR based molecular verification efficiently proved the genetic purity of the inter-specific hybrids. Morphological features such as epicotyls, colour, petiole length and germination habit are a good indicator of true hybridity. Adzukibean derived microsatellite markers can aid in genetic improvement of mungbean or other *Vigna* species by means of genomic studies for tagging and mapping agronomically important traits using marker-assisted breeding for desired traits. To overcome the limitations of the narrow genetic base of mungbean crop, conventional breeding approaches accompanied with biotechnological techniques will prove to be valuable. Last but not the least, F2 populations developed from the inter-specific hybridization can be very much useful in mapping population or developing recombinant inbred lines for the identification of gene/ quantitative trait loci for MYM disease resistance.

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Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

References

Adinarayananurty VV, Rao MVB, Satyanarayana A, Subramanyam D (1993) The crossability of *V. mungo* and *V.


Cooper DC, Brink RA (1940) Somatoplastic sterility as a cause of seed failure after interspecific hybridization. Genetics 25: 593.


Directorate of Economics and Statistics (DES - 2016) Available on http://eands.dacnet.nic.in/ access on 25.06.2017


Assessment of genetic purity in *Vigna radiata* and *Vigna umbellata*


ALLELOPATHIC PROPENSITY OF THE AQUEOUS LEAF EXTRACT AND LEAF LITTER OF *Melia dubia* CAV. ON PULSE CROPS

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ABSTRACT

Gas Chromatography Mass-Spectrometry (GC-MS) analysis revealed that leaf litter of *Melia dubia* contain phenolic acids and its derivatives, unsaturated fatty acid, alkaloids, methyl ketones (volatile allelochemical), aromatic ketone, chromene etc. Further it was reported that the aqueous leaf extracts (0, 25, 50, 75 and 100% concentration) and leaf litter (0, 5, 10, 15 and 20 g/pot) inhibited the germination, growth (shoot length, shoot length and vigour index) and initial biomass (shoot, root and total biomass) of green gram and black chickpea. Percentage of inhibition in germination and initial growth parameters increased with the increasing the concentration of aqueous extract or litter amount of *M. dubia*. However, pot experiments, carried out till crop maturity, revealed that there was no significant allelopathic effect on growth, biomass and grain yield of the test crops. This indicates that the allelochemicals present in *M. dubia* leaf litter are volatile in nature and their effect is transient in nature.

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1 Introduction

*Melia dubia* Cav. (Syn. *Melia composita* Willd.) is being planted in agroforestry system either in block plantation or along the farm boundary. Commonly known as Malabar neem/ Burma neem, is an industrially and economically important fast growing tree species, which can be harvested on a short rotation (Chauhan & Ritu 2005; Chavan et al., 2015). It is indigenous to the Western Ghats of Southern India and is common in moist deciduous forests of Kerala and outside India, it is found in Sri Lanka, Malaysia, Java, China and Australia (Saravanan et al., 2013). It is valued for its high-quality termite and fungus resistant timber for furniture, agricultural implements and house construction (Suprapti et al., 2004), as alternative pulp wood species, fuel wood and leaf used as a fodder (Parthiban et al., 2009) and has medicinal properties as well (Vijayan et al., 2004). The industrial and ecological importance of *M. dubia* has encouraged the farmers to take large scale plantations with different intercrops (Parthiban et al., 2009; Nuthan et al., 2009).

In some of the woody species, in agroforestry systems, allelopathic effects on under storey crops have been reported (Gupta et al., 2007; Narwal et al., 2011; Gunarathne & Perera, 2016). Hence to answer such queries of farmers who are interested to integrate this valuable species allelopathic investigations are to be done.

Thereby, keeping in view the importance and increasing popularity of this species, the present investigation was undertaken to investigate the allelochemicals in leaf litter of *M. dubia* to divulge the beneficial or antagonistic effect of aqueous leaf extract and leaf litter on germination, growth, biomass and yield of pulse crops in laboratory bioassay and pot culture.

2 Materials and methods

The present investigations were accomplished in the agroforestry laboratory as well as in the greenhouse complex of College of Forestry, Acharya agricultural University, Navsari, Gujarat, India (20.95° N latitude, 75.90° E longitude with an altitude of 10 m above MSL) during November 2014 to April 2015.

2.1 Leaf litter chemical analysis

The alleged allelopathic compounds in leaf litter samples of *M. dubia*, used in the present study, were detected through Gas Chromatography-Mass Spectrometry (GC-MS) as described by Murugesan et al. (2013).

2.2 Allelopathic studies

2.2.1 Plant material and preparation of aqueous extracts

The leaf litter (mixture of young and mature leaves showing signs of senescence) of *M. dubia* were collected from 3 year old plantations during October-November 2014. Leaf litter was initially dried at room temperature and later at 65°C in hot air oven until constant dry weight was reached (Perez-Corona et al., 2013). The dried leaf litter was stored at room temperature and was used for both petriplate and pot experiments bioassay.

Aqueous extracts were prepared by soaking 200g of grounded dried leaf litter in 1L distilled water. The solution was stirred and kept at room temperature (20-25°C) for 24 hours. The filtrate was centrifuged and supernatant was decanted (Prasad et al., 2011). The filtrate was defined as 100 per cent extract and was further diluted with distilled water at 25, 50, 75, 100 per cent concentrations (Nikneshan et al., 2011) while distilled water was used as control T1 (0 %) (Lawan et al., 2011).

2.3 Petridish bioassay experiment

The seeds (treated with Thirum @ 2g/kg) of green gram [*Vigna radiata* (L.)] and black chickpea (*Cicer arietinum* L.) were procured from Pulses and Castor Research Unit, Navsari Agricultural University, Navsari, Gujarat, India. In the laboratory experiment, five treatments of leaf aqueous extracts (0 to 100% concentration) of donor species were used with five replications for each. Each petridish of size 90 mm diameter was considered as replication. Total 50 seeds of both the test crops were placed on filter paper in sterilized petridishes and 5 ml of aqueous extract was applied on first day and after that, 2 ml was applied at alternate day to keep the filter paper moist till the completion of experiment (Bhat et al., 2011). Seeds were considered germinated upon radicle emergence. Daily germination count was made up to 9th day from day after extract treatment (DAET). Growth and biomass attributes were recorded by randomly selecting 10 seedlings from each replication on 11th DAET. Germination percentage and Germination Rate Index (GRI) were calculated following standard procedure (Anonymous 1983; Anonymous 1985). The shoot, root length and biomass were estimated randomly selecting ten seedlings from each replication on the 11th DAET. Root and shoot portion was dried separately in hot air oven at 60°C for 48 h and then samples were weighed using sensitive balance to estimate biomass.

2.4 Pot experiment

Pot experiments were conducted to investigate the effect of leaf litter of *M. dubia* on germination indices, initial growth and biomass of both the test crops. Leaf litter (course grounded mixture of young and mature leaves showing signs of senescence)
was used in five concentrations viz., T1 (Control), T2 (5 g), T3 (10 g), T4 (15 g) and T5 (20 g), these concentration were mixed in the upper soil layer of the pots of concern treatment (Thakur 2014). Soil without leaf litter was used as a control treatment. Each treatment was replicated five times. The litter treatments imposed were according to annual average litter fall (Li et al., 2013), where leaf litter fall of three months data was recorded by placing the 1 m² traps under 3 years old plantation of M. dubia and average leaf litter fall of three months (216.45 g/m²) was considered and extrapolated for pot with top area of 0.026 m² i.e. 5.63 g/pot. Hence, the leaf litter treatments were fixed within the range of average litter fall. Total 50 seeds of each crop were sown in the plastic pots [18 cm diameter × 16 cm height (4070 cc)] containing approximately 2.5 kg soil having N, P and K content of 84.82, 17.85 and 80.35 ppm, respectively. Pots were irrigated with tap water (pH 7.71 and electrical conductivity 1.752dSm/m) and seeds were sown after 24 hours. Daily germination count was made up to the last seed to germinate i.e., 8 days. Shoot and root length, and biomass were recorded on the 11th day randomly selecting 10 seedlings from each replication. Shoot and root samples were dried in hot air oven at 60°C for 48 h and then dry biomass was recorded. Germination percentage and Germination Rate Index (GRI) were calculated as per standard procedure followed in the laboratory bioassay.

2.5 Pot experiments (up to crop maturity)

In order to evaluate the allelopathic effect of leaf litter till crop maturity a separate experiment was laid out by following same procedure as adopted in pot experiment to study the germination, initial growth and biomass. Each litter treatment was replicated five times (three plants per replication). However, in each pot, only five seeds were sown and one seedling was retained after two weeks of sowing for further observations. The pots were kept in green house with 50 per cent relative shading. Growth parameters such as plant height, root length, no. of leaves, no. of branches, no. of flower, no. of pods per plant and average leaf area per plant was recorded. Furthermore, fresh and dry biomass of plant and grain yield was also recorded at crop maturity, when 80 percent of pods matured (3 months after sowing). Pods were separated from the plants and threshed to record the grains.

2.4 Statistical analysis

The experimental data of all the parameters studied in different experiments were subjected to the statistical analysis following completely randomized design (CRD) and F-test was done and ANOVA was constructed following Sheron et al. (1998). Treatment means were compared at P<0.05. Further, Duncan’s multiple range test (MRT) was used to compare the sets of means of each treatment.

3 Results and discussion

3.1 Leaf litter Phytochemicals

Gas Chromatography Mass-Spectrometry (GC-MS) screening of M. dubia leaf litter revealed the presence of 18 different types of phytochemicals (Table 1). Among the detected compounds most

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Compound name</th>
<th>Retention time</th>
<th>Area under Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ethanal 1 (2-amino)</td>
<td>5.47</td>
<td>131541</td>
</tr>
<tr>
<td>2</td>
<td>6 (3-hydroxy-4-methoxyphenyl) 3,5,7 Trithoxy 4-H-chroman-4-one</td>
<td>6.79</td>
<td>114900</td>
</tr>
<tr>
<td>3</td>
<td>Papaveroline 6-O-methyl Or 1,2-Benzenediol,4-(7-hydroxy-6-methoxy-1-isoquinolinyl) methyl</td>
<td>9.66</td>
<td>408430</td>
</tr>
<tr>
<td>4</td>
<td>5-methyl (5-8 dihydro 1-4 Naphthoquinone) or 1,4-aphthalenedione, 5,8 dihydro-5-methyl-</td>
<td>9.70</td>
<td>203565</td>
</tr>
<tr>
<td>5</td>
<td>2-propanone, 1,1-diehtoxy- or 1,1-diehtoxypropan-2-one</td>
<td>9.77</td>
<td>95117</td>
</tr>
<tr>
<td>6</td>
<td>2,3,4,5-tetrahydro-1-benzoepine</td>
<td>10.57</td>
<td>415469</td>
</tr>
<tr>
<td>7</td>
<td>1,5 Anhydro-3,6-di-O-acetyl, 2,4 di-O-methyl D-glucitol or D-Glucitol, 1,5-anhydro-2,4-di-O-methyl-, diacetate</td>
<td>11.30</td>
<td>696171</td>
</tr>
<tr>
<td>8</td>
<td>4-Piperidinol, 1-(2-phenoxymethyl)-4-phenyl</td>
<td>12.99</td>
<td>55688</td>
</tr>
<tr>
<td>9</td>
<td>2,4-Dimethyl-5,6-dithia-2,7-nonadienal, 5-oxide</td>
<td>14.79</td>
<td>348465</td>
</tr>
<tr>
<td>10</td>
<td>2-Methyl-3,5-dodecadiyne</td>
<td>15.14</td>
<td>608896</td>
</tr>
<tr>
<td>11</td>
<td>1,4 diethan-2-one-3-phenyl</td>
<td>15.39</td>
<td>417663</td>
</tr>
<tr>
<td>12</td>
<td>1,3-Dioxolane-4-methanol, 2-pentadecyl-, acetate, trans-</td>
<td>15.56</td>
<td>23571</td>
</tr>
<tr>
<td>13</td>
<td>2, methyl-2 phenyl-5 (1-4, dihydro-pyridine-4-yidene)-1,3-dioxan-4-6 dione</td>
<td>15.64</td>
<td>52239</td>
</tr>
<tr>
<td>14</td>
<td>Methyl 4-6 tetradecadiynoate</td>
<td>16.24</td>
<td>115370</td>
</tr>
<tr>
<td>15</td>
<td>1H-Purine-2,6-dione, 8-(1,2-dibromo-2-phenylethyl)-3,7-dihydro-1,3,7-trimethyl</td>
<td>16.95</td>
<td>347653</td>
</tr>
<tr>
<td>16</td>
<td>3,9 Epoxyregnane-11,14,18 triol-20-one 16 cyan-3methoxy, 11 acetate</td>
<td>20.74</td>
<td>280670</td>
</tr>
<tr>
<td>17</td>
<td>Eicosapentaenoic Acid or Icosapent</td>
<td>21.83</td>
<td>98308</td>
</tr>
<tr>
<td>18</td>
<td>Oxazole, 5-ethyl-2-methyl-4-benzoyl-</td>
<td>26.87</td>
<td>352502</td>
</tr>
</tbody>
</table>
Allelopathic propensity of *Melia dubia*

common are phenolic acids and their derivatives, omega-3 fatty acid, alkaloids, methyl ketones (volatile allelochemical), unsaturated fatty acids, aromatic ketone and chromene. Chromatograms showing the relative abundance, retention time and area under curve of chemical compounds detected and are presented in figure 1. The phytochemicals detected in this study through GC-MS have also been reported by Valentina et al. (2013) and Murugesan et al. (2013) in *M. dubia* extract. Further, the phytochemicals like 3, 4-Dihydroxyacetophenone, 4-hydroxybenzoic acid, Piperidinol, Tetradecanoic acid, 2,4-Dimethyl-5,6-dithia-2,7-nonadienal, 5-oxide, Icosapentaenoic acid, Naphthoquinone, Purine, D-Glucitol etc., detected in leaf

![Figure 1 GC-MS chromatogram showing retention time and peaks of different chemical compounds in *Melia dubia* leaf litter](image-url)
litter of *M. dubia* in this study, have been reported in other woody and non woody species and are alleged for their inhibitory allelopathic effect (extract or leaf litter) on germination and growth of various test crops (Suzuki et al., 1996; Duke et al., 2000; Kim & Kil 2001; Rezaeinodehi et al., 2006; Peneva 2007; Hongying & Hong 2008; Kato-Naguchi 2008; Koder 2011; Ruan et al., 2011; Jones et al., 2012; Aslani et al., 2014). However, this study has first report of allelopathic nature of leaf litter of *M. dubia*.

3.2 Laboratory and pot culture bioassays: Germination and its attributes

Laboratory and pot culture bioassays revealed that, aqueous leaf extract and leaf litter of *M. dubia* significantly (P<0.05) inhibited the germination (%) and germination rate index (GRI) of green gram and black chickpea (Table 2) relative to control (distilled water or no litter). The inhibitory effect gradually increased with incremental extract concentrations or leaf litter amount, over the control (Figure 2 A to D).

![Figure 2](image_url)

Figure 2 Showing the allelopathic influence of aqueous leaf extracts [0, (distilled water), 25, 50, 75 and 100%] and leaf litter [0 (no leaf litter), 5, 10, 15 and 20 g/pot] of *M. dubia* on germination and initial growth of green gram and black chickpea in laboratory (a and b) and pot culture bioassays (c and d), respectively.
The magnitude of per cent inhibition, over control, in all the germination parameters of green gram and black chickpea (Figure 3 to 6) increased with increase in extract concentration and leaf litter quantities with greatest at maximum concentration (100%) or litter amount (20 g/pot). The per cent reduction on germinations attributes was higher in laboratory bioassays as compared to pot culture.

3.3 Initial growth and biomass

The leaf aqueous extract under laboratory and leaf litter in pot culture, exhibited significant (P<0.05) inhibitory effect on growth parameters viz., shoot and root length of germinated seedling of green gram and black chickpea (Table 2 and 3). The data indicates that, growth parameters had gradual inhibitory effect as the extract concentration or litter quantity increased when compared with the control treatment. The magnitude of per cent inhibition in growth parameters of green gram (Figure 3 & 5) and black chickpea (Figure 4 & 6) over control, against aqueous extract and leaf litter, gradually increased with increase in extract concentration or litter amount with maximum at 100% extract concentration or maximum litter application i.e. 20 g litter/pot. The per cent reduction was more marked in root growth as compared to shoot except in black chickpea, where shoot length experienced little higher percent reduction, in laboratory bioassay. The reduction percentage was more in laboratory bioassay as compared to pot experiments.

The leaf extracts as well as leaf litter exhibited significant inhibitory effect on shoot, root and total dry biomass of germinated seedlings (Table 2 and 3). The magnitude of diminution progressed gradually with increase in leachate concentration or litter application over the control with maximum at 100% extract concentration and 20 g leaf litter/pot.

Intensity of per cent inhibition, over control, in growth traits of green gram and black chickpea (Figure 3 to 6), increased with increase in extract concentration or leaf litter quantities of *M. dubia*. All the biomass attributes experienced greater magnitude of reduction due to aqueous extracts compared to litter application in pots, over control treatments.

The magnitude of inhibition on germination indices, initial growth and biomass of seedlings increased with incremental aqueous extract concentration and leaf litter quantity. This showed concentration dependent effect of
aqueous extract and leaf litter. The findings are in congruence with earlier laboratory bioassays of *M. azedarach* on pulse crops (Phuwiwat et al., 2012; Akacha et al., 2013). This may be attributed to water soluble nature of allelochemicals (Table 4) in leaf litter (Rezaeinodehi et al., 2006). Petridish bioassay and pot culture experiments revealed that percent depression effect was more pronounced on root growth in laboratory bioassay as well as in pot experiments. Similar organ specific effects of *M. azedarach* leaf aqueous extracts have been reported earlier (Lungu et al., 2011; Phuwiwat et al., 2012; Akacha et al., 2013). This may be attributed to the fact that roots first come in contact with allelochemicals and are the first to absorb them from the environment in which they are growing and cell death and tissue browning frequently occur in the root apical zone, an area with active cell division (Rezaeinodehi et al., 2006; Ding et al., 2007).

Similar to present findings, it has been reported that young seedlings are more sensitive to allelopathicals compared to adult plants or other plant organs, especially the roots (Wu et al., 2007; Zhang et al., 2010).

![Figure 6 Per cent inhibition (over control) in germination, growth and biomass of black chickpea against leaf litter of *M. dubia*](image_url)

Table 2 Effect of aqueous leaf extract of *M. dubia* on germination traits, initial growth and biomass in bioassay culture

<table>
<thead>
<tr>
<th>Extract concentration (%)</th>
<th>Germination traits</th>
<th>Growth (cm)</th>
<th>Biomass (DM mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G (%)</td>
<td>GRI</td>
<td>Shoot length</td>
</tr>
<tr>
<td>Green gram</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (0%)</td>
<td>97.60 (77.17)*</td>
<td>91.36*</td>
<td>7.31*</td>
</tr>
<tr>
<td>25%</td>
<td>87.60 (69.29)b</td>
<td>63.16b</td>
<td>6.15b</td>
</tr>
<tr>
<td>50%</td>
<td>82.00 (65.04)c</td>
<td>51.86c</td>
<td>5.75c</td>
</tr>
<tr>
<td>75%</td>
<td>77.20 (60.95)d</td>
<td>47.14d</td>
<td>4.71d</td>
</tr>
<tr>
<td>100%</td>
<td>69.20 (55.19)e</td>
<td>42.19e</td>
<td>3.72e</td>
</tr>
<tr>
<td>CD (P ≤0.05)</td>
<td>2.99</td>
<td>2.45</td>
<td>0.20</td>
</tr>
<tr>
<td>SEM (±)</td>
<td>1.01</td>
<td>0.83</td>
<td>0.07</td>
</tr>
</tbody>
</table>

| Black chickpea            |                    |             |             |             |       |      |       |
| Control (0%)              | 97.20 (80.47)*     | 37.95*      | 3.40*        | 4.00*       | 23.33*| 21.25*| 44.80*|
| 25%                       | 80.80 (64.02)b     | 30.91b      | 2.50b        | 3.10b       | 21.07b| 19.25b| 40.32b|
| 50%                       | 63.60 (52.89)c     | 25.01c      | 2.10c        | 2.50c       | 18.60c| 17.00c| 35.60c|
| 75%                       | 60.00 (50.82)d     | 19.50d      | 1.70d        | 2.10d       | 16.01d| 14.63d| 30.63d|
| 100%                      | 48.00 (43.83)e     | 14.96e      | 1.40e        | 1.90e       | 13.08e| 11.95e| 25.03e|
| CD (P ≤0.05)              | 4.10               | 1.49        | 0.12         | 0.12        | 1.29 | 1.05 | 2.48  |
| SEM (±)                   | 1.38               | 0.50        | 0.04         | 0.04        | 0.44 | 0.35 | 0.84  |

*Figures in parenthesis are the transformed values; G=Germination; GRI=Germination Rate Index; MDG=Mean Daily Germination; DM=Dry Matter; CD=Critical difference; SEM=Standard error of mean, Letter different in same vertical column are significantly different according to Duncan’s multiple range test (P ≤ 0.05).
This study is the first to report the allelopathic nature of *M. dubia* leaf extract and leaf litter. Similar compounds have been detected in *M. azedarach*, member of same family, alleged for allelopathic influence on germination and initial growth and biomass of various crops (Mulatu et al., 2011; Shapla et al., 2011). The aqueous leaf extracts of plant species may hamper physiological processes of germinating seeds and growing seedlings. Such physiological hindrances might have resulted due to allelochemicals of *M. dubia* in present investigations. Phuwiwat et al. (2012) observed that water uptake and α-amylase activity of *Echinochloa crusgalli* was inhibited by aqueous extracts of young leaves (12.5 to 100 mg/mL) of *M. azedarach* and water soluble allelochemicals caused inhibition of both water uptake and α-amylase activity during germination process as compared to control. Metabolism activation within seed occurs in adequate moisture (Chong et al., 2002), which may be restricted in germinating seeds due to inhibition of specific enzymes. Germination inhibition could be the result of induction of oxidative stress (Javed, 2011).

All these findings may be ascribed to the inhibitory effect of *M. dubia* aqueous extracts on seed germination of green gram and black chickpea in the present study. Earlier studies showed that addition of leachates or incorporation of plant residues into the growth environment of another plant can result in inhibition effect on germination and growth due to depletion of the nitrogen content and impeding of the physiological processes of the seedlings growing in such environment (Al-Khatib et al., 1997). Similar effects might have resulted in reduced germination, growth and biomass of black gram against leaf mulch of *M. dubia* as compared to control in the present study. Akacha et al. (2013) reported that *M. azedarach* allelo-chemicals produced an imbalance in the oxidative status of cells and these allelochemicals made changes in activity of catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX) as well as in the levels of $\text{H}_2\text{O}_2$ and assimilatory pigments.

Allelochemicals have been alleged to decrease the stomatal conductance due to induced ABA production, which indirectly impact the photosynthesis, transpiration, respiration rates and

---

### Table 3 Effect of leaf litter of *M. dubia* on germination traits, initial growth and biomass of green gram and black chickpea in pot culture

<table>
<thead>
<tr>
<th>Leaf litter (g/pot)</th>
<th>Germination traits</th>
<th>Growth (cm)</th>
<th>Biomass (DM mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G (%)</td>
<td>GRI</td>
<td>Shoot length</td>
</tr>
<tr>
<td>No litter</td>
<td>94.80 (77.07)*</td>
<td>24.24*</td>
<td>15.50*</td>
</tr>
<tr>
<td>5 g</td>
<td>86.20 (68.18)*</td>
<td>23.44*</td>
<td>14.40*</td>
</tr>
<tr>
<td>10 g</td>
<td>78.20 (62.15)*</td>
<td>20.82*</td>
<td>13.60*</td>
</tr>
<tr>
<td>15 g</td>
<td>70.40 (57.03)*</td>
<td>18.05*</td>
<td>12.90*</td>
</tr>
<tr>
<td>20 g</td>
<td>62.20 (52.05)*</td>
<td>16.37*</td>
<td>12.30*</td>
</tr>
<tr>
<td>CD (P ≤0.05)</td>
<td>2.61</td>
<td>0.71</td>
<td>0.43</td>
</tr>
<tr>
<td>SEm (±)</td>
<td>0.88</td>
<td>0.24</td>
<td>0.14</td>
</tr>
</tbody>
</table>

*Figures in parenthesis are the transformed values; G=Germination; GRI=Germination Rate Index; MDG=Mean Daily Germination; DM=Dry Matter; CD= Critical difference; SEm= Standard error of mean, Letter different in same vertical column are significantly different according to Duncan’s multiple range test (P ≤0.05).
uncoupling oxidative phosphorylation (Yu et al., 2003; Bagavathy & Xavier, 2007). Multiple physiological effects, such as reduction in plant growth, absorption of water and mineral nutrients, ion uptake, leaf water potential, shoot turgor pressure, physiological drought and osmotic potential have been attributed to reduction in germination, growth and biomass of seedlings both in laboratory bioassay and pot experiments (Barkosky & Einhellig 2003; Rezaeinodehi et al., 2006).

3.4 Allelopathic effect of leaf litter on growth, biomass and grain yield at harvesting

The data on growth, biomass and grain yield (3 months after sowing) attained by green gram and black chickpea (Table 4) expressed that there was no significant effect of leaf litter of *M. dubia* applied @ 0, 5, 10, 15 and 20 g/pot, on growth, biomass and grain yield both the test crops.

Despite validation of allelochemicals in *M. dubia* through GC-MS analysis, the leaf mulch treatments did not exhibit inhibitory or stimulatory effect on later stage of growth of green gram and black chickpea in the present study. In contrary to present findings, Shapla et al. (2011) reported that *M. azedarach*, sister species of *M. dubia*, mulch application @ 20 gm/pot inhibited the growth (shoot and root length, number of leaves) and biomass (shoot, root and total fresh and dry) of mung bean and soybean. Similar adverse effects of leaf mulch of fruit and timber tree species on other pulse and cereal crops have also been reported (Sale & Oyun 2013; Thakur, 2014). Studies on pot culture carried out by Divya et al. (2004) and Hossain et al. (2002) are also divergent to the present findings. These studies have reported inhibitory effect of leaf litter application only up to a month or so. However, in this study, results of growth, biomass and yield are reported till maturity of the test crops.

This may be attributed to faster mulch decomposition, leaching out of allelo-chemicals due to frequent irrigation done to maintain the moisture in the pots, ephemeral nature of allelo-chemicals, loss from soil through volatilization, especially phenolics (Ampofo 2009; Narwal et al. 2011). Management practices like frequent watering may have resulted in faster decomposition of leaf mulch of *M. dubia*, hence did not exhibited any significant inhibitory effect on growth, yield and dry matter production of pulse crops in present study. The mulch used in the present study was crushed and reduced in size before application, which might have resulted in quick decomposition, thus, alleviating the allelochemicals. These evidences may be attributed to non-significant effect of mulch treatments of *M. dubia* on growth, biomass and grain yield of test crops in the present study.

Laboratory bioassay and pot culture studies divulged that, the leaf litter of *M. dubia* contain different types of phytotoxic chemicals, as evident from the GC-MS analysis, with putative inhibitive potential on seed germination, initial growth and biomass of green

### Table 4 Effect of leaf litter of *M. dubia* on growth, biomass and grain yield (3 MAS) of green gram and black chickpea in pot culture

<table>
<thead>
<tr>
<th>Leaf litter (g/pot)</th>
<th>Plant Height (cm)</th>
<th>Collar diameter (mm)</th>
<th>Root length (cm)</th>
<th>Grain yield (g/plant)</th>
<th>Biomass (DM g/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green Gram</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No litter</td>
<td>54.43</td>
<td>2.62</td>
<td>21.40</td>
<td>2.20</td>
<td>7.69</td>
</tr>
<tr>
<td>5 g</td>
<td>48.94</td>
<td>2.71</td>
<td>20.90</td>
<td>2.23</td>
<td>7.11</td>
</tr>
<tr>
<td>10 g</td>
<td>49.93</td>
<td>2.70</td>
<td>22.93</td>
<td>2.18</td>
<td>7.76</td>
</tr>
<tr>
<td>15 g</td>
<td>50.94</td>
<td>2.38</td>
<td>21.91</td>
<td>2.50</td>
<td>7.70</td>
</tr>
<tr>
<td>20 g</td>
<td>49.52</td>
<td>2.52</td>
<td>20.35</td>
<td>2.01</td>
<td>6.30</td>
</tr>
<tr>
<td>CD (P ≤0.05)</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>SEM (±)</td>
<td>2.50</td>
<td>0.19</td>
<td>2.64</td>
<td>0.20</td>
<td>1.08</td>
</tr>
<tr>
<td>Black chickpea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No litter</td>
<td>35.25</td>
<td>2.83</td>
<td>6.78</td>
<td>*</td>
<td>4.72</td>
</tr>
<tr>
<td>5 g</td>
<td>33.43</td>
<td>2.80</td>
<td>7.02</td>
<td>*</td>
<td>4.33</td>
</tr>
<tr>
<td>10 g</td>
<td>34.33</td>
<td>3.23</td>
<td>7.19</td>
<td>*</td>
<td>4.68</td>
</tr>
<tr>
<td>15 g</td>
<td>32.01</td>
<td>2.78</td>
<td>6.03</td>
<td>*</td>
<td>4.36</td>
</tr>
<tr>
<td>20 g</td>
<td>34.74</td>
<td>2.82</td>
<td>7.03</td>
<td>*</td>
<td>4.61</td>
</tr>
<tr>
<td>CD (P ≤0.05)</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>*</td>
<td>N.S.</td>
</tr>
<tr>
<td>SEM (±)</td>
<td>1.98</td>
<td>0.29</td>
<td>0.42</td>
<td>*</td>
<td>0.24</td>
</tr>
</tbody>
</table>

MAS= Months after sowing; DM=Dry Matter; *Grain formation did not occur; CD= Critical difference; SEM= Standard error of mean.
gram and black chickpea. However, pot culture studies, revealed that there was no significant allelopathic effect on later growth, biomass and grain yield of both the test crops. The second investigation brought out that allelochemicals in leaf litter of *M. dubia* are of ephemeral nature and their effect got alleviate over of time.

**Conflict of Interest**

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

**References**


Allelopathic propensity of *Melia dubia*


EFFECT OF POTASSIUM NITRATE ON SEED QUALITY ENHANCEMENT IN DIFFERENT AGED SEEDS OF BOTTLE GOURD
[Lagenaria siceraria (Molina) Standl]

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ABSTRACT

In the present investigation, three bottle gourd varieties i.e., AB-1, Pusa Santusti and Pusa Navin were subjected to seven different osmopriming chemical treatments of KNO₃ at three storage periods. The data obtained from various observations were analyzed by using Factorial Completely Randomized Design (FCRD). The better seed quality was recorded when the seeds stored for 3 months under ambient condition. Among the studied varieties Pusa Navin stored for 3 months and treated with 150 ppm KNO₃ recorded significantly highest seed germination as well as other seed quality parameters. The results depicted that the seed quality of bottle gourd was significantly influenced by varieties, storage periods and osmopriming treatments.

KEYWORDS
Bottle gourd
Seed
Priming
Germination
KNO₃

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1 Introduction

Bottle gourd [Lagenaria siceraria (Molina) Standl] is an important cucurbitaceous vegetable crop grown for its fleshy fruits in tropical and subtropical regions. It is cultivated both in kharif and summer season in western part of the country; whereas in tropical regions it is cultivated round the year under mild temperature conditions (Desai et al., 2016). Germination percentages of several vegetable species have been shown to increase after seed treatment with chemicals and various osmotica (Malik et al., 2001). In many seeds crops, particularly vegetables and small seeded grasses, seed priming has been successfully used to improve germination and emergence (Gharahlar et al., 2009). There are several methods of seed priming which includes hydropromining, halopromining, osmopromining and nano priming. The priming with nitrate solutions stimulates the germination which might be due to nitric oxide (NO) synthesis (Lara et al., 2014).

Storing and preserving the quality seed stock till the next season is equally important as producing quality seeds but some time seeds lose their viability during storage period condition. The seed deterioration during storage leads to various changes apart from quantities losses viz., change or shift in metabolic activity, changes in composition, decrease or change in enzymatic activities, morphological, cellular changes. Since the seeds have the capacity to absorb water the viability and vigour of the seeds are regulated by various physicochemical factors, initial seed quality, package materials etc (Arvindkumar et al., 2014).

Poor seedling emergence and lower seedling vigor cause poor establishment in crop stand. For this different seed treatment practices have been adopted for different crops. Post-harvest seed enhancement treatments improve germination and seedling vigor. Seed pre-soaking causes hydration of membrane proteins and initiation of several metabolic processes and re-drying of seeds arrest the process (Maiti et al., 2011).

In light of the above facts, the present research work was conducted in order to study the effect of potassium nitrate on different stored seeds of Bottle gourd.

2 Materials and Methods

The experiment was carried out at the Department of Seed Science and Technology, B. A. College of Agriculture, Anand Agricultural University, Anand, Gujarat, India during 2013-14 and 2014-15 with three varieties of bottle gourd i.e., AB-1 (V1), Pusa Santusti (V2) and Pusa Navin (V3). The experiment was conducted for three different stored seeds viz 3, 6 and 9 months (P1, P2, and P3). The seeds were exposed to seven different duration treatments i.e. Control (T1), 100 ppm KNO₃ for 12 hrs (T2), 100 ppm KNO₃ for 18 hrs (T3), 150 ppm KNO₃ for 12 hrs (T4), 150 ppm KNO₃ for 18 hrs (T5), 200 ppm KNO₃ for 12 hrs (T6) and 200 ppm KNO₃ for 18 hrs (T7) and kept for germination by using between the paper method as per ISTA procedure. Ten normal seedlings were randomly selected from each replication and observations were recorded for germination percentage, root length (cm), shoot length (cm), dry weight of seedling (gm) and vigor index. The data obtained from various observations were analyzed by using Factorial Completely Randomized Design (FCRD). The method used in the present study was indigenously developed by Department of Statistics, B. A. College of Agriculture, Anand Agriculture University, Anand.

3 Results and Discussion

The duration of germination process varies according to the type of seed and the local environmental conditions to which the seed is exposed (Kevin et al., 2015). The seed quality of bottle gourd was significantly influenced by variety, storage period and priming treatments. Three months of storage in ambient condition recorded highest seed quality. Significantly higher germination percentage as well as other seed quality parameters was recorded for the same storage period. It was observed that as storage period increased, there was concomitant reduction in germination percentage and other seed quality parameter. Potassium nitrate has been used extensively in the seed testing laboratories for many years. It has been reported that seed priming with KNO₃ showed enhancement in seed germination, seedling emergence, vigour index in different vegetable crops (Nath & Dekha, 2015). The priming treatments involving different concentrations and durations with KNO₃ was found to influence the germination potential as well as other quality parameters of bottle gourd. Among the priming treatments the bottle gourd seeds primed with 150 ppm for 12 hrs recorded significantly higher germination percentage as well as other seed quality parameters viz., root length, seedling fresh weight, seedling dry weight and vigor index I & II. Pusa Navin was recorded highest seed germination of 82.51% and this was followed by AB-1 (80.45%) and Pusa Santusti (79.11%) but these three treatments are not significantly different. The other parameters viz., root length, shoot length, seedling fresh weight, seedling dry weight, vigor index I and vigor index II also followed similar trend (Table 1). Pusa Navin stored for 3 months and primed with 150 ppm KNO₃ for 12 hrs recorded significantly higher germination percentage (99.77%). The similar treatment combination (V3×P1×T3) recorded significantly higher vigour index I (3472) and other parameters (Table 2). The result were in accordance with the work of Farooq et al., (2007) where they reported that osmopriming with KNO₃ not only improved
germination of seeds but also improved the seedling emergence rate and early seedling growth. It was reported by Abnavi & Ghobadi (2012) that the superiority in speed of germination of KNO₃ priming was related to more nitrogen and potassium accumulation in seeds. The increase in the germination may be due to the higher activity of α-amylase during osmopriming. Seed priming with KNO₃ might have resulted in enhancement of nutrient supply (K⁺ and NO₃⁻) toward the developing seedling that results in higher weight. Also, Tian (2009) reported an increase in the germination of red clover seeds by treating the seeds with 0.2% KNO₃ solution soaking for 6 or 12 hour.

In light of the present investigation it can be concluded that pre treating bottle gourd seeds with 150 ppm KNO₃ for 12 hrs can be beneficial for seed quality enhancement at different storage intervals and slowdown the seed quality deterioration process.

### Table: 1 Seed quality parameters as influenced by potassium nitrate treatments in different aged seeds of three varieties of Bottle Gourd (pooled for two years)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Germination (%)</th>
<th>Root length</th>
<th>Shoot length</th>
<th>Seedling length</th>
<th>Seedling fresh weight</th>
<th>Seedling Dry weight</th>
<th>Vigour index I</th>
<th>Vigour index II</th>
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<td>V1</td>
<td>80.45*</td>
<td>9.81*</td>
<td>13.76*</td>
<td>23.10*</td>
<td>9.15*</td>
<td>0.36*</td>
<td>1889.1*</td>
<td>30.5</td>
</tr>
<tr>
<td>V2</td>
<td>79.11*</td>
<td>9.29*</td>
<td>13.39*</td>
<td>23.18*</td>
<td>8.76*</td>
<td>0.33*</td>
<td>1895.3*</td>
<td>30.6</td>
</tr>
<tr>
<td>V3</td>
<td>82.51*</td>
<td>11.33*</td>
<td>14.92*</td>
<td>26.30*</td>
<td>9.62*</td>
<td>0.39*</td>
<td>2201.2*</td>
<td>33.8</td>
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<td>CD₀.₀⁵</td>
<td>0.80</td>
<td>0.59</td>
<td>0.77</td>
<td>0.43</td>
<td>0.64</td>
<td>0.01</td>
<td>72.17</td>
<td>NS</td>
</tr>
<tr>
<td>S.Em.</td>
<td>0.206</td>
<td>0.151</td>
<td>0.196</td>
<td>0.111</td>
<td>0.163</td>
<td>0.005</td>
<td>18.383</td>
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<td>87.59*</td>
<td>12.04*</td>
<td>16.00*</td>
<td>28.05*</td>
<td>11.25*</td>
<td>0.48*</td>
<td>2470.2*</td>
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<td>P2</td>
<td>81.99*</td>
<td>10.10*</td>
<td>14.41*</td>
<td>24.52*</td>
<td>9.02*</td>
<td>0.33*</td>
<td>2024.3*</td>
<td>29.5*</td>
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<tr>
<td>P3</td>
<td>72.48*</td>
<td>8.33*</td>
<td>11.67*</td>
<td>20.01*</td>
<td>7.26*</td>
<td>0.27*</td>
<td>1491.2*</td>
<td>20.8*</td>
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<tr>
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<td>2.12</td>
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<td>2.35</td>
<td>2.42</td>
<td>1.09</td>
<td>0.03</td>
<td>277.93</td>
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<tr>
<td>S.Em.</td>
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<td>0.227</td>
<td>0.599</td>
<td>0.617</td>
<td>0.279</td>
<td>0.010</td>
<td>70.798</td>
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<td>6.91*</td>
<td>11.72*</td>
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<td>6.87*</td>
<td>0.22*</td>
<td>1182*</td>
<td>14.4*</td>
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<tr>
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<td>15.88*</td>
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<td>9.89*</td>
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<td>2225.1*</td>
<td>36.6*</td>
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<tr>
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<td>81.67*</td>
<td>10.24*</td>
<td>14.09*</td>
<td>24.34*</td>
<td>9.30*</td>
<td>0.36*</td>
<td>1997.8*</td>
<td>30.8*</td>
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<td>T6</td>
<td>76.74*</td>
<td>9.61*</td>
<td>13.56*</td>
<td>23.17*</td>
<td>8.61*</td>
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<td>1792.3*</td>
<td>25.3*</td>
</tr>
<tr>
<td>T7</td>
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<td>8.90*</td>
<td>12.99*</td>
<td>21.90*</td>
<td>7.78*</td>
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<td>0.224</td>
<td>0.203</td>
<td>0.282</td>
<td>0.002</td>
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</tr>
</tbody>
</table>

*, P<0.05 (Significant at 5% level)
Effect of potassium nitrate on seed quality enhancement in Bottle gourd

Table 2 Seed quality parameters as influenced by different treatments in three varieties of Bottle Gourd (pooled for two years)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Germination (%)</th>
<th>Root length</th>
<th>Shoot length</th>
<th>Seedling length</th>
<th>Seedling fresh weight</th>
<th>Seedling Dry weight</th>
<th>Vigour index I</th>
<th>Vigour index II</th>
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<td>8.*</td>
<td>14.3*</td>
<td>22.6*</td>
<td>8.3*</td>
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<td>1584*</td>
<td>22*</td>
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<td>V₁×P₁×T₂</td>
<td>96.4*</td>
<td>13.7*</td>
<td>16.7*</td>
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<td>0.587*</td>
<td>2861*</td>
<td>57*</td>
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<td>V₁×P₁×T₃</td>
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<td>13.1*</td>
<td>0.671*</td>
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<td>Treatment</td>
<td>Germination (%)</td>
<td>Root length</td>
<td>Shoot length</td>
<td>Seedling length</td>
<td>Seedling fresh weight</td>
<td>Seedling Dry weight</td>
<td>Vigour index I</td>
<td>Vigour index II</td>
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<td>-----------------</td>
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Effect of potassium nitrate on seed quality enhancement in Bottle gourd

Acknowledgement:

The authors’ are thankful to Department of Agricultural Statistics, B. A. College of Agriculture, Anand Agricultural University, Anand for Statistical analysis of the data.

References


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*, P<0.05 (Significant at 5% level)
IN VITRO REGENERATION AND TRANSFORMATION OF APPLE
(Malus domestica Borkh.) ROOTSTOCK MALLING 7 USING
RICE CHITINASE GENE

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ABSTRACT

In most of the fruit crops, the low frequency of Agrobacterium mediated genetic transformation hampers the respective transgenics production. Hence, it is pre-requisite to understand and optimize the different parameters involved directly or indirectly in Agrobacterium mediated transformation. A. tumefaciens strain LBA4404 harboring the transforming vector pCAMBARchi11 containing the rice chitinase gene (chi11) and hygromycin phosphotransferase (hpt) and phosphinothricin acetyl transferase (bar) genes was used for carrying out genetic transformation of apple (Malus x domestica Borkh.) rootstock M7. Two explants viz. leaf and internodal segments were involved in regeneration and resulted 24.37% and 60.58% shoot regeneration, respectively. The obtained putative transformants were selected on full strength MS medium containing 5 mg l⁻¹ hygromycin and 500 mg l⁻¹ cefotaxime. Two days pre-culturing and 96 hours co-cultivation was found effective for procuring maximum number of hygromycin resistant shoots. Putative transgenic shoots were multiplied separately and rooted on root induction medium containing 5 mg l⁻¹ hygromycin. Further, PCR analysis was done using chitinase gene specific primers and two apple transgenic lines confirmed the integration of chi11 gene by yielding 237 bp and 584 bp amplified products, respectively.

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1 Introduction

The domesticated apple is one of the most important fruit crops of the colder and temperate parts of the world. It belongs to the family Rosaceae, subfamily Maloideae, and the farmed apple tree with a pomaceous fruit is known as *Malus x domestica* Borkhausen (Harris et al., 2002). The long juvenile period, high levels of heterozygosity, long hybrid evaluation time and the process of releasing new cultivars are the critical factors which slow down the pace of various conventional apple breeding programmes. So there is an urgent need to adopt modern biotechnological tools which could help to accelerate this process.

Plant genetic transformation using desirable genes has geared up and facilitated various apple crop improvement activities by targeting resistance to fungal or bacterial diseases, improved fruit quality, or root stocks with better rooting or dwarfing ability (Igarashi et al., 2016). Among fruit crops, domesticated apple attains the distinctiveness because of the availability of a wide range of clonal rootstocks, thereby permitting the development of a ‘designer tree size’ appropriate for high density plantations. The inherent characteristics of these rootstocks especially disease resistance, growth and flowering habits etc. has remarkable impression on scion varieties. In Indian conditions, rootstock like Malling 7 (M7) is recommended as it is virus-free, and has proven itself to be one of the most popular rootstocks in the commercial industry, mostly due to its capability of producing a semi-dwarf tree i.e. approximately 50-60% of standard size. It is tolerant to collar rot, a major soil-borne disease of apple. While, it shows susceptibility to a serious soil borne disease ‘white root rot’ caused by the fungus *Dematophora necatrix* Hartig.

There are number of limiting factors which restricts various plant disease management practices to some extent. Plant genetic transformation has emerged as a powerful and attractive strategy for the genetic improvement of fruit trees constrained by their reproductive biology and high levels of heterozygosity. This strategy has emerged as a promising tool and addressed various problems while opening new avenues for crop modification (Rao et al., 2009). *Agrobacterium*-mediated transformation is widely used gene transfer method for introducing foreign genes into dicotyledonous plants. In this process, type of *Agrobacterium* strain, disarmed binary vector and explant (leaf, hypocotyl or callus cultures) are the limiting factors for an efficient transformation. Reliable and highly efficient regeneration system is pre-requisite base for the development of successful transformation protocol in any crop. Hence, these biotechnological based strategies can be considered as an important supplement along with existing technologies to speed up various fruit crop improvement programmes.

Innate defense mechanisms assist plants to respond towards various biotic stresses. Several researchers have demonstrated that many defense-related (DR) proteins, such as pathogenesis-related proteins (PR) and anti-microbial peptides (AMP) have been identified in different plants during biotic stress. Chitinases are the enzymes that rupture chitin i.e. the primary component of cell walls of several types of fungi and exoskeletons of invertebrates by hydrolyzing the β-1,4-linkage among N-acetylglucosamine units (Datta et al., 1999).

For enhancing disease resistance in apple, a chitinase gene from different sources was introduced for the first time against apple scab causal organism (Wong et al., 1999; Bolar et al., 2000). An efficient plant regeneration system along with gene transfer and selection system guarantees the genetic advancement of apple through transgenic production. However, low regeneration frequency as compared to those of herbaceous species keeps genetic transformation studies in apple lacked behind. Therefore, considering such kind of key limiting factors, we aimed at developing a simple and reliable genetic transformation protocol using antifungal chitinase gene in commercially important apple rootstock M7 rootstock.

2 Materials and Methods

2.1 Explants used

4-5 weeks old *in vitro* proliferating cultures (raised from axillary buds) of apple rootstock M7 were used for procuring two explants i.e. leaves and internodal segments.

2.2 Shoot regeneration experiments

The selected explants i.e. leaves and internodal segments were excised, wounded and then inoculated on solid shoot regeneration medium (RM) containing MS salts and vitamins (Murashige & Skoog, 1962) fortified with varying concentrations (mg l⁻¹) of cytokinins as mentioned in Tables 1 and 2. Further, regenerated shoots were multiplied on shoot multiplication medium (MM) i.e. MS salts and vitamins supplemented with benzyl adenine (BA) 0.5 mg l⁻¹, gibberellic acid (GA₃) 0.5 mg l⁻¹, indole-3-butyric acid (IBA) 0.1 mg l⁻¹ for multiplication. 2.0-3.0 cm long regenerants were cut and induced to root by dipping in half strength MS liquid medium containing 0.5 mg l⁻¹ thiamine, 20 g l⁻¹ sucrose and 0.3 mg l⁻¹ IBA. These were incubated in dark for initial first week and then transferred to basal solidified medium and then kept in light. For hardening, rooted shoots were washed gently. After four weeks, well established plantlets were transferred to earthen pots containing sand, soil and FYM (1:1:1 w/w). The plants were acclimatized by gradually exposing to natural conditions.
Table 1 Effect of growth regulators on leaf explants for callus induction and adventitious shoot regeneration

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<td>-</td>
</tr>
<tr>
<td>35</td>
<td>RM35</td>
<td>-</td>
<td>0.8</td>
<td>-</td>
</tr>
<tr>
<td>36</td>
<td>RM36</td>
<td>-</td>
<td>1.0</td>
<td>-</td>
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</table>

\( \text{CD}_{\text{95}} \) 3.36 (2.34) * 8.40 (0.65) **

\( \text{SE (m)} \) 1.18 (0.83)* 2.97 (0.23)**

(Values in parentheses are - *angular and **square root transformed values)
In vitro regeneration and transformation of apple by using rice chitinase gene

2.3 Genetic transformation experiments

The chitinase gene construct harboring plasmid pCAMBIA bar-ubi-chi II (13.8 kb, Figure 1) with chitinase (chi-II, 1.1 kb) gene driven by ubiquitin-I (Ubi-I) promoter and two selectable marker genes i.e., hygromycin phosphotransferase (hpt) & phosphinothricin acetyltransferase (bar) under the control of CaMV35S was obtained from Dr S. Muthukrishnan, KSU, USA. Further, plasmid DNA was transferred into A. tumefaciens strain LBA4404 via triparental mating by Sharma et al. (2012) for carrying out co-cultivation experiments.

Table 2 Effect of growth regulators on internodal explants for callus induction and adventitious shoot regeneration

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Medium code</th>
<th>Plant growth regulators (mg L⁻¹)</th>
<th>Frequency of callus induction (%)</th>
<th>Frequency of shoot regeneration (%)</th>
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<tr>
<td></td>
<td>TDZ</td>
<td>NAA</td>
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</tr>
<tr>
<td>1</td>
<td>RM1</td>
<td>0.2</td>
<td>0.5</td>
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<td>17.83 (24.96)</td>
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<td>RM4</td>
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<td>15.33 (23.04)</td>
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<td>RM5</td>
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<td>0.5</td>
<td>12.50 (20.68)</td>
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<td>RM6</td>
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<td>1.0</td>
<td>40.50 (39.50)</td>
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<td>0.4</td>
<td>1.0</td>
<td>37.75 (37.89)</td>
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<td>8</td>
<td>RM8</td>
<td>0.6</td>
<td>1.0</td>
<td>31.50 (34.12)</td>
</tr>
<tr>
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<td>RM10</td>
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<td>21.33 (27.47)</td>
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</table>

CDₐ₀₆₅ 2.67 (1.80)* 2.33 (1.52)*
SE (m) 0.89 (0.60)* 0.78 (0.51)*

(Values in parentheses are angular transformed values)

Figure 1 Map of pCAMBIA bar-ubi-chi I transforming vector
2.4 Strain maintenance

The *A. tumefaciens* strain LBA4404 was regularly maintained on YMB (Yeast extract 1.0 g l⁻¹, Mannitol 10.0 g l⁻¹, Dipotassium phosphate 0.5 g l⁻¹, Magnesium sulphate 0.2 g l⁻¹, Sodium chloride 0.1 g l⁻¹, Calcium carbonate 1.0 g l⁻¹, Final pH 7.0) medium containing filter sterilized streptomycin (25 mg l⁻¹) and kanamycin (50 mg l⁻¹) (Hi-Media) respectively. To achieve proper growth, incubation at 28°C under dark conditions for 3-4 days and further storage at low temperature (4 ± 2°C) was followed.

2.5 Bacterial suspension preparation

For co-cultivation, fresh cultures of *Agrobacterium* strain LBA4404 were prepared in 25 ml liquid YMB medium containing 50 mg l⁻¹ kanamycin (filter sterilized) and incubated overnight at 28 °C in an orbital shaker at 200 rpm. Bacterial suspension was centrifuged at 6,000 rpm for 5 minutes and the pellet was resuspended in MS basal liquid medium to a O.D. = 0.520 at 540 nm pertaining to 5x10⁶ cells per ml.

2.6 Antibiotics used

Two antibiotics i.e. hygromycin for transformants selection and cefotaxime for controlling *A. tumefaciens* growth were used. 5mg l⁻¹ Hygromycin and 500 mg l⁻¹ cefotaxime were added to pre-sterilized molten MS regeneration medium (RM) fortified with 0.8 mg l⁻¹ TDZ and 1.0 mg l⁻¹ NAA by filter sterilization through 0.22µm pore size membrane filter to carry out the respective transformation experiments.

2.7 Pre-culturing and co-cultivation experiments

Both types of explants were pre-cultured for 2 days in light prior to infection on RM and then further, co-cultivated on selective RM supplemented with 5mg l⁻¹ Hygromycin and 500 mg l⁻¹ cefotaxime. The pre-cultured explants were immersed in bacterial suspension for 15 minutes with gentle pricking and stirring for infection with *Agrobacterium*. Explants were further blotted on sterilized filter paper to remove the excess of bacteria and placed on basal MS medium after *Agrobacterium* infection. The cultures were co-cultivated/incubated for 96 hours in dark and observations were recorded on the basis of morphological evidences i.e. *Agrobacterium* growth and the average number of calli or shoots formed from survived explants after four weeks, respectively. Both types of explants were transferred to selective RM medium containing 500 mg l⁻¹ cefotaxime to eliminate the bacteria and 5 mg l⁻¹ hygromycin to select the putative transformed shoots following 96 hours (dark) of co-cultivation. Explants were washed with sterile distilled water containing 250 mg l⁻¹ cefotaxime, blotted on sterilized filter paper and then transferred on fresh selective RM to check the excessive bacterial growth. The performance of putative transformed cells or shoots was compared with control on selective medium as well as on non-selective medium. Depending upon the growth of *Agrobacterium*, the concentration of cefotaxime was decreased gradually from 500 to 250 mg l⁻¹. 26 putative regenerants of length (≥ 0.5 cm) were separated off from the explant and multiplied on selective MM supplemented with 5 mg l⁻¹ hygromycin and 250 mg l⁻¹ cefotaxime. Of these, only 18 selected putative shoots (about 0.5 - 1.0 cm) were rooted and hardened.

2.8 Molecular level confirmation

Genomic DNA from leaves of 2 untransformed control shoots and 18 hygromycin resistant shoots were isolated following CTAB method and further quantitative and qualitative analysis was done using UV spectrophotometer (Bio-Rad). Two sets of specific primers for the amplification of rice endochitinase class I (*chil1*) gene were designed with their product size 237 bp (forward primer- GGACGCAGTCCTCCTTAGA, reverse primer- AGTGCAGTAGCGCTTGA) and 584 bp (forward primer- GCTTCTACACTACGACGCCT, reverse primer- GTAGCGTGTAGAAACCGCAG), respectively to check the integration of T-DNA in the genome of putative transformants. PCR conditions were standardized for these primers by using isolated DNA along with two PCR controls i.e, positive control (plasmid DNA) and negative control (reaction mix with water except template DNA) in a thermal cycler (Eppendorf). 25 µl PCR cocktail consisted of 3U/reaction Taq DNA polymerase, 10x Taq DNA polymerase buffer, 1.5 mM MgCl₂, 10 pmol/reaction each specific chitinase primers (forward and reverse), 2.5 mM each dNTPs (deoxynucleotide triphosphate) and 50 ng/reaction template DNA respectively. Amplified PCR products were visualized and photographed under UV light using alpha imager® EC gel documentation system (Biosis) after electrophoresis on a 1.2 % (w/v) agarose gel containing 0.5 µg ml⁻¹ ethidium bromide.

2.9 Data analysis

The statistical analysis based on mean values per treatment was made using ANOVA technique for completely randomized design (CRD).

3 Results

3.1 Adventitious shoot regeneration studies

Instead of various other auxin and cytokinin combinations, adventitious shoots emerged only in TDZ and NAA combinations in case of both the explants (leaf and internodal segments). In case of leaf explants, from 16 different combinations of BA and NAA/IAA used, only RM7 and RM16 combinations produced adventitious shoots with a frequency of 4.16% and 1.38% respectively (Table 1). Likewise, out of 20 different combinations of TDZ and NAA/IAA, an average frequency of adventitious
In vitro regeneration and transformation of apple by using rice chitinase gene

Shoot regeneration of 24.37%, 18.05% and 9.71% was achieved in RM25 (0.8 mg l\(^{-1}\) TDZ and 1.0 mg l\(^{-1}\) NAA), RM20 (0.8 mg l\(^{-1}\) TDZ and 0.5 mg l\(^{-1}\) NAA) and RM19 (0.6 mg l\(^{-1}\) TDZ and 0.5 mg l\(^{-1}\) NAA) respectively (Table 1). On the other hand, TDZ and IAA combinations failed to produce adventitious shoots. Only RM25 (0.8 mg l\(^{-1}\) TDZ and 1.0 mg l\(^{-1}\) NAA) showed both direct and indirect organogenesis (Figure 2). While assessing the frequency of adventitious shoot regeneration, internodal segments were found better than the leaf explants. The most significant treatment was 0.8 mg l\(^{-1}\) TDZ and 0.5 mg l\(^{-1}\) NAA with a maximum of 60.58% regeneration in case of internodal segments (Table 2, Figure 3). Although both leaf and internodal explants showed good regeneration rates with TDZ and NAA combinations but later showed relatively higher regeneration rate. Regenerated shoots were propagated on shoot multiplication medium (MM). About 70-75% rooting was noted in the regenerants with 50-60% hardening rate.

3.2 Effect of antibiotics

Optimization of antibiotics dosage is compulsory as it determines the basic pre-requisites like normal regeneration of putative transgenic shoots and controlled Agrobacterium growth. Cefotaxime @ 500 mg l\(^{-1}\) controlled the excessive Agrobacterium growth effectively after co-cultivation along with less regeneration frequency. While lower concentrations of cefotaxime i.e. 200-300 mg l\(^{-1}\) triggered the regeneration. On the other hand, 5 mg l\(^{-1}\) hygromycin proved effective for the selection of putative transformants after co-cultivation. In this study, data with respect to antibiotics standardization have not shown.

3.3 Genetic transformation experiments

Following 15 minutes infection of explants with Agrobacterium cell suspension (5x10\(^{8}\) cells/ml) with gentle stirring, co-cultivation for 96 hours in all the precultured leaf and internodal explants (Tables 3 & 4) had a strong impact on the regeneration frequency i.e. 1.75 & 7.66 as well as the number of putative transformed shoots i.e. 1.33 & 3.33, respectively. A total of 26 putative transgenic shoots from both the explants i.e. leaves and internodes (Figure 4) were obtained and maintained separately on selective (5 mg l\(^{-1}\) hygromycin and 250 mg l\(^{-1}\) cefotaxime) MM. Upon subculturing, the non-transformed tissues or the escapes shoots, which became necrotic or white, were removed. The putative transformed shoots revealed a steady growth pattern as compared to control. In present study, leaf explants predominantly generated
Table 3 Effect of pre-culturing and co-cultivation durations on regeneration of leaf explants

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Pre-culturin (Days)</th>
<th>Co-cultivation (Hours)</th>
<th>Total no. of explants cultured</th>
<th>Frequency of shoot regeneration (%)</th>
<th>Average no. of shoots/ explant</th>
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<tbody>
<tr>
<td>1</td>
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<td>24</td>
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<td>90</td>
<td>0.00 (1.00)*</td>
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</tr>
</tbody>
</table>

CD<sub>0.05</sub> 0.19 (0.06)* 0.15 (0.05)*

SE(m) 0.06 (0.02)* 0.05 (0.01)*

(Values in parentheses are square root transformed values)

Figure 4: Putative transformed shoot regeneration on selective medium (a-c) through leaf explants (d-f) through internodes

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3.4 Molecular level confirmation

Integration of the chitinase gene in 18 putative transgenic shoots was checked by PCR analysis. Only two lines (T3 and T9) out of 18 were proved PCR positive by amplifying 237 bp and 584 bp fragments (Figure 5a & 5b) respectively. Consequently, the presence and integration of chitinase gene in the genome of apple rootstock M7 was demonstrated by PCR analysis. Hardening was attempted in case of rooted transgenic lines (Figure 6).

3.5 Transformation efficiency

In the present studies, the observed transformation efficiency with respect to the type of explants viz. leaf and internode was 0.32% and 1.70%. Out of two transgenic lines, one arose from leaf explants and another from internodes. Correspondingly, the actual transformation frequency was 0.05% and 0.08% in leaf and internodal explants.

4 Discussion

With the emergence of genetic transformation method the chances for the development of resistant cultivars have been increased in

Table 4 Effect of pre-culturing and co-cultivation durations on regeneration of internodal segments

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Pre-culturing (Days)</th>
<th>Co-cultivation (Hours)</th>
<th>Total no. of explants cultured</th>
<th>Frequency of shoot regeneration (%)</th>
<th>Average no. of shoots/explant</th>
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<td></td>
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<td>0.32 (0.09)*</td>
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(Values in parentheses are square root transformed values)

the putative shoots directly i.e. without callus phase. On the other hand, internodal segments generated the putative shoots indirectly i.e. through callus phase.

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case of important commercial apple cultivars and rootstocks vulnerable to various fungal diseases. Genetic engineering widens the swiftness of genes by transferring them to plants from any other source like plants, animals or microorganisms which is usually limited in conventional breeding programs (Polanco et al., 2010). Many of the species and cultivars of apple perform poorly towards the development of transgenic tissues because of inefficacious regeneration system (Akdemir et al., 2012. Increased leaf regeneration efficiency remains to be the most imperative factor for development of a transformation system in apple while using A. tumefaciens vectors. Axenically raised shoot tissue cultures serves as a perfect source for explants like expanded leaves or stems. For adventitious shoot bud/shoots induction from leaves and internodes TDZ was preferred over BA in our experiments. Regarding remarkable effect of TDZ in shoot bud induction our results are in concordance with Gercheva et al., 2000; Dobranszki et al., 2002; Qin et al., 2002; Ou et al., 2008. 

Fundamentally, the transfer of bacterial T-DNA [positioned on its tumor-inducing (Ti) plasmid] into the plant nuclear genome is plant genetic transformation. For many fruit species Agrobacterium mediated transformation relies chiefly upon the type of genotype. Conversely to all other strains, A. tumefaciens LBA4404 strain could be adequately removed off from apple explants post co-cultivation with low amounts of antibiotics (Komori et al., 2009; Modgil & Sharma 2009).

Attributes of antibiotics like vast dimension for bacterial infection and low eukaryotic toxicity allows the widespread use of cefotaxime and carbenicillin to eliminate A. tumefaciens from plant cultures after co-cultivation. Findings of present study are coherent with Modgil & Sharma (2009) regarding remarkable effect of cefotaxime on bacterial growth elimination (@ 500 mg l⁻¹) and its prompt response towards shoot regeneration (@ 200-300 mg l⁻¹). The most significant step in any transformation procedure

Figure 5 (a) 237 bp & (b) 584 bp amplification in T3 and T9 transgenic lines.

Figure 6 Rooting in (a) control (b) transgenic line.
is the discrimination of transformed regenerants from non transformed ones. Miki & McHugh (2004) reported broad spectrum activity of hygromycin against prokaryotes and eukaryotes and toxicity in plants. Nevertheless, the screening for transgenic regenerated shoots is often partial and difficult due to regeneration of escapes and chimeras. In this study 5 mg l⁻¹ hygromycin was used as a selection pressure for the co-cultivated explants which strengthened the previous findings (Kumar et al., 2004; Modgil & Sharma 2009, Sharma et al., 2012).

Pre-treatment allows the conditioning of the explants prior to A. tumefaciens infection which particularly improves the porosity of cell walls thereby enhancing the transformation rate (Gill et al., 2004). Further, it was reported that 15 minutes Agrobacterium cell suspension infection of explants is quite productive. On the contrary, many reports showed that the infection time and temperature are genotype dependent, while gentle shaking of leaves for different durations at room temperature was preferred to obtain maximum transformation efficiency in M. domestica cv. ‘Jonagold’ and rootstock MM106 respectively (De Bondt et al., 1994; Sharma et al., 2012).

Developed organogenesis protocols or regeneration systems are the backbone of competent genetic transformation methods. Presence of escapes (non-transformed tissues) in this studies is consistent with Radchuck & Korkhovoy 2005; Flachowsky et al., 2008; Sharma et al., 2012 in case of apple. Usually, the participation of few cells in the origin of new adventitious shoots enhances the likelihood of chimera development (George et al., 2008). Among the developed putative transformed regenerants, few can be chimeras i.e. a mix of transformed and non-transformed cells in the tissues (Hanke et al., 2007). In most transformation systems, the generation of a number of escapes is expected. Regeneration of escapes and chimerical shoots at high frequencies has been reported in some species, although their importance and frequency have been underestimated. The problem of chimiserism seems to be more frequent than originally thought and it has been reported in several herbaceous species and woody fruit trees (Padilla & Burgos, 2010).

Though the adventitious shoot regeneration from wounded leaf segments was reported by Norelli et al., 1996 and Sharma et al., 2012, however, a proficient system of transgenic shoots production via apical internodal explants from etiolated ‘Royal Gala’ apple shoots was cited by Liu et al., 1998.

PCR has many potentialities in the various fields of biology. Among these, it is one of the diagnostic tool for transformed cells detection. Likewise, in the present investigations the presence of rice chitinase gene (chiII) in transformed plants was confirmed by amplifying 584 bp and 237 bp size fragments through PCR as previously reported by Ganeshan et al., 2009; Sharma et al., 2012 who also used the same construct in case of cotton and apple rootstock ‘MM106’ respectively.

Though the genetic transformation efficiency is significantly lower in present study but first time it helps in developing a simple and reliable chitinase gene transfer protocol for the apple rootstock M7 by using A. tumefaciens. For the improvement of existing trusted rootstocks, transgenic technology can be applied by incorporating the useful foreign genes of interest. Further studies are required for the validation of transgene performance towards resistance to white root rot (Dematophora necatrix) in apple rootstock M7. The results obtained from this research would contribute for possible development of fungal resistant transgenic lines.

**Conflict of Interest**

Authors would hereby like to declare that there is no conflict of interest that could possibly arise.

**Acknowledgements**

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**References**


the morphogenetic activity of *in vitro* leaves. Acta Agronomica Hungarica 50:117-126


DO MARKETING OBJECTIVES AFFECT MARKETING EFFICIENCY?
A CASE STUDY OF DATES MARKETING IN SAUDI ARABIA

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ABSTRACT

Saudi Arabia has dates as the most important agriculture crop, from both production and area harvested sides, its area was 155 thousand hectares and production was 991 thousand tons in 2010. Even that, the post production stage for dates did not developed to offer the efficient marketing services. Technical and cost efficiency estimation of date marketing became a necessary step to develop date marketing services. This study explores the relationship between, date marketing efficiencies, technical (TE) and cost (CE), and date marketing objectives, with special reference to date marketing scales (Y), date marketing margin (MM), and date marketing classical efficiency as ratio between date marketing margins to date marketing costs (MM/MC). Results of this case study indicated that technical efficiency, based on dates marketing scale objective, gets the highest value at Madena (0.80) while lowest was at Al-Hassa (0.68). While cost efficiency did not change much as it was 0.23 at Al-Hassa and 0.20 at the other 3 provinces. While for date marketing margin objective, the technical and cost efficiencies are the same, 0.7 and 0.2, respectively for all the studied four provinces. The ratio of market margin to marketing costs (MM/MC), as an objective, has its impact on technical efficiency for date marketing was about 0.7, for 3 provinces except at Riyadh, it was 0.6. The dates marketing cost efficiencies for all 4 provinces have the same value of 0.2.

KEYWORDS
Technical Efficiency
Cost Efficiency
Date marketing
Marketing Margin

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All rights reserved.
1 Introduction

Dates is the most important crop in Saudi Arabia, from both production and area harvested sides, as its area increased from 142 to 162 thousand hectares from 2000 to 2009. Its production increased from 734 to 991 thousand tons at the same period. Even that, the post production stage for dates did not developed to offer marketing services of that production. Technical and cost efficiency of date marketing, and its efficiency determents became a necessary step to develop date marketing sector. This study explores the relationship between, date marketing efficiencies, such as, technical and cost efficiencies, and dates marketing goals and/or objectives. Research in Marketing has been focused on the marketing performance which is related to the marketing efficiency. Charnes et al. (1978) define the efficiency as the comparison among firms of the ratio of outcomes over the inputs required to achieve them. On the other hand, Sheth et al. (2002) define marketing efficiency as the ratio of marketing output revenues over input costs. These both definitions are combinedly used in marketing research. Sheth et al. (2000) and Sheth & Sisodia (1995), in referring to their definition of marketing productivity, include two dimensions, efficiency as well as effectiveness, i.e. getting loyal customers at low marketing costs. On the other hand, Rust et al. (2004) use the term marketing productivity to refer how marketing activities are linked to short-term and long-term profits. Charnes et al. (1985) first suggested applying DEA to gain insights into efficiency of marketing efforts. Since then, there have been some marketing studies that used the DEA as a methodology. Kamakura et al. (1988) used DEA to measure welfare loss and market efficiency. Mahajan (1991) studied a DEA model for assessing the relative efficiency of sales units that simultaneously incorporates multiple sales outcomes, controllable and uncontrollable resources, and environmental factors. Kamakura et al. (1996) evaluated multiple retail stores (branches) for their efficiency using DEA and multiple translog cost function estimation, while Donthu & Yoo (1998) compared the results obtained in the evaluation of multiple retail stores (restaurant chain) using DEA and regression. Fare et al. (1985) use techniques from the efficient measurement literature to evaluate the performance of six United States beer firms in terms of their ability to translate advertising messages into sales. Luo & Donthu (2001) demonstrate the application of DEA to benchmark advertising efficiency and to estimate the relative efficiency of advertising campaigns characterized by multiple inputs and multiple outputs. Heskett et al. (1994) propose the framework for the Service-Profit Chain for linking service operations, employee assessments, and customer assessments to firm’s profitability, and Kamakura et al. (2002) develop an approach to assess it. The approach combines data such as measures of operational inputs, customers’ perceptions and behaviors, and financial outcomes from multiple sources. They use DEA for the operational analysis. Donthu et al. (2005) try to fill the gap in research of the lack of appropriate methodological tools for analyzing the benchmarking process in marketing. DEA is suggested to aid traditional benchmarking activities and is useful in identifying the best performing units to be benchmarked against, as well as in providing actionable measures for improvement of a company’s marketing performance. On the other hand, Keh et al. (2006) intend to answer how a service firm can help in the marketing expenses and yet strive to maximize revenue. They employ a DEA model with total expenses as the raw input, marketing expenses as intermediate output/input and revenues from room rentals, and food and beverages as final outputs.

2. Material and Methods

2.1 Data Envelopment Analysis: The Model

The DEA model involves optimizing a scoring function (H) which Coelli (1996), defined as the ratio of the weighted sum of date marketing objectives (Y1,Y2, andY3), as output, and the weighted sum of date marketing cost functions (x1, x2, x3, x4, and x5), as an inputs, subject to the constraints that the similar ratios for every DMU be less or equal to one, implying that efficient units will have a score of one. Decision making unit (DMU) outputs includes marketed date quantity in ton (Y1), dates marketing margin in Saudi Riyals (Y2), and ratio of date marketing margin to date total cost of marketing (Y3). Inputs of DMU used in marketing dates are, x1, x2, x3, x4, x5 as cost of labor, transportation, grading, storage, and other cost including advertising and commissions, respectively. For each i-th DMU, the linear problem is the following (Farrell 1957):

\[
\begin{align*}
\max_{\mu, v} & \quad H = \left( \mu' y \right) / \left( v' x \right) \\
\text{s.t.} & \quad \left( \mu' y_j / v' x_j \right) \leq 1, \quad j = 1,2,...,N \\
& \quad \mu, v \geq 0
\end{align*}
\]

Where \( \left( \mu' y / v' x \right) \) is the scoring function (where \( u \) is an Mx1 vector of output (Y) weights and \( v \) is an Kx1 vector of input (x) weights). The goal is to find values for \( u \) and \( v \) that maximize the efficiency score of the i-th DMU subject to the constraint that all the efficiency measures must be less than or equal to one. This ratio formulation ensures that 0 < Max H ≤ 1; a unit will be efficient if and only if this ratio equals unity otherwise it is considered as relatively inefficient.

In order to identify the technical efficiency of DMU in our study, we solve the following linear programming problem:

\[
\text{Max } y_j \lambda_j^2, \ldots, \lambda_k \quad (1)
\]
where \( y \) is an optimal level of output or date marketing objective (\( Y_i \)), \( y_k \) denotes the output of the \( k \)th DMU, \( x_n \) denotes the level of the \( n \)th input used on DMU \( k \), \( x_n = 0 \) is the \( n \)th input used on the DMU whose efficiency is being tested, and \( \lambda k \) is the weight given to DMU \( k \) in forming a convex combination of the input vectors. The resulting technical efficiency index is calculated as a ratio between the observed level of output on the DMU being tested (\( y0 \)) and the optimal level of output (\( y \)). Technically efficient DMU’s are those with an efficiency index equal to one. Technically inefficient DMU’s are those with an index strictly lower than one.

Cost efficient DMU’s (under the assumption of variable returns to scale) are identified by solving:

\[
\begin{align*}
\min_{\alpha_i - \alpha_j, \gamma - \gamma'} & \sum_{n=1}^{n} W_n x_n \\
\text{s.t.:} & \sum_{k=1}^{K} y_k \lambda_k \geq y, \quad k = 1, 2, \ldots, K \\
& \sum_{k=1}^{K} x_n \lambda_k \leq x_n, \quad \text{for } 1 \leq n \leq t, \quad k = 1, 2, \ldots, K, \\
& \sum_{k=1}^{K} x_n \lambda_k \leq x_n, \quad \text{for } n > t, \quad k = 1, 2, \ldots, K, \\
& \sum_{k=1}^{K} \lambda_k = 1.
\end{align*}
\]

where \( W_n \) is the cost of the \( n \)th \((n=1,\ldots,t)\) input used by the DMU whose efficiency is being tested, \( \lambda_k \) is the weight given to DMU \( k \) in forming a convex combination of the output or input vectors, \( x_n \) denotes the optimal amount of input \( n \) \((n=1,\ldots,t)\), and \( \alpha_i \) denotes the output of DMU \( k \) \((k=1,\ldots,K)\), \( \gamma 

### 2.2 Dates marketing efficiency determinants

In the two-stage method, a DEA problem is solved in the first stage of analysis, involving only the traditional inputs and outputs. In the second stage, the efficiency scores are regressed upon the explanatory variables (Coelli et al., 1998). In this study, the two-stage approach is used to assess the influences of various factors on technical and cost efficiencies. This approach has several advantages, such as not requiring prior assumptions regarding the direction of influence and the ability to accommodate more than one variable with continuous or categorical variables. A Tobit regression approach will be used for estimating the potential determinants of technical and economic efficiencies, because the dependent variables lie in the interval of \((0-1)\) (Binam et al., 2004; Chavas et al., 2005; Cinempre et al., 2006), which are given as:

\[
\begin{align*}
EE_i & = \beta_{0} + \sum_{j=1}^{n} \beta_{j} V_{ij} + u_{i} \quad \text{if } \mu_{i} > -\beta_{0} - \sum_{j=1}^{n} \beta_{j} V_{ij} \\
EE_i & = 0 \quad \text{if } \mu_{i} \leq -\beta_{0} - \sum_{j=1}^{n} \beta_{j} V_{ij}
\end{align*}
\]

where

- \( EE_i \) = The measure of Economic or Cost efficiency for date marketing unit (DMU) \( i \)
- \( V_{ij} \) = Explanatory variables that influence the economic efficiencies of the date marketing units
- \( \beta \) and \( u \) = Parameters of the model and the random error term, respectively

In order to determine the factors contributing to technical and cost efficiencies, the following model was formulated and estimated using the computer software DEAP (Coelli, 1996).
2.3 Study Data

The two stage analysis require collecting data for each stage, the first stage concerns the efficiency of date marketing unit (DMU). Data includes dependent variables (Yi), which include date quantity in tons (Y1), marketing margin in 1000 SR (Y2), and the ratio of marketing margin to total marketing costs (Y3). The independent variables (Xi) required estimating technical efficiency (TEi) includes costs of labor (X1), shipping (X2), grading (X3), storage (X4), and packing for advertising (X5). For cost efficiency (CE) estimation, the costs of last marketing functions are needed per ton of date (Wi), then multiplied by date quantity per tons which has used in each marketing function, x11, x22, x33, x44, x55. Then the parameter relation forms:

\[
\begin{align*}
TE_i &= Y_i = f(X1, X2, X3, X4, X5) \\
CE_i &= Y_i = f(W1X11, W2X22, W3X33, W4X44, W5X55)
\end{align*}
\]

Following efficiency scores will be regressed against the set of date marketing unit DMU specific factors to obtain the determinants for Technical and cost efficiencies. Where, TEi, CEi are the technical and cost efficiency of the i-th date marketing unit DMU:

\[
\begin{align*}
TE_i &= a_0 + a_1H1 + a_2H2 + a_3H3 \\
CE_i &= a_0 + a_1C1 + a_2C2 + a_3C3
\end{align*}
\]

Where manager and owner characteristics are education (H1), experience (H2), and job as father (H3). Efficiency determents for the DMU will include trade type (C1), starting date (C2), ratio of marketing date to other crops marketing (C3). While \(a_0, a_1, a_2, ..., a_3\) are regression parameters estimated by the Tobit model, using Maximum Likelihood Estimator (MLE). Simply because limited dependent variable of efficiency score with a range 0-1, and will not fit ordinary least square (OLS) assumptions.

3 Results

The efficiency of date marketing is estimated for the Kingdom and its four main provinces, Al-Hassa, Madena, Qaseem, and Riyadh. Results include technical (TE) and cost efficiency (CE) of date marketing based on the three main marketing objectives. Date marketing objectives are classified into the marketing date quantity (tons), date marketing margin (SR), and the ratio of date marketing margin to the date marketing costs. Dates marketing efficiency is based on five main marketing function costs, including labor, transportation and distribution, grading and backing, regular and cold storage, and requirements for advertising and commission in buying and selling dates.

3.1 Dates Marketing Efficiency, based on marketing objectives at the studied four Provinces

As mentioned before, developing date marketing sector in the Kingdom need more details about that activity in its main provinces', so policies for developing that sector would be different among these provinces based on its date market characteristics. The study has collected data from the main 4 provinces including Riyadh, Qaseem, Madena, and Al-Hassa on the production and marketing of dates. This section of the study includes four subsections to show the impact of date marketing objectives on the dates marketing efficiency.

It is important to show the differences among Kingdom provinces in date marketing as long as policies to develop that sector need to distinguish among each characteristic. The differences in date marketing efficiencies are shown in table 1, based on marketing scale (tons). To compare date marketing efficiencies among provinces, the study results showed that Madena Province lead others. For technical efficiency, with the assumption of constant return to scale, it is 0.34, 0.29, 0.27, and 0.21 for Madena, Qaseem, Riyadh, and Al-Hassa respectively. For Cost efficiency of dates marketing among 4 provinces, results show that it is 0.23, 0.22, 0.20, and 0.20 for Riyadh, Al-Hassa, Qaseem, and Madena respectively.

At Riyadh province, both technical efficiency, with assumptions of decreasing scale of date marketing, i.e., large scale date marketing are more efficient than that of small scale date marketing (Table 1). Cost efficiency decreased from 0.55 to 0.25 as date marketing scale decreased from 1511 to 4 tons on average. While in case of Qaseem Province, 77% of the studied samples (56 date traders), have date marketing scale of 18 tons on average. Technical efficiency of date marketing, with assumption of constant return to scale, indicate the reduction from 0.63 to 0.29 as marketing scale decreased from 193 to 1 ton on average. The same results are sharing with cost efficiency of date marketing too, where it decreased from 0.38 to 0.18.

Further, at Madena Province, 37 date traders (37%) have 193 tons on an average dates marketing; they have 0.54 and 0.75 as technical efficiency with constant and variable return to scale respectively. As marketing scale of dates decreased at Madena, from 1645 to 8 tons on average, the economic efficiency of date marketing decreased from 0.56 to 0.09. Results of Al-Hassa Province revealed minimum technical efficiency, 0.21 and 0.68, at the kingdom, even that it has the highest cost efficiency of date marketing with Riyadh province, 0.22 and 0.23. These results support the starting of marketing subsidy policies at Al-Hassa for improving the technologies of date marketing. The most of date trader's at Al-Hassa (55%), has 19 tons on average of marketing scale. While it has the greatest average of date marketing scale (4667 tons), in the kingdom, with full technical efficiency with assumptions of constant and variable return to scale, see table. No other provinces reach the full technical efficiency except Al-Hassa, with 0.22 as cost efficiency, these results would direct policies of improving date market at Al-Hassa.
3.2 The Impacts of date marketing objectives on date marketing efficiency estimations

The objectives of date marketing and its efficiency need to be explained, as one of main goals of this study. Table 2 indicates the difference in technical and the cost efficiencies among 4 provinces at the Kingdom, with special reference to selected four objectives of date marketing viz. marketing scale, marketing margin, the ratio of market margin to marketing costs and last three objectives at once. In general, increasing date marketing objectives lead to higher efficiency of date marketing, except at Riyadh Province. Result of study revealed that at Al-Hassa, technical efficiency was 0.68 at marketing scale (tons), and it increased to 0.81 as date marketing objects included three goals at once. Also, at Madena, the cost efficiency of date marketing increased from 0.2 to 0.4 as marketing objectives increased from one goal (marketing scale) to three goals (Table 2).

3.2.1 Dates marketing scale (tons):

Technical efficiency gets the highest value at Madena (0.80) while it was reported lowest at Al-Hassa (0.68). While cost efficiency did not change much as it was 0.23 at Al-Hassa and 0.20 at the other 3 provinces (Table 2).
Do Marketing Objectives affect Marketing Efficiency? A Case Study of Dates marketing

3.3. The Determents of date marketing technical and cost efficiencies

There are two groups of factors that could affect cost efficiency of date marketing, one group factors is related to manager of date marketing unit (DMU), the other one is related to the main characteristics of date marketing unit, see study methodology. Table 3 shows the impact of these two groups on the cost efficiency at different provinces.

3.3.1 Date marketing at Al-Hassa province

Table 3 shows that personnel characteristics (education, experience, and as father job) of date marketing unit manager (owner) would not affect cost efficiency of date marketing. For date marketing unit (DMU) characteristics, include trade type, date share in marketing (%), and date marketing income share (%). The only factor which affecting cost efficiency of date marketing is date marketing share in Al-Hassa province. The negative impact (0.0038), is significant at 5%. Results of study suggested that increasing share of other crops marketing activity would increase the cost efficiency of date marketing. To explain the negative relation between cost efficiency of marketing date and its share in all marketed crop, the experience of marketing different crops and availability of more areas and equipments used for other crops would have positive impact on date marketing cost efficiency (Table 4 & 5).

3.3.2 Date marketing at Madena province

Manager or owner personnel characteristics (education, experience, and as father job) of date marketing unit would not affect cost efficiency of date marketing at Madena. While Trade type of DMU has a significant positive impact (0.04), on the cost efficiency of date marketing, i.e., when DMU extent its date marketing activity to include wholesaling and export would increase cost efficiency of date marketing at Madena. This result support the recommendation to increase scale of date marketing and extend date marketing activities to include , in addition to retailer, wholesaler and date export (Table 4 & 5).

3.3.3 Date marketing at Qaseem province

The results presented in table 3, show the negative impact of experience and being as father job, -0.0061 and -0.0004, with cost efficiency of date marketing. These relations were significant at levels of 10% and 5% respectively. The DMU characteristics of date marketing

Table 2 The Impacts of date marketing objectives on date marketing efficiency

<table>
<thead>
<tr>
<th>Marketing Objectives</th>
<th>Provence</th>
<th>Al-Hassa</th>
<th>Riyadh</th>
<th>Qaseem</th>
<th>Madena</th>
</tr>
</thead>
<tbody>
<tr>
<td>Market Scale (tons)</td>
<td>0.68</td>
<td>0.37</td>
<td>0.23</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Market Margin (1000SR)</td>
<td>0.68</td>
<td>0.37</td>
<td>0.22</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>MM/MC</td>
<td>0.67</td>
<td>0.36</td>
<td>0.21</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Y_MM_MM/MM/MC</td>
<td>0.81</td>
<td>0.43</td>
<td>0.36</td>
<td>0.7</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Source: Data analysis of the study
Table 3 Cost Efficiency Determinants of Date Marketing

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SD</th>
<th>T</th>
<th>Prob.(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>0.127235</td>
<td>0.109764</td>
<td>1.159</td>
<td>0.2464</td>
</tr>
<tr>
<td>Education</td>
<td>XA2</td>
<td>0.058199</td>
<td>0.045494</td>
<td>1.279</td>
</tr>
<tr>
<td>Experience</td>
<td>XA51</td>
<td>0.003277</td>
<td>0.004611</td>
<td>0.711</td>
</tr>
<tr>
<td>As father job</td>
<td>XA6</td>
<td>-0.07593</td>
<td>0.067515</td>
<td>-1.125</td>
</tr>
<tr>
<td>Constant</td>
<td>0.696593</td>
<td>0.164476</td>
<td>4.235</td>
<td>0.0000</td>
</tr>
<tr>
<td>Trade Type</td>
<td>XA1</td>
<td>-0.00499</td>
<td>0.022318</td>
<td>-0.224</td>
</tr>
<tr>
<td>Date marketing%</td>
<td>XA4</td>
<td>-0.0038</td>
<td>0.001813</td>
<td>-2.094</td>
</tr>
<tr>
<td>Date income %</td>
<td>XA7</td>
<td>-0.00155</td>
<td>0.001538</td>
<td>-1.007</td>
</tr>
<tr>
<td>Constant</td>
<td>0.218231</td>
<td>0.049304</td>
<td>4.426</td>
<td>0.0000</td>
</tr>
<tr>
<td>Education</td>
<td>XA2</td>
<td>-0.00089</td>
<td>0.031632</td>
<td>-0.028</td>
</tr>
<tr>
<td>Experience</td>
<td>XA51</td>
<td>-0.00378</td>
<td>0.002384</td>
<td>-1.586</td>
</tr>
<tr>
<td>As father job</td>
<td>XA6</td>
<td>-0.960238D-04</td>
<td>0.000179</td>
<td>-0.536</td>
</tr>
<tr>
<td>Constant</td>
<td>0.062326</td>
<td>0.04328</td>
<td>1.44</td>
<td>0.1499</td>
</tr>
<tr>
<td>Trade Type</td>
<td>XA1</td>
<td>0.040318</td>
<td>0.014842</td>
<td>2.717</td>
</tr>
<tr>
<td>Date marketing%</td>
<td>XA4</td>
<td>2.79029D-04</td>
<td>0.000179</td>
<td>0.156</td>
</tr>
<tr>
<td>Date income %</td>
<td>XA7</td>
<td>3.20443D-04</td>
<td>9.18630D-04</td>
<td>0.349</td>
</tr>
<tr>
<td>Constant</td>
<td>0.265177</td>
<td>0.049754</td>
<td>5.33</td>
<td>0.0000</td>
</tr>
<tr>
<td>Education</td>
<td>XA2</td>
<td>0.176668D-04</td>
<td>0.00019</td>
<td>0.093</td>
</tr>
<tr>
<td>Experience</td>
<td>XA51</td>
<td>-0.00608</td>
<td>0.003789</td>
<td>-1.604</td>
</tr>
<tr>
<td>As father job</td>
<td>XA6</td>
<td>-0.0004</td>
<td>0.000185</td>
<td>-2.183</td>
</tr>
<tr>
<td>Constant</td>
<td>0.399778</td>
<td>0.240377</td>
<td>0.165</td>
<td>0.8686</td>
</tr>
<tr>
<td>Trade Type</td>
<td>XA1</td>
<td>-0.00948</td>
<td>0.02071</td>
<td>-0.458</td>
</tr>
<tr>
<td>Date marketing%</td>
<td>XA4</td>
<td>0.004161</td>
<td>0.002191</td>
<td>1.899</td>
</tr>
<tr>
<td>Date income %</td>
<td>XA7</td>
<td>-0.00257</td>
<td>0.00146</td>
<td>-1.759</td>
</tr>
<tr>
<td>Constant</td>
<td>0.165981</td>
<td>0.182028</td>
<td>0.912</td>
<td>0.3619</td>
</tr>
<tr>
<td>Trade Type</td>
<td>XA1</td>
<td>0.017127</td>
<td>0.021665</td>
<td>0.791</td>
</tr>
<tr>
<td>Date marketing%</td>
<td>XA4</td>
<td>0.00012</td>
<td>0.002048</td>
<td>0.059</td>
</tr>
<tr>
<td>Date income %</td>
<td>XA7</td>
<td>-1.27745D-04</td>
<td>0.00024</td>
<td>-0.053</td>
</tr>
<tr>
<td>Constant</td>
<td>0.267263</td>
<td>0.047364</td>
<td>5.643</td>
<td>0.0000</td>
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<tr>
<td>Education</td>
<td>XA2</td>
<td>-0.00015</td>
<td>0.000248</td>
<td>-0.61</td>
</tr>
<tr>
<td>Experience</td>
<td>XA51</td>
<td>-0.00347</td>
<td>0.002922</td>
<td>-1.188</td>
</tr>
<tr>
<td>As father job</td>
<td>XA6</td>
<td>-0.00484</td>
<td>0.060671</td>
<td>-0.08</td>
</tr>
</tbody>
</table>

Source: Analysis of study data

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Table 4 Cost Efficiency Deterents in the studied province

<table>
<thead>
<tr>
<th>Deterrents</th>
<th>Personal Characteristics</th>
<th>Date Marketing Unit (DMU) Characteristics</th>
<th>Model/ Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trade Type</td>
<td>Starting Date</td>
</tr>
<tr>
<td>Riyadh</td>
<td></td>
<td>Constant</td>
<td>Experience</td>
</tr>
<tr>
<td>Coefficient</td>
<td>0.240333</td>
<td>-0.03444</td>
<td>-0.0035</td>
</tr>
<tr>
<td>T test</td>
<td>0.719</td>
<td>-0.709</td>
<td>-1.038</td>
</tr>
<tr>
<td>Prob.</td>
<td>0.4721</td>
<td>0.4784</td>
<td>0.2994</td>
</tr>
<tr>
<td>Madena</td>
<td></td>
<td>Constant</td>
<td>Education</td>
</tr>
<tr>
<td>Coefficient</td>
<td>0.2020677</td>
<td>-0.00052</td>
<td>-0.00262</td>
</tr>
<tr>
<td>T test</td>
<td>0.846</td>
<td>-0.016</td>
<td>-1.05</td>
</tr>
<tr>
<td>Prob.</td>
<td>0.3976</td>
<td>0.9873</td>
<td>0.2935</td>
</tr>
<tr>
<td>Qaseem</td>
<td></td>
<td>Constant</td>
<td>Experience</td>
</tr>
<tr>
<td>Coefficient</td>
<td>7.186001</td>
<td>.462513D-05</td>
<td>-0.00541</td>
</tr>
<tr>
<td>T test</td>
<td>0.409</td>
<td>0.023</td>
<td>-0.454</td>
</tr>
<tr>
<td>Prob.</td>
<td>0.6829</td>
<td>0.9816</td>
<td>0.6498</td>
</tr>
<tr>
<td>Al-Hassa</td>
<td></td>
<td>Constant</td>
<td>Experience</td>
</tr>
<tr>
<td>Coefficient</td>
<td>-29.2282*</td>
<td>0.047068</td>
<td>0.010743</td>
</tr>
<tr>
<td>T test</td>
<td>-1.737</td>
<td>0.895</td>
<td>0.98</td>
</tr>
<tr>
<td>Prob.</td>
<td>0.0823</td>
<td>0.3706</td>
<td>0.3272</td>
</tr>
</tbody>
</table>

* Significant at 1%, ** Significant at 5%, Significant at 10%; Source: Data analysis of the study
Table 5 Technical Efficiency Determents in Date marketing

<table>
<thead>
<tr>
<th>Determents</th>
<th>Constant</th>
<th>Personal Characteristics</th>
<th>Date Marketing Unit (DMU) Characteristics</th>
<th>Model/ significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riyadh</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient</td>
<td>0.480299</td>
<td>0.057775</td>
<td>-0.0027</td>
<td>0.355141</td>
</tr>
<tr>
<td>T test</td>
<td>1.009</td>
<td>0.813</td>
<td>-0.56</td>
<td>9.23</td>
</tr>
<tr>
<td>Prob.</td>
<td>0.3131</td>
<td>0.4161</td>
<td>0.5752</td>
<td>0.000</td>
</tr>
<tr>
<td>Madena</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient</td>
<td>0.54229</td>
<td>-0.08231</td>
<td>-0.00258</td>
<td>0.309485</td>
</tr>
<tr>
<td>T test</td>
<td>1.258</td>
<td>-1.381</td>
<td>-0.556</td>
<td>11.277</td>
</tr>
<tr>
<td>Prob.</td>
<td>0.2083</td>
<td>0.1673</td>
<td>0.5783</td>
<td>0.000</td>
</tr>
<tr>
<td>Qaseem</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient</td>
<td>23.5912</td>
<td>.627496D-04</td>
<td>-0.02277</td>
<td>0.393291</td>
</tr>
<tr>
<td>T test</td>
<td>0.598</td>
<td>0.144</td>
<td>-0.848</td>
<td>8.321</td>
</tr>
<tr>
<td>Prob.</td>
<td>0.5497</td>
<td>0.8851</td>
<td>0.3964</td>
<td>0.000</td>
</tr>
<tr>
<td>Al-Hassa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient</td>
<td>-10.4908</td>
<td>0.074718</td>
<td>0.004573</td>
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</tr>
<tr>
<td>T test</td>
<td>-0.555</td>
<td>1.306</td>
<td>0.358</td>
<td>5.845</td>
</tr>
<tr>
<td>Prob.</td>
<td>0.5786</td>
<td>0.1915</td>
<td>0.7201</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* Significant at 1%, ** Significant at 5%, Significant at 10%; Source: Data analysis of the study
share in other crops marketing, and date marketing income share of total marketing income have different impacts on cost efficiency of date marketing, +0.004 and – 0.0025 respectively. Note that increasing share of date marketing quantity by 10% among other crops marketing, would increase cost efficiency of date marketing by 0.04 at significant level of 5% (Table 4 & 5). While the negative impact of dates marketing income share on cost efficiency of date marketing is less significant (10%).

3.3.4 Date marketing at Riyadh province

The determents of efficiency have different impacts, but all of impacts were non-significant, so study would not depend on dates marketing policy improvements (Table 4 & 5).

4 Discussions

The date marketing efficiency at the Kingdom, based on five categories of marketing scales, with three marketing objectives was estimated. Results, in addition to the Kingdom, include four main provinces, Al-Hassa, Madena, Qaseem, and Riyadh. The study results include the impact of marketing objectives of date marketing on efficiency estimations. Also, results include date marketing efficiency for the labor (management and technical labors) used in marketing dates. The area used for date grading, backing, regular storage, and cold storage for date marketing at the 4 provinces at the Kingdom are used for estimating its technical efficiency. Finally, study results include technical and cost efficiency determents for date marketing. Efficiency determents include two groups of variables, one for the manger or owner main characteristics, and the other is for date marketing unit characteristics. The significant of such impacts are discussed too. For the Kingdom, based on marketing scale of date, study results show that technical efficiency (TE) and cost efficiency (CE) decreases as date marketing scale decrease i.e., more date scale mean they are more professional in marketing date with few exceptions. Also, economies of scale would decrease input use and marketing costs per unit, which increase its TE and CE. Lower efficiency of date marketing would result in sharing marketing of other crops at the same time, so it’s hard to distinguish between costs of marketing date and marketing other crops. The last consideration would explain over estimation of marketing date, which is not the case of TE and CE.

Study results of date marketing efficiency, based on 4 main provinces in the Kingdom, Al Hassa, Madena, Qaseem, and Riyadh, have supported last results of decreasing TE, with constant and variable returns to scale, and CE as scale of marketing date decreases. Even that Madena province has highest TE, 34% and 75% for constant and variable return to scale, but it has the lower CE (20%), among 4 provinces. That result show us how costs of marketing affect its cost efficiency of marketing with assumption of having good experience of marketing date, as they have a relatively higher TE. It is important to show the impacts of date marketing goals on its marketing efficiency, the differences among date traders and their objectives would give us an indicator of degree of maturity of marketing policies. The first objective would be the scale (Y0), then the market margin (MM), and its ratio to marketing costs (MM/MC). The final objective is including the three goals at once. For TE, it will increase from 68% to 81% at Al Hassa, and from 72% to 74% at Qaseem, as date marketing goals included all objectives. In case of CE, it was 23%, 20%, 20%, and 20% at Al Hassa, Riyadh, Qaseem, and Madena respectively with only scale of date marketing goal. Including all goals of date marketing will increase CE to 36%, 30%, 24%, and 40% respectively. The knowledge and vision to date marketing activities need to be supported through different program and extension tools by policy makers. The labor technical and cost efficiency for marketing date have been estimated, based on 3 marketing goals of Y, MM, and MM/MC at 4 provinces. Madena has the highest labor efficiency in marketing date, full 100%, for both TE and CE. In general, the third goal of date marketing (MM/MC) would affect the efficiency negatively with exception of Riyadh province, i.e., including marketing cost has a negative impact on efficiency estimation at other provinces. The mean cost efficiency of labor were 0.62, 0.65, and 0.45 for marketing goals Y, MM, and MM/MC respectively. Al Hassa has the most negative impact, were TE and CE decreased from full efficiency,100%, in case of Y goal, to only 17% in case of MM/MC goal of marketing date.

The availability of areas for date grading, backing, and storage would affect the date marketing efficiency. Technical efficiency of using such areas, with respect to 3 date marketing goals, Y,
MM, and MM/MC, are estimated for the 4 provinces. The mean TE are 60%, 60%, and 48%, based on date marketing goals Y, MM, MM/MC respectively.

Note that, Madena has full TE, 100%, of area used in dates marketing, same as labor use efficiency. Al Hassa TE of area use in date marketing decreased from 68% to 10% as marketing goal changed from Y to MM/MC. While, in case of Riyadh province it increased from 25% to 57%. So, date marketing policies will differ among provinces, based on its sensitivity to include date marketing cost in date marketing goals. Date marketing efficiency determinants are estimated, based on owner (manager) and date marketing unit (DMU) characteristics, using Tobit Model and MLE for estimating regression parameters. The significant positive impacts of dates marketing ratio (%) relative to marketing other crops are observed at Al Hassa and Qaseem provinces, where increasing that ration by 10% would increase CE by 3% and 4% respectively. Trade type for date marketing which include, in addition to retailer, wholesaler and exporting of date has a positive impact, (+4%) and (+3%), on CE and TE of date marketing at Madena.

Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

References


RATIONAL SYNCHRONOUS ELECTRIC DRIVE OF AGRICULTURAL PUMPS

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ABSTRACT

This article discusses design and mathematical description of rational synchronous electric drive of portable irrigation pumps. This electric drive makes it possible to decrease wasteful energy loss for irrigation due to improvement of energy performances of electric drive. Electric drives of irrigation pumps are the most energy intensive power consumers in agricultural industry, which often operate from long distance power transmission line, improvement in their energy performances decreases power loss both on the drive itself and in supply line. Power supply line for irrigation purposes usually operates with the voltage of 10 kV, whereas the voltage of rational motors for pump drives is below 1000 V. Under such conditions it would be reasonable to operate transformer–motor assemblies, which optimize control and protection of pump assemblies. Such assemblies enable rationalization of control device of synchronous motor in pump drive by simplifying exciter with retention of automatic excitation control in function of mechanical load and motor supply voltage. This article presents mathematical description of transient and steady electromagnetic processes in transformer–motor assemblies of the considered design.

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1 Introduction

Nearly all Russian agricultural regions are characterized by unsteady moistening stipulated by deficit in natural precipitations or existence of waterlogged soils. Under such conditions artificial irrigation and reclamation of lands are highly efficient approach to agriculture (Shchedrin, 2004). This is aided by portable pumps with diesel or electric drive. Such pumps are also used as reserve ones for emergency water supply of residential areas and industrial facilities, for their fire protection and other purposes.

According to data by US Hydraulic Institute and European National Pump Association, about 20% of global electric power is consumed by pump stations (Shchedrin, 2004). If all expenses for procurement and operation of pump assemblies are set to 100%, then the main expenses for procurement, maintenance, electric power, without costs of water in 10 years of operation will be distributed as 85% power costs; 10% maintenance and remaining 5% for the procurement of equipment (Shchedrin, 2004).

Since the most expensive portion is the power consumption by pump station, then one of the most efficient ways of their improvement is increase in their power performances: either in efficiency or in power factor (Oskin, 1996). In addition, the assemblies should be equipped with reliable, easily controllable electric drive characterized by required electromechanic properties, high power performances and possibly low cost. Insufficient reliability of drives is related with significant economic costs (Kopylov, 2000). Non-adjustable alternative current drives are widely applied in irrigation, including asynchronous and synchronous, each of them is characterized by known advantages (Syromyatneykov, 1963; Beglyarov & Strizhkov, 2010). The first ones are used up to 100 kW, and the latter above 500 kW. At the range of 100-500 kW both drive types are used and it is exactly the range which is required for portable pump stations (Oskin & Didych, 2011; Oskin, 2014). Present study aim to discuss the design and mathematical description of rational synchronous electric drive of portable irrigation pumps.

2 Materials and Methods

New design of synchronous motor for pumps is proposed by Kuban state agrarian university, Russia (Fig. 1) It differs from conventional synchronous motors by the connection circuit of stator (armature) winding, which in addition to common functions acts as compound chain of motor excitation system instead of expensive external exciters aiming at provision of preset sequence of automatic adjustment of excitation, thus resulting in simpler and less expensive motor (Strizhkov et al., 2006a; Strizhkov et al., 2006b; Strizhkov et al., 2009; Strizhkov et al., 2012a). Peculiar feature of armature winding makes it possible to solve efficiently the issue of reduction of starting current using starting of section motor (Strizhkov et al., 2010a). Using the proposed motor as pump drive of stationary facilities makes it possible to combine high power performances and relatively low cost of the drive. It is known that economic efficiency of serial synchronous motors is usually limited by 100 kW, and in actual agricultural irrigation the lower limit of pumps with synchronous drive is 200 kW.

Engineering and economic analysis revealed that at present the use of synchronous motor with twin armature winding (SMTW) for agricultural purposes allows to decrease this limit to 60 kW, and in some cases even to 50 kW (Beglyarov & Strizhkov, 2010). This is achieved due to the various advantages of SMTW, among these some common are (i) decrease in drive dimensions and weight in comparison to serial motor, (ii) availability of automatic adjustment of excitation, (iii) decrease in starting current to 1.25-1.75-fold upon switching of armature winding sections for the period of asynchronous start and (iv) holding of high overload and compensating ability upon possible non-emergency voltage decrease in supply circuit.

Modern electric engineering proposes various approaches to study and simulation of machinery and valves. The most widely used is the method of mathematical description of electromagnetic processes by equilibrium equations of currents and voltages of electric circuits included in the considered object in combination with motion equation of electric drive. The theory of synchronous motors is based on generalized electromechanic transformation of coordinates upon its mathematical description (Kopylov, 2000). This theory is discussed in details with regard to synchronous motors with several magnetically coupled three-phase windings.
on stator (Strizhkov, 2012). This article presents results of mathematical description of electric equipment of transformer–SMTW assembly in d, q coordinates rigidly coupled with rotor. The mentioned coordinates are common rational system for description of synchronous machines. This work uses relative units, common for transformer and motor, the basic principles of the units are described in Strizhkov (2013). Mathematical descriptions of the assembly are given in dynamic modes in the form of non-linear differential equations and in static modes in the form non-linear algebraic equations.

3 Results

General mathematical description of the assembly is comprised of equations of transformer and motor, given in unified system of relative units with basic voltage and current, equating to nominal values for motor. While solving problems of analysis electromagnetic processes in electric motor, it is reasonable to present transformer by equivalent circuit (Kopylov, 2000), which significantly simplifies the mathematical model and provides acceptable error of calculations. Herewith, transformer idle current is neglected; it amounts to about 3% of nominal value and does not significantly influence currents in motor windings, thus, it is reasonable to introduce assumption about proportionality of voltages on outputs of transformer secondary winding. The error resulting from neglecting processes in magnetizing branch is lower by an order of magnitude than the error added by assumptions idealizing motor and transformer. If required, the transformer idle current can be taken into account as the current of independent branch ported out to terminals of power source (circuit).

While solving the problems of analysis of processes in transformer windings as well as while developing universal mathematical model of transformer–SMTW assembly, it is required to use the equations of transformer voltage equilibrium. In this case the set of equations of mathematical description of the assembly is as follows (Strizhkov, 2012; Strizhkov, 2013; Strizhkov et al., 2010b):

\[
\begin{align*}
\frac{d\psi_{yd}}{dt} &= -r_{yd}\psi_{yd}; \\
\frac{d\psi_{yq}}{dt} &= -r_{yq}\psi_{yq}; \\
\frac{d\omega}{dt} &= (\psi_{d1}\psi_{q1} + \psi_{d2}\psi_{q2} - \psi_{d1}\psi_{q2} - m_c) / J; \\
\frac{dy}{dt} &= \omega; \\
u_d &= \eta_1 T_{d1} + \eta_2 T_{d2} + \eta_3 T_{d3}; \\
u_{d1} &= \eta_1 T_{d1} - \eta_{d1} T_{d1}; \\
u_{d2} &= \eta_2 T_{d2} - \eta_{d2} T_{d2}; \\
u_{d3} &= \eta_3 T_{d3}; \\
u_{q1} &= \eta_1 T_{q1} + \eta_{q1} T_{q1}; \\
u_{q2} &= \eta_2 T_{q2}; \\
u_{q3} &= \eta_3 T_{q3}; \\
\psi_d1 &= x_d1 i_d1 + x_d2 i_d2 + x_d3 i_d3; \\
\psi_d2 &= x_d1 i_d1 - x_d2 i_d2 + x_d3 i_d3; \\
\psi_f &= x_f1 i_f1 + x_f2 i_f2; \\
\psi_d &= x_d1 i_d1 + x_d2 i_d2 + x_d3 i_d3; \\
\psi_f &= x_d1 i_d1 - x_d2 i_d2 + x_d3 i_d3; \\
u_d &= U_m \sin \gamma; \\
u_q &= U_m \cos \gamma; \\
u_{d1} &= -\eta_{d1} i_d1 + \omega \psi_{d1}; \\
\frac{d\psi_{d1}}{dt} &= i_d1 - \eta_{d1} i_d1 + \omega \psi_{d1}; \\
\frac{d\psi_{d2}}{dt} &= i_d2 - \eta_{d2} i_d2 + x_f2 i_f2 + \omega \psi_{d2} - \frac{i_d2 \cos \phi_f - i_d2 \sin \phi_f \psi_{d2}}{\beta_f} - \frac{i_d2 \sin \phi_f - i_d2 \cos \phi_f \psi_{f2}}{\beta_f}; \\
\frac{d\psi_{d3}}{dt} &= i_d3 - \eta_{d3} i_d3 - x_f d i_f d - \omega \psi_{d3} - \frac{i_d3 \cos \phi_f - i_d3 \sin \phi_f \psi_{d3}}{\beta_f}; \\
\frac{d\psi_{q1}}{dt} &= i_q1 - \eta_{q1} i_q1 - \omega \psi_{q1}; \\
\frac{d\psi_{q2}}{dt} &= i_q2 - \eta_{q2} i_q2 - x_f2 i_f2 - \omega \psi_{q2} - \frac{i_d2 \sin \phi_f - i_d2 \cos \phi_f \psi_f2}{\beta_f}; \\
u_{q1} &= x_y d i_q1 + x_y d i_q2 + x_y d i_q3 + x_y d i_f2; \\
u_{q2} &= x_y d i_q1 + x_y d i_q2 + x_y d i_q3 + x_y d i_f2; \\
u_{q3} &= x_y d i_q1 + x_y d i_q2 + x_y d i_q3 + x_y d i_f2; \\
\psi_y &= x_y d i_d1 + x_y d i_d2 + x_y d i_d3; \\
\psi_y &= x_y d i_q1 + x_y d i_q2 + x_y d i_q3 + x_y d i_f2; \\
\psi_y &= x_y d i_d1 + x_y d i_d2 + x_y d i_d3; \\
\psi_y &= x_y d i_q1 + x_y d i_q2 + x_y d i_q3 + x_y d i_f2.
\end{align*}
\]

where \( U_m \) is the voltage amplitude of supply circuit; \( u_d, u_{d1}, u_{d2}, u_q, u_{q1}, u_{q2} \) are the voltages on transformer primary winding, winding W1 and winding W2, respectively, along longitudinal axis (d) and
transversal axis \((q)\) of orthogonal coordinates \(d, q\); \(i_d, i_q, k_d, k_q\), \(i_q\) are the currents on transformer primary winding, winding \(W1\) and winding \(W2\), respectively, along longitudinal and transversal axes; \(r_{1T}, r_{2T}, r_{T}\) are the active resistances of primary and secondary transformer windings; \(x_{d1}, x_{d2}, x_{d3}, x_{d12}, x_{d13}, x_{d23}\) are the induction resistances of self-induction and mutual induction of primary and secondary transformer windings, respectively; \(r_1, r_2, r_T\) are the active resistances of windings \(W1, W2\) and excitation winding, respectively; \(x_{p1}, x_{p2}, x_{q1}, x_{q2}, x_{q3}\) are the induction resistances of self-induction and mutual induction of synchronous motor; \(\omega\) is the angular rotor frequency; \(\gamma\) is the load angle; \(\beta_d, \beta_q, \beta_i\) are the coefficients of bridge rectifier with regard to voltage, current, and resistance, respectively (Strizhkov et al., 2012b); \(\psi_{f}, \psi_{d1}, \psi_{q1}, \psi_{d2}, \psi_{q2}, \psi_{q3}\) are the linkage of windings \(W1, W2,\) excitation winding and damper winding, respectively, along coordinate axes \(d, q\).

One of properties of asynchronous motor determining its operation performances is the law of the angular excitation adjustment. The most reasonable for irrigation pump drive at free standing pump stations is the law of excitation adjustment with regard to constant zero reactive power.

Parameters of transformer and motor providing for preset excitation law are determined on the basis of equations of static operation mode of the drive.

Secondary transformer voltages \(U_1\) and \(U_2\) in static modes without great error can be considered as independent from motor load mode (with regard to current in windings). No electromagnetic processes occur in electric circuits of damper (starting) winding, since electromotive force (EMF) in these windings along \(d\) and \(q\) axes is not induced and damper currents are zero. Taking into account these assumptions the equations of static mode are as follows:

\[
\begin{align*}
U_{d1} &= r_2 I_{d1} - \psi q_1 = -U_1 \sin \Theta; \\
U_{d2} &= r_3 I_{d2} - \psi q_1 = -U_2 \sin \Theta; \\
U_{d1} &= R_2 I_{q1} + \psi d_1 = U_1 \cos \Theta; \\
U_{d2} &= R_2 I_{q2} + \psi d_2 = U_2 \cos \Theta; \\
\psi d_1 &= x d1 I_{d1} + x d3 I_{d2} + x d1 f \phi; \\
\psi d_2 &= x d3 I_{d1} + x d2 I_{d2} + x d1 f \phi; \\
\psi q_1 &= x q1 I_{q1} + x q3 I_{q1} + x q1 f \phi; \\
\psi q_2 &= x q3 I_{q1} + x q2 I_{q2} + x d1 f \phi; \\
I_f &= \beta q_1 I_{d2} + I_{q2}; \quad U_2 = k_1 U_1; \\
M &= \psi d1 I_{q1} + \psi d2 I_{q1} - \psi q1 I_{d1} - \psi q2 I_{d2}
\end{align*}
\]

In the equations the motor variables are denoted as: \(R_2 = r_2 + r_1\) is the cumulative active resistance of through stator winding, bridge rectifier, and excitation winding (that is, electric circuit of through winding); \(x_{q2} = x q1 + x q1\) is the cumulative induction resistance of through stator winding, bridge rectifier, and excitation winding in the coordinated; \(x_{q2} = x q1 + x q1\) is the cumulative induction resistance of through stator winding, bridge rectifier, and excitation winding in the coordinate \(q; k_1\) is the ratio of secondary transformer voltages upon idle run; the variables are denoted by capital letters, the same as in equation 1.

Rectifier–excitation winding assembly in the equations of static mode is presented by passive assembly with active induction resistance (parameters \(r_1\) and \(x_1\)).

### 4 Discussion

The set of equations 2 contains non-linear equations of momentum and excitation current, this set is non-linear and has no standard solutions. It can be solved by particular solution algorithm and by methods of mathematical simulation. At present applied simulation proposes various forms and simulating software for electromechanic systems. The transformer–SMTW assembly could be most reasonably studied using Matlab–Simulink (Strizhkov & Beglyarov, 2009; Strizhkov et al., 2012a; Strizhkov et al., 2013; Oskin, 2014; Strizhkov et al., 2014; Strizhkov & Chesnyuk 2015).

The variables \(P, Q = f (\Theta)\) in the theory of synchronous machines are considered as angular properties. Comparison of experimental data with simulated data (Figure 2) in the range of \(0 < \Theta < 0.5\) revealed deviation in active power not higher than 5% and in reactive power not higher than 7% of experimentally measurements.

![Figure 2 Angular properties of motor at various voltages](http://www.jebas.org)
Conclusion

This article presented mathematical description of rational electric drive of portable irrigation pumps on the basis of transformer–synchronous motor assembly, which allows to determine the required practical properties of the considered drive using mathematical methods or to construct mathematical model of electric drive in static and dynamic operation modes.

For pump drive of portable pump assemblies intended for artificial irrigation or reclamation of agricultural or other lands in the districts with well-developed power supply overhead lines with the voltage above 1000 V it would be reasonable to use the proposed electric drive with transformer–synchronous motor assembly developed by Kuban state agrarian university. The design of the electric drive allows to use the set of electric control and protection equipment with the voltage below 1000 V and provides minimization of current in supply line as a function of operation of pump assembly and minimization of power loss in supply line and own equipment of pump assembly.

References


Oskin SV (1996) Povyshenie nadezhnosti elektroprivoda sel'skokhozyaistvennykh mashin [Reliability improvement of electric drive of agricultural machinery]. Mehanizatsiya i Elektrifikatsiya Sel'skogo khoziaistva 3: 19


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MOLECULAR IDENTIFICATION AND GROWTH INHIBITION OF SOME HUMAN PATHOGENIC BACTERIA ISOLATED FROM KING FAHAD GENERAL HOSPITAL, JEDDAH

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KEYWORDS
Antibacterial activity
Actinomycetes
Streptomyces
Human pathogenic bacteria
Saudi Arabia

ABSTRACT

Antimicrobial properties of bacterial antagonist against human pathogenic bacteria have become a field of increasing importance in the medical sector. The present study has been carried out to identify the antimicrobial properties of actinomycetes bacteria against five human pathogenic bacteria viz., Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Acinetobacter baumannii and Escherichia coli isolated from many wound swabs, from different service units of King Fahad General Hospital (KFGH), Jeddah. These isolated bacterial isolates were identified by using morphological and physiological characters along with molecular techniques. The antibacterial activities of five actinomycetes species along with Ampicillin (5µg/ml) as positive control were also determined against the multidrug resistant S. aureus, S. pyogenes, P. aeruginosa, A. baumannii and E. coli by using disc diffusion assay. Result of study revealed that among the tested five actinomycetes species, Streptomyces 5 (St 5) was highly effective against P. aeruginosa, S. aureus, S. pyogenes and E. coli while it showed weak activity against A. baumannii. As compared to this, Streptomyces 2, 3 and 4 showed moderate antibacterial activities while Streptomyces 1 showed the lowest activity. Further, all the tested Streptomyces extracts and Ampicillin were found weak against the multi-drug resistant A. baumannii. In conclusion, actinomycetes especially genus Streptomyces can be used as a safe and effective source against multidrug resistant bacteria.

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Molecular identification of some human pathogenic bacteria

1 Introduction

Excess use of antibiotic, not only developed multidrug resistance in human pathogenic microorganisms. Indiscriminate uses of these drugs were leading to the selection of the bacterial pathogens, resistant to multiple antibiotics (Grasso et al., 2016). Resistance in bacteria against multiple drugs was due to the accumulation of multiple resistance genes against different drugs on the plasmids and/or the increase in gene expression that code for multidrug efflux pumps (Service, 1995, Nikaido, 2009). In addition to this, sometimes excess use of antibiotics are associated with various adverse effects such as hypersensitivity, immune-suppression and allergic reactions (Ahamed et al., 1998). Further, certain bacterial strains developed mechanisms or produced substances which block the action of antibiotics or change their target or ability to penetrate cells (Ali et al., 1995). Therefore, disease causing microbes that have become resistant to antibiotics are increasing public health problems and this forced scientists to search alternative to antibiotics which have good bactericidal activities.

Actinomycete metabolites have great bactericidal effects and therapeutic values against wide range of microorganisms. Further, antimicrobial activities of these actinomycetes are depending on the method of extraction. These metabolites can be used as alternative drugs with antibacterial or antifungal or antioxidant activities which protect the host from cellular oxidation (Singh et al., 2014). Zothan et al. (2017) reported that methanolic extract of S. cyaneofuscatus inhibited the growth of E. coli, P. aeruginosa, Micrococcus luteus S. aureus, and Candida albicans with IC50 ranged from 2.1-43.63 μg/ml. This antimicrobial activity may be due to the interaction of these metabolites with bacterial cell surface and causing structural changes, damaging, disturbing vital cell functions such as permeability, depressing the activity of respiratory chain enzymes, and finally leading to cell death (Demain, 1999, Ueda & Beppu, 2017).

The aim of the present study was to evaluate the antimicrobial activities of some actinomycetes against multidrug resistant bacteria, isolated from local hospital.

2 Materials and Methods

All the required chemicals for this study were purchased from Merck, Germany and Sigma– Aldrich, USA.

2.1 Collection and isolation of bacterial isolates:

Sterile cotton swabs, moistened with sterile saline, were used to obtain samples from different patient wounds at King Fahad General Hospital, Jeddah, Saudi Arabia. Preliminary experiments were carried out to select antibiotics resistant bacteria, which have resistant against at least 3 most commonly used antibiotics. Wound samples were collected in two swabs, among these, one swab was used for Gram staining and the other one was inoculated on Mac-conkey and blood agar plates for isolating the different bacterial pathogens. Isolated Acinetobacter baumannii was identified by culturing this bacterium on Leeds Acinetobacter medium (Hardy Diagnostics, USA, catalog no. G261). The inoculated plates were incubated at 37°C overnight and examined for growth. Isolated colonies were identified on the basis of morphological characteristic and biochemical tests including Catalase, Oxidase, Urease, Indole, Methyl Red, Voges Proskauer and Citrate Utilization tests as described by Koneman et al., (2005). The pure cultures of the tested bacteria were maintained on Nutrient agar slants at 4°C and in Glycerol Broth (16 ml glycerol + 84ml nutrient broth) at -70°C.

2 Molecular identification of the bacterial isolates

For PCR, required DNA templates were obtained from overnight bacterial cultures that were collected, re-suspended in 200 ml of sterile distilled water, and boiled for 1 minute (Usein et al., 2009). Species specific gene uid A for E. coli, ecfX for Pseudomonas aeruginosa, oxa-51 for A. baumannii and 16S rRNA for Staphylococcus aureus and Streptococcus pyogenes were used for the identification the detection particular bacterium (Clifford et al., 2012). Primers for uidA (E. coli) were prepared as according to the method described by Moyo et al. (2007), while oxa-51 (A. baumannii) primers were according to Brown & Amyes (2005), and ecfX primers (P. aeruginosa) were according to Clifford et al. (2012). The universal 16S rRNA primers were prepared as according to Tork et al., (2016). PCR performed in the Takara thermal cycler (Takara, Tokyo, Japan) and the PCR products were separated by using 1.5% agarose in Tris–acetate–EDTA buffer at 100V and visualized. O’Range Ruler™ 100+500 bp DNA Ladder, ready-to-use was included in each run.

2.3 Isolation of actinomycetes

The five Actinomycete isolates were obtained from Department of Botany, Faculty of Science, Tanta University, Egypt. These collected isolates were identified as Streptomyces exfoliatus (St 1) and S. niveus (St 2) as per guide line given by Agwa et al. (2000), while S. anulatus SM 21 (St 3) and S. coelicolor SM 1 (St 4) were identified as guideline given by Aly et al., (2011). Further, S. exfoliates LP10 (St 5) was identified as method given by Aly et al. (2012).

2.4 Estimation of Antimicrobial activity by agar well diffusion method

Standard agar well diffusion method was carried out to detect the activity of Actinomycete filtrates against some selected
Each actinomycete isolate was cultured in 50 ml of starch nitrate broth for 2 days at 25°C and healthy cells were collected and used to inoculate in 50 ml of production broth medium, composed of (g/l) 10 g glucose, 1.0 g K$_2$HP0$_4$, 1.0 g MgSO$_4$, 7H$_2$O, 1.0 g NaCl, 1.2 g NH$_4$N0$_3$, and 2.0 g CaCO$_3$ (Agwa et al., 2000). After 7 days, cell free culture filtrate was collected for each organism and extracted with the same volume of methanol (V/V). The methanol extract was dried, dissolved in DMSO and the antibacterial activities were determined on nutrient agar on which 100 µl of the overnight suspension of each bacterial pathogen was spread. Using sterile cork borer (8 mm), agar well was made and filled with 50µl of the prepared actinomycete extract in DMSO or 5µl (5 mg/ml) of the standard antibiotic (positive control) under aseptic conditions. Then, the plates were kept in refrigerator for 2 hours before incubation to permit diffusion of the extract and incubated at 37°C for 24 hr, this was followed by the examination of antibacterial activity (diameter of inhibition zone, mm). Duplicate plates were used for each tested bacterial pathogen.

### 3 Results and Discussion

In wound infections, many aerobic and anaerobic bacteria are found which lead to morbidity or prolonged hospitalization (Bowler et al., 2001). From last few decades, the emergence of antibiotic resistance in pathogenic isolates of human pathogenic bacteria is a dangerous threat to worldwide public health. This became more serious in case of Gram-negative bacteria such as A. baumannii, E. coli and P. aeruginosa and Gram-positive S. aureus which were associated with pus and wound infections, due to extensive prescription and inadequate dose of antibiotics (Rice, 2006, Misic et al., 2014). Rapid spread of multidrug resistant bacteria poses a serious threat to public health due to the limited treatment options and the decrease in the discovery of new classes of antibiotics (Iredell et al., 2016).

Bacterial identification is very important to reduce morbidity and mortality in patients. Good identification of the bacterial pathogens improves treatments options and lead to successful therapy (Barenfanger et al., 1999). In this study, five human pathogenic bacteria viz., Staphylococcus aureus, Streptococcus pyogenes, P. aeruginosa, A. baumannii and E. coli were isolated from wound of regular visiting patients of KFGH and identified by using conventional as well as molecular identification techniques. Among the isolated microorganisms, 16S rRNA primer was used for the identification of S. pyogenes and S. aureus while ecfX, uida and bla oxa-51 were used for identification of P. aeruginosa, E. coli and A. baumannii respectively (Figures 1 and 2). The results of bacterial identification by using 16S rDNA are in agreement with the findings of Weisburg et al. (1991). Similar findings was reported by Zhang et al. (2014) those who reported that predominance of E. coli, S. aureus, and P. aeruginosa in pus samples from patients with severe intra-abdominal infection. In another study, S. aureus was identified as the dominant bacterial species from wounds and this was followed by P. aeruginosa, P. mirabilis, E. coli (Lorrot et al., 2014). Acinetobacter baumannii is a nosocomial pathogen which affects critically ill patients and has increased importance. Further, Aly et al. (2014) recorded resistance nature of A. baumannii against various commonly used antibiotics.

![Figure 1 A: Agarose gel electrophoresis of PCR product of amplified uida gene (623 bp). Lane M: Marker, Lane 1 positive control, Lane 2-5: positive strains for uida gene. B: Agarose gel electrophoresis of PCR product of amplified ecfX gene. Lane M: Marker, Lane 1: positive control for ecfX gene. Lane 2-3 positive strains for ecfX gene.](image1)

![Figure 2 A: Agarose gel electrophoresis of amplified PCR product of oxa-51 gene B: PCR product 16S rRNA gene. Lane M: Marker, Lane 1 positive control, Lane 2-5: positive strains for 16S rRNA gene.](image2)
Molecular identification of some human pathogenic bacteria

Antibiotics. There is an evidence that carbapenemase gene was naturally occurred in *A. baumannii* (Al Masoudi et al., 2013).

Actinomycetes have highly significant roles in drug discovery and have provided bioactive secondary metabolites with interesting activities such as antimicrobial, antiviral and anticancer. An antimicrobial activity of actinomycete extracts varies with the tested bacterial isolates and the used pathogens (Bruntnor et al., 2005, Bhave et al., 2013). In this study, methanolic extracts of the five *Streptomyces* species were found affective against the tested resistant bacteria (Figure 3) and *S. exfoliates* (St 5) was the most affective one with highest inhibition zone against *P. aeruginosa* (27 mm), *E. coli* (25 mm), *A. baumannii* (18mm), *S. pyogens* (25 mm) and finally *S. aureus* (24 mm) as shown in Table 1. The growth and morphology of the *S. exfoliates* were shown in Figure 4. From past seven decades, the antibiotics obtained from actinomycetes have many successes. Actinomycetes are a sustained mine of new antibiotics with many mode of action that kills the pathogens without harming the host. Further, erythromycin, tetracyclines, aminoglycosides, daptomycin, tigecycline are the most common antibiotics which obtained from various actinomycetes. Among the identified bioactive compounds that have been obtained so far from microbes, 45 % are produced by actinomycetes while 38 % by fungi and 17 % by unicellular eubacteria (Mahajan & Balachandran, 2012). Result of study revealed that all the tested methanolic extracts of *Streptomyces* have good antibacterial activity against all tested bacteria except for *A. baumannii* and among the tested microbes, maximum antibiotic activity was reported against *P. aeruginosa* and *S. pyogenes* while in case of conventional antibiotic, maximum activity of Ampicillin was reported against *S. pyogenes* with inhibition zone of 19 mm. The antimicrobial ability of *Streptomyces* might be referred to their effect on the cell wall of the bacteria which finally causing the destruction of cell wall and death of bacteria. Also, the extracts interact with the building elements of the outer membrane and might cause structural

Figure 3 The effect of methanol extracts of the five tested *Streptomyces* species on *S. aureus* (A), *E. coli* (B) and *S. pyogenes* (C).

Figure 4 The selected *Streptomyces* isolate (St 5) under light microscope (A), scanning electron microscope (B) and on starch nitrate agar (C).
changes; degradation and finally cell death. Also, antibacterial activities of the extracts could be due to the susceptibility of pathogens cell wall and toxicity in addition to the change in membrane potential, inhibition of ATP syntheses and levels leading to collapse of all biological process (Cui et al., 2012). Similar results were reported by Srinivasan et al. (2009) who reported strong antibacterial activity against Gram-positive than the gram-negative bacteria, this may be due to the structure differences in cell wall structure, where Gram negative has outer membrane which block the penetration of antibiotics and plant extracts and making them resistant against various antibiotic substances. Extract of Strepomyces 5 was more effective as compared to other methanolic extracts of actinomycetes and it is equally effective against all the tested microbes. Further, moderate antibacterial activities were recorded for the methanolic extracts of Streptomyces 2, Streptomyces 3 and Streptomyces 4 while Streptomyces 1 showed the least antibacterial activity. Antibiotics from actinomycetes may affect essential processes in bacterial cell wall biosynthesis and change bacterial structures and functions. Antibiotics essentially target cell membrane protein translation, RNA transcription, DNA replication and synthesis (Ueda & Beppu, 2017). Glycopeptides are a class of drugs produced by Actinomycetes bind to the dipeptide D-alanyl–D-alanine of cell wall of Gram-positive bacteria preventing the addition of new units to the peptidoglycan and inhibiting the peptidoglycan synthesis (Demain, 1999, Ueda & Beppu, 2017).

In conclusion, the antibiotics from actinomycetes must be intensively studied, their sources, structures, activities, and mode of actions and further research is required to use these safe and effective extracts in alternative medicines.

### Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

### References


Table 1 The antibacterial activity (diameter of inhibition zone mm) of the methanolic extracts of five actinomycete isolates against some bacterial pathogens

<table>
<thead>
<tr>
<th>Tested actinomycetes</th>
<th>Zone of inhibition (mm) ± SEM in various bacterial isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td>Streptomyces 1</td>
<td>14±2.1</td>
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<tr>
<td>Streptomyces 2</td>
<td>25±1.7*</td>
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<tr>
<td>Streptomyces 3</td>
<td>20±1.5*</td>
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<tr>
<td>Streptomyces 4</td>
<td>14±2.3</td>
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<tr>
<td>Streptomyces 5</td>
<td>27±1.6*</td>
</tr>
<tr>
<td>Ampicillin (control)</td>
<td>17±1.6</td>
</tr>
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</table>

*: Significant results compared to control


SELECTING FOR FOOD-FEED TRAITS IN EARLY AND LATE MATURING LENTIL GENOTYPES (*Lens culinaris*)

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KEYWORDS
Lentil, Straw
Nutritive value
Genotypic variation
Food-feed traits

ABSTRACT

To explore genetic and environmental variability of food-feed traits in lentil genotypes, straws of 78 elite genotypes and 4 checks of early and late maturing lentil types were evaluated for their nutritive value and potential trade-offs of the nutritive parameters with straw yield and grain yield. Further, effects of genotypic and environmental sources on variation in the nutritive value were also determined. Straw nutritive traits were analyzed by a combination of conventional laboratory techniques and Near Infrared Reflectance Spectroscopy. Results from eight trials carried out across 3 different sites in Ethiopia showed highly significant genotypic variation (P<0.05) in grain yield, straw yields and straw nutritive traits. This confirmed the existence of exploitable genetic variation in these traits. Similarly, the relationship between grain yield and straw yield was positive. The correlation between grain yield and nutritive parameters of straw was insignificant or negative. The correlation between maturity types and straw traits was either neutral or negative. Genotype by environment interactions were significant (P<0.05) for straw yield and nutritive traits indicating that variation in the traits is dependent of environment. It is possible to develop genotypes with a combination of food-feed traits from early and late maturing lentil types to address the high demand for grain and livestock fodder in various agro ecological zones in mixed crop-livestock farming systems using appropriate breeding approaches.

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1 Introduction

Lentil (Lens culinaris) is an annual cool-season legume primarily used for human consumption and the straw used as livestock feed. The major lentil-growing countries of the world are Canada, India, Turkey, Australia, USA, Nepal, Bangladesh, China and Ethiopia (FAOSTAT, 2014). Ethiopia covers 69.4% of the areas devoted to lentil and 81.5% of the total production in Africa (FAOSTAT, 2014). In 2016, lentil was produced by over 850,000 small holder households and covered about 114,000 ha with an average productivity of 1.5t/ha (CSA, 2017). The International Center for Agricultural Research in the Dry Areas (ICARDA) has a world mandate for lentil improvement and is working with national programs across several countries to enhance production and productivity, increase incomes of farmers and provide lentil to consumers for food and nutritional security. Understanding of genotype by environment interactions, local constraints to production and consumer requirements for seed as food and straw as feed, has been a guide to the national and international breeding programs to develop new genetic materials for various agro-ecologies in West Asia, North and East Africa region. In the predominant mixed crop-livestock systems of Ethiopian highlands, lentil straw is among fibrous crop residues from cereals and legumes that constitute large proportions of livestock feeds, particularly, during dry seasons (Valbuena et al., 2012). Lentil straw has been reported to have better degradation in the rumen (Singh et al., 2011) and higher concentrations of crude protein and digestible energy (Hadjipanayiotou, 1997) than cereal straws routinely used as fodder. Similarly, high acceptability and digestibility of lentil straw in ration of livestock was also reported by Abbeddou et al. (2011). Since lentil straw is valorized mainly in ruminant nutrition, it is important that its nutritive value is as high as possible. This offers an opportunity for livestock nutritionists and lentil breeders to collaboratively explore the feasibility of genetic enhancement of not only grain traits but also straw yield and its nutritive value. Exploitable genetic variability among lentil genotypes has been reported for straw yield (Kusmenoglu & Muehlbauer, 1998) and nutritive value (Erskine et al., 1990). However, these studies were undertaken in single environments, thus, there is need to support them with additional studies across various locations and populations of different maturity types so as to draw concrete conclusions on genetic variability and food-feed trait relationships. Differences in phenological development in lentil contributes 45-60% of the variation in grain yield (Siddique et al., 1998; Shrestha et al., 2006). Ghanem et al. (2015) indicated that selection and breeding for lentil accessions should consider changes in plant phenology and/or sowing dates. Therefore, the current study aimed to determine the variability of straw traits and food-feed relationships in early and late maturing lentil genotypes developed for locations with varying rainfall patterns in Ethiopia. Results from this study can be further explored to select for

<table>
<thead>
<tr>
<th>Trial Code</th>
<th>Location</th>
<th>Populations</th>
<th>N Blocks</th>
<th>Genotypes</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVT-LMS-DZ*</td>
<td>DebreZeit</td>
<td>Early maturing</td>
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<tr>
<td>NVT-PE-AK*</td>
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<td>16</td>
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<tr>
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<td>DebreZeit</td>
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<td>PVT-PE-DZ†</td>
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<td>Late maturing</td>
<td>3</td>
<td>25</td>
<td>72</td>
</tr>
</tbody>
</table>

* † ‡ §: trials with different symbols differ in genotypes, PVT: preliminary variety trial; NVT: national variety trial, LMS: low moisture stress environments, PE: potential environments, DZ, DebreZeit, AK: Akaki, CD, ChefeDonsa.
2 Materials and methods

2.1 Experimental layout and lentil genotypes

Eight field trials (Table 1) were conducted in 3 sites namely Akaki (AK; 08°53’N 38°49’E), DebreZeit (DZ; 08°44’N 38°58’E), and Chefe Donsa (CD; 08°57’N 39°06’E) in Ethiopia. Akaki, DebreZeit and Chefe Donsa are located in Central Ethiopian Highlands at altitudes of 2200, 1900 and 2400 m.a.s.l and average annual rainfall reported to be 1025 mm, 851 mm and 878 mm respectively. Soils of the studied sites are vertisols. All the experimental sites and trials were preceded by wheat crops. Trials were undertaken during the main rainy season of 2014/15 cropping seasons. Elite genotypes were collected from 2014 preliminary variety trials (PVT) and national variety trials (NVT) of the Ethiopian Lentil Improvement Program. These genotypes were selected on the basis of their high grain yield, agronomic traits in potential environments (PE) and low moisture stress environments (LMS). These genotypes were selected from ICARDA breeding lines sent to the Ethiopian Institute for Agricultural Research through its international nurseries platform. A total of 82 lentil genotypes (78 elite lines and 4 checks) were evaluated for the study on food and feed traits. The 8 trials are identified by their codes (Table 1) which indicate which variety trials the genotypes were drawn from (PVT, NVT) or the Ethiopian Lentil Improvement Program. Each trial was analyzed separately according to the following environment they were planted in (PE, LMS) and the locations where they were planted (AK, DZ, CD). The design was randomized complete block design with either three or four replications (Table 1) depending on the trial. The plot size was 3.2m2 with four rows in all trials. At physiological maturity, two middle rows were manually harvested. The biomass was air-dried in the field and threshed for seed and straw yields. Straw yield was calculated based on harvest index. Sub-samples of 500 g of representative straw were taken from each plot for chemical composition and digestibility analyses.

2.2 Nutritive analysis of straw

Laboratory analyses were undertaken at the Animal Nutrition Laboratories of the International Livestock Research Institute (ILRI), Addis Ababa, Ethiopia and Patancheru, India. After oven-drying at 100°C for 24 h, straw samples were ground to pass through a 1 mm sieve mesh. The samples were analyzed using Near Infrared Reflectance Spectroscopy (NIRS) and conventional wet chemistry. The NIRS instrument, Foss Forage Analyzer 5000 with the software package WinISI II in the 1108-2492 nm spectra range was used to scan lentil straw samples and a good-of-fitness lentil NIRS equation was used for the prediction of dry matter (DM), nitrogen, neutral detergent fiber (NDF) and in vitro digestibility (IVOMD). Validation of the NIRS equation was undertaken using conventional wet chemistry, whereby 20% representative samples were analyzed for DM and crude protein (CP) according to the methodology of AOAC (2000). Dry matter was determined by oven drying at 105°C overnight (method 934.01). Ash was determined by burning in a muffle furnace at 500°C overnight (method 942.05). Nitrogen content was determined by Kjeldahl method using Kjeldahl (protein/nitrogen) Model 1026 (Foss Technology Corp.), (method 954.01). A conversion factor of 6.25 was used to convert nitrogen to crude protein. Neutral detergent fiber, acid detergent fiber (ADF) and lignin were determined as described by Van Soest & Robertson (1985). Neutral detergent fiber did not involve use of heat stable amylase and the result was expressed exclusive of residual ash. Acid detergent fiber was expressed without residual ash. Lignin was determined by solubilisation of cellulose with sulphuric acid. In vitro organic matter digestibility was measured in rumen microbial inoculum using in vitro gas production technique. The buffer solution was prepared according to the method described by Menke & Steingass (1988). Rumen fluid was collected prior to morning feeding using a vacuum pump from three ruminally cannulated cows fed a total mixed ration of grass hay (790 g/kg), wheat bran (203 g/kg), salt (3.2 g/kg) and a mineral and vitamin mixture (4.6 g/kg) on a DM basis. Use of cows was assessed and approved by the Environmental and Occupational Health and Safety Unit of ILRI. The rumen fluid from the cows was composited (1:1, v/v), filtered through four layers of cheesecloth, and added to the buffer solution (1:2, v/v), which was maintained in a water bath at 39°C under continuous flushing with CO2. The buffered rumen fluid (30 ml) was pipetted into 100 ml syringes containing 0.2 g of sample and immediately placed into a water bath at 39°C. Gas production was recorded after 24 hours of incubation and used to calculate IVOMD according to Menke et al. (1979) equations suitable for legume hays as follows:

\[ \text{IVOMD (g/kg)} = 14.88 + 0.889\text{GP} + 0.45\text{CP} + 0.0651\text{XA} \]

Where GP: 24 h net gas production (ml/200 mg); CP: Crude protein (g/kg DM); XA: Ash content (g/kg DM).

2.3 Calculations and statistical analysis

A general linear model was used to test the effect of variety on grain yield, straw yield and nutritive value parameters of straw. Each trial was analyzed separately according to the following model (Ertilo et al., 2013):

\[ Y_{ij} = \mu + B_i + G_j + E_{ij} \]
Where: \( Y_{ijk} \): grain/straw traits, \( \mu \): overall mean, \( B_i \): effect of the block i, \( G_j \): effect of the genotype j, \( E_{ij} \): random error. To evaluate the effect of location and genotype-location interaction (GxL), data from all trials combined and analyzed according to the following model (Ertiro et al., 2013):

\[
Y_{ijk} = \mu + G_i + L_j + GL_{ij} + B(L)_{ij} + E_{ijk}
\]

Where: \( Y_{ij} \): grain/straw traits, \( \mu \): overall mean, \( G_i \): effect of the genotype i, \( L_j \): effect of location j, \( GL_{ij} \): effect of interaction between the genotype and location, \( B(L)_{ij} \): effect of block i within location j, \( E_{ijk} \): random error. Relationships between grain and straw traits were calculated separately for each trial using Pearson's correlation.

Principal component analysis (PCA) was used to simultaneously evaluate mean differences to quantify the contribution of each constituent to the variation in nutritive values. Crude protein, NDF, and IVOMD are major determinants of nutritive value of straw, thus were included in PCA. Neutral detergent fiber is reported to negatively correlate to dry matter intake (Horrocks & Vallentine, 1999), while IVOMD positively correlates to ME. The sign and magnitude of eigenvectors were examined for their relevance in explaining the nutritive value of straw. All eigenvectors were standardized to unite the variance. Principle component analysis was performed for each group of genotypes separately.

All statistical procedures were carried out using Statistical Analysis System software (SAS, 2012).

### 3 Results

#### 3.1 Variations of genotypes for time to maturity, grain and straw yields

Grain and straw yields of individual trials showed significant genotypic variations (P<0.05) in all trials (Table 2). Mean squares for location and GXL interaction are presented in Table 4. The effect of location and GXL interaction on grain and straw yields was significant (P<0.05) in trials of late maturing genotypes (Table 4). The highest grand means for both grain (2.82 t/ha) and straw yields (6.43 t/ha) were from PVT-PE-CD. The lowest grand means for grain (0.982 t/ha) and straw yields (3.07 t/ha) were from NVT-PE-DZ and NVT-PE-AK respectively. Considering genotypes in individual trials, the widest range of grain yield from 0.28 t/ha to 2.61 t/ha was from NVT-PE-DZ while PVT-PE-CD showed the widest range of straw yield from 3.22 t/ha to 9.33 t/ha.

### Table 2 Means and ranges of grain yield, straw yield and days to maturity of lentil genotypes in 8 trials across 3 locations.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Grain yield (t/ha)</th>
<th>Straw yield (t/ha)</th>
<th>Maturity (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Early maturing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NVT-LMS-DZ</td>
<td>0.482-2</td>
<td>1.37</td>
<td>0.08</td>
</tr>
<tr>
<td>PVT-LMS-DZ</td>
<td>0.74-2.04</td>
<td>1.25</td>
<td>0.16</td>
</tr>
<tr>
<td>Late maturing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NVT-PE-AK</td>
<td>0.72-2.72</td>
<td>1.65</td>
<td>0.20</td>
</tr>
<tr>
<td>NVT-PE-CD</td>
<td>1.62-3.11</td>
<td>2.24</td>
<td>0.10</td>
</tr>
<tr>
<td>NVT-PE-DZ</td>
<td><strong>0.28-2.61</strong></td>
<td>0.982</td>
<td>0.15</td>
</tr>
<tr>
<td>PVT-PE-AK</td>
<td>0.404-2.32</td>
<td>1.62</td>
<td>0.16</td>
</tr>
<tr>
<td>PVT-PE-CD</td>
<td>1.91-3.71</td>
<td>2.82</td>
<td>0.32</td>
</tr>
<tr>
<td>PVT-PE-DZ</td>
<td>0.401-2.01</td>
<td>1.1</td>
<td>0.16</td>
</tr>
</tbody>
</table>

†: P>0.05 otherwise P<0.05, PVT: preliminary variety trial; NVT: national variety trial, LMS: low moisture stress environments, PE: potential environments, DZ, DebreZeit, AK: Akaki, CD, ChefeDonsa.
Maturity type showed significant genotypic variation in all trials except NVT-PE-AK. The effect of the location on maturity type was significant in PVTs and NVTs. Considering genotypes in individual trials, the widest range of maturity was found in NVT-PE-DZ (40 days).

3.2 Variations in straw nutritive traits

Table 3 shows trial means and ranges for straw nutritive traits (CP, NDF and IVOMD). The variation among genotypes for CP, NDF and IVOMD was significant (P<0.05) in all trials except NVT-LMS-DZ and PVT-PE-AK. The effect of location and GXL on nutritive traits was significant (P<0.05) for late maturing genotypes (Table 4). The means of trials for CP ranged from 57.4 g/kg DM in PVT-PE-CD to 114 g/kg DM in PVT-PE-DZ. The highest range within trials was found in PVT-PE-DZ with 64.5 g/kg DM. The genotype with highest (148 g/kg DM) and lowest CP (38.2 g/kg DM) was observed from NVT-PE-DZ and PVT-PE-CD respectively. The grand means of trials for IVOMD ranged from 55% in NVT-LMS-DZ to 58.4% in NVT-PE-AK (Table 3). The highest range within trials was found in PVT-PE-AK from 52.3% to 61.9%. The genotypes with highest (62.4%) and lowest (50.2%) IVOMD were observed from NVT-PE-AK.

Table 4 Mean squares of location and genotype by environment interaction for grain yield, straw yield and nutritive traits of straws in 6 late maturing genotypes trials

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Source of variance</th>
<th>Location</th>
<th>G×L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grain yield</td>
<td>63.8</td>
<td>0.594</td>
<td></td>
</tr>
<tr>
<td>Maturity</td>
<td>18726</td>
<td>2398†</td>
<td></td>
</tr>
<tr>
<td>Straw yield</td>
<td>257</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>77073</td>
<td>327</td>
<td></td>
</tr>
<tr>
<td>NDF</td>
<td>59668</td>
<td>2519</td>
<td></td>
</tr>
<tr>
<td>IVOMD</td>
<td>3517</td>
<td>1362</td>
<td></td>
</tr>
</tbody>
</table>

G×L: genotype-location interaction, CP: crude protein, NDF: neutral detergent fiber, IVOMD: in vitro organic matter digestibility, †: P<0.05 otherwise P≥0.05.
Selecting for food-feed traits in lentil genotypes

Table 5 Principle component analysis of nutritive parameters of straw

<table>
<thead>
<tr>
<th>Type</th>
<th>Statistics</th>
<th>P1</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early maturing</td>
<td>Eigenvalue (variation explained)</td>
<td>2.24(0.75)</td>
<td>0.66(0.22)</td>
</tr>
<tr>
<td></td>
<td>Eigenvectors</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>0.524</td>
<td>0.744</td>
</tr>
<tr>
<td></td>
<td>NDF</td>
<td>-0.551</td>
<td>0.667</td>
</tr>
<tr>
<td></td>
<td>IVOMD</td>
<td>0.648</td>
<td>-0.034</td>
</tr>
<tr>
<td>Late maturing</td>
<td>Eigenvalue (variation explained)</td>
<td>1.99(0.66)</td>
<td>0.879(0.29)</td>
</tr>
<tr>
<td></td>
<td>Eigenvectors</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CP</td>
<td>0.358</td>
<td>0.918</td>
</tr>
<tr>
<td></td>
<td>NDF</td>
<td>-0.641</td>
<td>0.373</td>
</tr>
<tr>
<td></td>
<td>IVOMD</td>
<td>0.678</td>
<td>-0.131</td>
</tr>
</tbody>
</table>

Table 6 Pearson correlation between grain yield and days to maturity with straw traits

<table>
<thead>
<tr>
<th>Trials</th>
<th>Straw yield</th>
<th>Grain yield</th>
<th>Days to maturity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CP</td>
<td>NDF</td>
</tr>
<tr>
<td>Early maturing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NVT-LMS-DZ</td>
<td>0.391</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PVT-LMS-DZ</td>
<td>0.372</td>
<td>ns</td>
<td>-0.672</td>
</tr>
<tr>
<td>Late maturing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NVT-PE-AK</td>
<td>ns</td>
<td>-0.772</td>
<td>-0.722</td>
</tr>
<tr>
<td>NVT-PE-CD</td>
<td>ns</td>
<td>-0.473</td>
<td>-0.512</td>
</tr>
<tr>
<td>NVT-PE-DZ</td>
<td>0.413</td>
<td>-0.381</td>
<td>-0.652</td>
</tr>
<tr>
<td>PVT-PE-AK</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PVT-PE-CD</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PVT-PE-DZ</td>
<td>0.452</td>
<td>ns</td>
<td>-0.691</td>
</tr>
</tbody>
</table>

CP: crude protein, NDF: neutral detergent fiber, IVOMD: in vitro organic matter digestibility, ns: non-significant at P<0.05 otherwise P≤0.05, PVT: preliminary variety trial; NVT: national variety trial, LMS: low moisture stress environments, PE: potential environments, DZ: DebreZeit, AK: Akaki, CD, ChefeDonsa.

and NVT-PE-DZ respectively. The grand means of trials for NDF ranged from 445 g/kg DM in NVT-PE-AK to 521 g/kg DM in NVT-LMS-DZ. The highest range within trials was found in NVT-PE-DZ with 182 g/kg DM. The genotype with highest (617 g/kg DM) and lowest NDF (390 g/kg DM) was from NVT-PE-DZ and NVT-PE-AK respectively.

### 3.3 Principle component analysis

Results of PCA are showed in Table 5 where PC1 and PC2 cumulatively accounted for more than 90% of the variation. In all populations, PC1 accounted for 64% to 76% of the variance, which was the highest among the PCAs. An examination of the eigenvectors showed that PC2 consistently showed negative sign for IVOMD and a positive sign for NDF in all genotypes, whereas PC1 showed a positive sign for IVOMD and a negative sign for NDF. In all genotypes populations, IVOMD had the highest magnitude among the eigenvectors followed by NDF then CP.

### 3.4 The correlation between maturity, grain and straw yields

The correlation between grain yield and straw yield was not consistent across trials as shown in Table 6. Grain yield correlated moderately and positively to straw yield in early maturing
genotypes. In late maturing genotypes, grain yield related moderately and positively to straw yield in DebreZeit location. The correlation between grain yield and IVOMD was negative and strong in NVT-PE-AK and negative and moderate in NVT-PE-CD and NVT-PE-DZ. Crude protein correlated strongly and inversely to grain yield in PVT-LMS-DZ, NVT-PE-, NVT-PE and PVT-PE-DZ. The correlation between grain yield and CP was inverse and moderate in NVT-PE-CD. Grain yield correlated inversely and moderately to NDF in PVT-LMS-DZ, NVT-PE-AK and NVT-PE-CD. A weak and positive correlation between grain yield and NDF was found in PVT-PE-DZ. Maturity correlated strongly to straw yield in PVT-LMS-DZ, NVT-PE-CD and PVT-PE-CD. PVT-PE-AK showed moderate and positive correlation between maturity and straw yield. Maturity showed either insignificant or positive correlation with CP and IVOMD.

4 Discussion

4.1 Grain and straw yield

In mixed crop-livestock systems, increasing crop residue biomass tends to improve milk and meat production of livestock. The wide genetic variation in grain and straw yields in all trials in this study are in agreement with what was reported by Bidinger et al. (2010) in pearl millet and Ertri et al. (2013) in maize. The results of this study showed that yields of grain and straw in late maturing lentil genotypes were affected by location. Ertri et al. (2013) showed similar GXL interactions in grain and stover yield of maize. It implies the possibility of increasing both grain and straw yield of lentil by improving agronomic practices. Though lentil improvement programs target grain yield as a primary trait, the large variability that exists among genotypes for both grain and straw yield as shown in this study suggests that breeding programs can exploit the variability for improvement of grain and straw production. However, such programs need to be location-specific. The GXL interaction in early maturing genotypes of lentil was not studied in this study. Thus, further studies need to identify the relationship between the environment and grain and straw production of early maturing genotypes of lentil.

4.2 Straw nutritive traits

All genotypes in the two maturity groups showed a wide range in nutritive value parameters of straw. Others studies in maize (Tolera et al., 1998; Ertri et al., 2013), durum wheat (Tolera et al., 2008), pearl millet (Blummel et al., 2007; Blummel et al., 2010a) and sorghum (Blummel et al., 2010b) also showed wide variations in straw nutritive values. Crude protein content in feeds is important to achieve optimal rumen activity. Risco & Melendez (2011) recommended that a minimum of 70-80 g/kg and 100-110 g/kg of CP in rations of non-lactating and lactating animals respectively, are required to sustain rumen fermentation. The grand means of CP in 7 trials out of 8 were higher than 70 g/kg. Moreover, genotypes with the lowest CP across 5 trials had values higher than 70g/kg while grand means of CP of 3 trials exceeded the minimum recommendation for lactating animals. The nutritive value of lentil straw was reported to be greater than vetch and wheat straw and close to the nutritive value of alfalfa hay when fed to Awassi sheep (Haddad & Husein, 2001).The highest CP content in this study (148 g/kg) is equivalent to that of good quality hay. The wide genetic variation in NDF is indicative of wide variation in potential dry matter intake as NDF and dry matter intake of feed are closely related. Accordingly, the nutritive value of lentil straw can be improved by exploiting the natural genotypic variations. The significant effect of location on nutritive value of straw suggests the possibility to improve nutritive value of lentil straw by manipulating agronomic factors. All nutritive parameters had significant GXL interactions indicating that the genotypic variation in the traits is dependent of environment. This interaction should be considered in any improvement program that targets increasing the nutritive value of lentil straw.

4.3 Principle component analysis

Principle component analysis was carried out to investigate the possibility of minimizing parameters representing nutritive value of lentil straw. Crude protein and IVOMD are expected to contribute positively to nutritive value and are, therefore, expected to have positive signs. Similarly, NDF is expected to have a negative sign because it contributes negatively to nutritive value. PCI showed that NDF and IVOMD can represent the nutritive value of both early maturing and late maturing genotypes as their eigenvectors were both of high magnitude and had negative and positive signs respectively. However, NDF is preferred because the procedure to determine it in the laboratory is simpler compared to IVOMD. Therefore, NDF can represent the nutritive value of lentil straw and can be used for screening genotypes for nutritive value. The nutritive value of lentil straw can be improved by breeding genotypes which have straw with low NDF content. Our results agree with Alkhtib et al. (2016) who found out that principle component analysis score can be used to summarize the nutritive value of faba bean straw.

4.4 Relationship between grain and straw traits

Grain yield is the main trait targeted by improvement programs of lentil. Thus, it is imperative that selection of lentil genotypes for better straw traits does not compromise grain yield. The
correlation between grain yield, straw yield and nutritive value of straw was not consistent across trials. However, no negative correlation between grain yield and straw yield was found in any trial. That means there are no tradeoffs between grain yield and straw yield, thus, breeding lentil for increased grain production would not have detrimental effects on straw yield. However, straw yield cannot be predicted from grain yield because the correlations were moderate ($R^2 \leq 0.2$). The correlations between grain yield and nutritive value parameters were also inconsistent. There were trials which exhibited insignificant correlations with grain yield indicating that there is potential to identify genotypes within particular environments that have no tradeoffs between increasing grain yield and nutritive value of straw. These results concur with findings of previous studies reported in faba bean (Alkhtib et al., 2016), pearl millet (Blummel et al., 2010a) and sorghum (Blummel et al., 2010b) and which reported that selection of genotypes to achieve high grain yield did not depress the digestibility and energy content of straw. Most of the trials showed positive and high correlations between maturity type and straw yield as well as maturity type and nutritive value. That means breeding for early maturity could be associated with a decline in straw yield and nutritive value parameters. However, neutral relations in some of the trials indicate the possibility of finding early maturing genotypes with superior food-feed traits.

Conclusion

Mixed crop-livestock systems, the backbone of both animal and crop agriculture in developing countries, are characterized by smallholder farmers. In these closely integrated production systems, particularly those in the highlands, cereal and legume straws are predominantly used to supplement feed for livestock. As important as it is to increase straw production, is it critical to enhance the nutritive value of the straw. Nutritive value is likely to exert effects on voluntary intake and digestibility of dry matter in livestock. Therefore, incorporating straw nutritive traits in lentil breeding programs and varietal release criteria holds promise for enhanced livestock productivity in smallholder mixed farming systems.

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Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

References


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STANDARDIZATION OF HEAVY METALS AND OIL CONTENT BY BIOLOGICAL INDICATORS IN THE SOUTHERN CHERNOZEMS OF THE TAMAN

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ABSTRACT

This paper provides an assessment of the resistance of Taman chernozems to contamination with heavy metals (Cr, Cu, Ni, Pb) and oil by biological parameters. As a rule, upon contamination of the Taman chernozems, a significant reduction was observed in the total bacterial count, activity of catalase and dehydrogenase, cellulolytic activity, abundance of the Azotobacter genus bacteria and in the radish germination intensity. The extent of the deterioration in biological properties was determined by two factors: the chemical nature of the metal and its amount in the soil. As a rule, a direct relationship was observed between the concentration of the pollutant and the degree of deterioration of the soil properties in this study. The metals studied had shown different ecotoxicity with respect to the chernozems of Taman: Cr > Cu ≥ Ni = Pb Regional norms for the maximum allowable content of Cr, Cu, Ni, Pb, and oil in the Taman southern chernozems based on a disturbance of the ecological functions of soils were proposed.

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1 Introduction

In December 2018, the construction of an automobile and railway bridge between the Taman and the Crimean Peninsula through the Kerch Strait, connecting the Azov and Black Seas, will be ended. A sharp increase in traffic flow through Taman to the Crimea and the development of associated road and resort infrastructure can cause increased pollution of the soils of the Taman Peninsula with heavy metals (HMs), oil and petroleum products.

Taman has unique soils that have no analogues in the world (Val’koy et al., 2008). Before 1977, they were called chestnut chernozem, according to the ecological and genetic classification of Russian soils of 1977 - southern chernozems (Classification and diagnosis of soil of USSR, 1977). In the substantive-genetic classification of Russian soils of 2000, there was no place for Taman chernozems (Val’koy et al., 2006). According to the World Reference Base for Soil Resources (WRB) classification, these soils are called Chernozems Calcic (World Reference Base for Soil Resources, 2006), which does not reflect their genetic and ecological characteristics.

The chernozems of Taman noticeably differ in their properties from other types and subtypes of black and other soils in southern Russia (Kazee & Kolesnikov, 2015; Kuzina et al., 2015). These differences in soil genetic characteristics determine their different resistance. However, to date, the limits of their resistance to chemical pollution have not been established.

The purpose of this work is to assess the resistance of the southern Taman chernozems to the contamination with HMs (Cr, Cu, Ni, Pb) and oil by biological indicators; and to determine quantitative guidelines for the development of regional standards for the maximum permissible concentration (MPC) of Cr, Cu, Ni, Pb and oil in the southern Taman chernozems based on the violation of ecological and agricultural soil functions.

2 Materials and Methods

Contamination with HMs and oil has been modeled in laboratory conditions. The southern chernozem (chestnut) of southern European facies (Russia, Krasnodar Region, Temryuk District, 2 km from the city of Taman to the south, 45° 10'51.73 "N, 36 ° 41'30.47" E) has been used as the object of the study. The soil for model experiments has been selected from the top layer of 0-25 cm, since this is the layer where most of the soil pollutants accumulate.

The soil under study is characterized by an average humus content in the upper horizon of 3.2%, neutral reaction of the medium of pH 7.7, heavy loam granulometric composition, high absorption capacity, good structural stability, oxidative conditions, sufficiently high biological activity (the total bacterial count – 4.2 bln/g of soil, catalase activity - 7.3 ml O2/g of soil per 1 min, dehydrogenase activity - 16.5 mg TPF/10g of soil for 24 hours, and an abundance of the Azotobacter genus bacteria - 100% fouling mass) These properties have been defined using traditional methods (Soil Microbiology and Biochemistry Methods, 1991; Kazeev et al., 2016).

Cr, Cu, Ni, and Pb have been chosen as pollutants, since the soils in southern Russia are largely polluted by these HMs (Dyachenko & Matasova 2016). Moreover, the HMs selected are interesting for comparison their maximum permissible concentration (MPC) (100 mg/kg of soil). In present study, MPC values developed in Germany (Kas’yanenko 1992) have been used, because there is no MPC has been reported from the soil of Russia (the gross content of copper, nickel and other components are less than the required for the estimation of MPC). The concentration of oil in the soil was expressed as a percentage. This is due to the fact that the oil MPC in the soil has not been developed so far.

HM was applied to the soil in the amount of 1, 10, 100 MPC (100, 1000 and 10,000 mg/kg, respectively), oil in the amount of 1, 5, 10% of the soil mass. The HM content up to 100 MPC or even greater is often found in areas soil near metallurgical, chemical and fuel industries. In addition to these sources, soil contamination up to 10 MPC is usually caused by road transport and/or as a result of agricultural activities: mineral fertilizers, pesticides, and seed disinfectants. Soil contamination with oil of up to 10% of the soil mass is more common in areas with oil production, transportation and refining (Kabata-Pendas 2010).

HM were introduced into the soil in the form of oxides: CrO3, CuO, NiO, PbO. There are two reasons for this. Most of the HM enters the soil in the form of oxides (Kabata & Pendas 2010). The use of oxides eliminates the influence of anions on the soil, as in the case of the use of salts. Contaminated soil was incubated in three replications for 30 days at a temperature of 20-22°C and a humidification of 60% of the field moisture capacity.

Biological properties of soil have been determined 30 days after contamination. This period is the most informative in assessing the chemical effect on the biological state of the soil (Kolesnikov et al., 2000).

The total bacterial count, the abundance of the bacterial genus Azotobacter, the activity of catalase and dehydrogenase, cellulolytic activity, phytotoxic properties of soils and other indices have been by using conventional methods (Soil Microbiology and Biochemistry Methods, 1991; Kazeev et al., 2010).

To combine a large number of indicators, special methodology has been developed for determining the integral index of the
biological state of the soil (IIBS) (Kazeev et al., 2016). This technique allows assessing the biological state of the soil as a whole.

3 Results and Discussion

As a result of the study, it has been found that the pollution of the southern chernozems of the Taman with Cr, Cu, Ni, Pb, and oil leads to deterioration of its state (Table 1). In most cases, the values of all the biological indicators studied have been reliably reduced. The values of the dehydrogenase activity, the length of the radish roots, and the cellulosolytic activity have been declining. The abundance of Azotobacter bacteria, the total number of bacteria, and the catalase activity decreased to a lesser extent.

Table 1 The impact of chemical aggression on biological properties of the southern chernozems of the Taman

<table>
<thead>
<tr>
<th>Element (substance)</th>
<th>Control</th>
<th>1 MPC (1 %)</th>
<th>10 MPC (5 %)</th>
<th>100 MPC (10 %)</th>
<th>LSD05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacterial count, billion per 1 g of soil (n = 720: 3 incubation vessels with soil x 3 soil samples x 4 square centimeters on specimen slides x 20 fields of view)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>4.2</td>
<td>2.9</td>
<td>1.6</td>
<td>1.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Cu</td>
<td>4.2</td>
<td>4.1</td>
<td>2.7</td>
<td>1.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Ni</td>
<td>4.2</td>
<td>4.3</td>
<td>2.8</td>
<td>1.9</td>
<td>0.2</td>
</tr>
<tr>
<td>Pb</td>
<td>4.2</td>
<td>4.1</td>
<td>3.3</td>
<td>3.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Oil</td>
<td>4.2</td>
<td>2.9</td>
<td>2.4</td>
<td>2.2</td>
<td>0.4</td>
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<tr>
<td>LSD05</td>
<td></td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Catalase activity, ml O₂ per 1 g soil for 1 min (n = 36: 3 incubation vessels with soil x 3 soil samples x 4 analytical replications)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cr</td>
<td>7.3</td>
<td>6.8</td>
<td>4.6</td>
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</tr>
<tr>
<td>Cu</td>
<td>7.3</td>
<td>7.9</td>
<td>6.8</td>
<td>5.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Ni</td>
<td>7.3</td>
<td>7.7</td>
<td>7.1</td>
<td>6.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Pb</td>
<td>7.3</td>
<td>7.5</td>
<td>7.0</td>
<td>6.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Oil</td>
<td>7.3</td>
<td>6.7</td>
<td>2.3</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>LSD05</td>
<td></td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Activity of dehydrogenase, mg TTF per 10 g soil for 24 hours (n = 36: 3 incubation vessels with soil x 3 soil samples x 4 analytical replications)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>16.5</td>
<td>12.8</td>
<td>10.2</td>
<td>3.2</td>
<td>1.4</td>
</tr>
<tr>
<td>Cu</td>
<td>16.5</td>
<td>13.7</td>
<td>12.6</td>
<td>7.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Ni</td>
<td>16.5</td>
<td>16.5</td>
<td>12.2</td>
<td>8.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Pb</td>
<td>16.5</td>
<td>15.8</td>
<td>10.6</td>
<td>6.9</td>
<td>1.7</td>
</tr>
<tr>
<td>Oil</td>
<td>16.5</td>
<td>4.2</td>
<td>1.4</td>
<td>0.8</td>
<td>0.8</td>
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<tr>
<td>LSD05</td>
<td></td>
<td>1.4</td>
<td>1.1</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Cellulosolytic activity,% of control (n = 9: 3 incubation vessels with soil x 3 pulp webs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>100</td>
<td>35</td>
<td>15</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Cu</td>
<td>100</td>
<td>98</td>
<td>82</td>
<td>61</td>
<td>6</td>
</tr>
<tr>
<td>Ni</td>
<td>100</td>
<td>98</td>
<td>73</td>
<td>55</td>
<td>4</td>
</tr>
<tr>
<td>Pb</td>
<td>100</td>
<td>100</td>
<td>86</td>
<td>68</td>
<td>7</td>
</tr>
<tr>
<td>Oil</td>
<td>100</td>
<td>63</td>
<td>39</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>LSD05</td>
<td></td>
<td>8</td>
<td>9</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>
Since the MPC of all the four studied HMs is the same (100 mg/kg), it is possible to correctly compare their toxic effect with respect to the biological parameters studied. The results obtained from this study indicated that the chromium had most significant negative effect. Three other HMs (lead, copper and nickel) showed lesser impact as compared to this. Accordingly, by the degree of negative impact on the chernozem of the southern Taman series of HMs looks as follows: Cr>Cu ≥ Ni = Pb. A similar pattern was obtained in the studies carried out using the same method with other soils in the south of Russia: ordinary chernozem, leached, typical, compacted, mountain (Kolesnikov et al., 2014), chestnut, brown semidesert, solonets, sandy (Kolesnikov et al., 2011), brown (Kolesnikov et al., 2016a), brown forest (Kolesnikov et al., 2016b), solonchaks (Kolesnikov et al., 2016c) etc.

However, such a sequence of HMs by their environmental hazard for soils does not always coincide with the previously obtained data on other types of soils (Van de Plassche & De Bruijn, 1992; Crommentuijn et al., 1997; Vodyanitsky 2012). It is possible that the higher toxicity of chromium in chernozems is due to the fact that chromium is more mobile in higher alkaline and oxidizing conditions (Zachara et al., 1989), and low toxicity of lead is due to a higher content of humic acids in chernozems, which bind to the lead more strongly than copper (Morin et al., 1999; Manceau et al., 2002).

It is not advisable to compare the toxic effects of HMs and oil especially by using genetic methods (Trushin et al., 2013), since it is impossible to correctly compare their concentrations in the soil. As a rule, there is a direct relationship between the concentration of the pollutant in the soil and the degree of decrease biological parameters.

The biological indicators used in the study (the bacterial count, the activity of catalase and dehydrogenase, cellulolytic ability, the abundance of the bacterial genus Azotobacter, the length of radish

<table>
<thead>
<tr>
<th>Element (substance)</th>
<th>Control</th>
<th>1 MPC (1 %)</th>
<th>10 MPC (5 %)</th>
<th>100 MPC (10 %)</th>
<th>LSD05*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abundance of the Azotobacter genus bacteria, % of fouling mass (n = 241: 3 incubation vessels with soil x 3 soil samples in Petri cups x 25 fouling clusters)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr 100</td>
<td>100</td>
<td>69</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Cu 100</td>
<td>100</td>
<td>92</td>
<td>88</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Ni 100</td>
<td>100</td>
<td>100</td>
<td>93</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Pb 100</td>
<td>100</td>
<td>85</td>
<td>81</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Oil 100</td>
<td>100</td>
<td>91</td>
<td>86</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>LSD05 12</td>
<td>12</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Length of radish roots (phytotoxicity), % of control (n = 241: 3 incubation vessels with soil x 3 soil samples in Petri dishes x 25 radish seeds) |
|---------------------|---------|-------------|--------------|----------------|
| Cr 100 | 46 | 13 | 0 | 9 |
| Cu 100 | 90 | 58 | 31 | 4 |
| Ni 100 | 89 | 63 | 33 | 5 |
| Pb 100 | 99 | 64 | 28 | 6 |
| Oil 100 | 74 | 43 | 36 | 9 |
| LSD05 11 | 8 | 6 | |

| Integral indicator of the biological state of the soil (IIBS), % of control |
|---------------------|---------|-------------|--------------|
| Cr 100 | 70 | 43 | 14 |
| Cu 100 | 96 | 77 | 56 |
| Ni 100 | 99 | 79 | 61 |
| Pb 100 | 99 | 79 | 64 |
| Oil 100 | 71 | 52 | 35 |

* Note: If the difference between the test cases (for example, between Control and 1 MPC, or Control and 10 MPC, etc.) is greater than LSD05, then the impact of contamination is significant.
roots) had confirmed their compliance with the necessary requirements for indicators used to monitor, diagnose and normalize chemical contamination of soils. They are distinguished by high informativeness and sensitivity, sufficient reproducibility, allowable variation of the indicator, small error of the experiment, simplicity, low laboriousness and high speed of determination methods, abundance of methods, etc.

The study made it possible to establish quantitative benchmarks to develop regional standards for the MPC of Cr, Cu, Ni, Pb, and oil in chernozems of the southern Taman, based on a disturbance of the ecological and agricultural functions of soils.

Further, it was reported by Kolesnikov et al. (2002) that the disturbance of the ecological functions of the soil occurred in a certain order. Ecosystem functions are initially violated and followed by biochemical, physico-chemical and chemical ones. Physical functions are violated aftermost, already with very strong soil contamination (Classification of ecosystem functioning of soil is provided as per the Dobrovolsky & Nikitin, 1990). This pattern can be used for environmental regulation of soil pollution. It is useful to use IIBS to assess the violation of certain eco-functions. It has been established that when IIBS values have decreased by less than 5%, the soil normally fulfills its ecological functions while this reduction in IIBS values reached by 5-10% violation of informational eco-functions reached by 10-25% violation in biochemical, physicochemical, chemical and holistic functions and 25% violation was reported in physical functions (Kolesnikov et al., 2002). Using the results of the study, regression equations were constructed between the number of pollutant in the soil and the IIBS. The regression equations have determined the concentrations of pollutants that cause a violation of certain soils eco-functions (Table 2).

The suggested approach and the quantitative values of pollutant content in the soil that cause disturbance of different groups of ecological functions seem appropriate to be used in ecological standardization, where the main goal should be to preserve the ecological functions of the soil.

### Conclusion

Pollution of southern chernozems of the Taman with oxides of Cr, Cu, Ni, Pb, oil leads to the deterioration of its biological properties, the total bacterial count, as well as the activity of catalase and dehydrogenase, cellulyotic ability, abundance of the Azotobacter genus bacteria decrease, and the germination and initial radish growth deteriorate. The extent of the deterioration of biological properties is determined by two factors: the chemical nature of the metal and its amount in the soil.

In most cases, a direct relationship between the pollutant content in soil and the degree of decrease in biological indices was recorded for all the HMs and oil studied. The investigated HMs form the following series in terms of the degree of negative effect on the biological properties of the southern chernozem (a series is averaged over the pollutant doses): Cr > Cu ≥ Ni = Pb.

### Table 2 Scheme of ecological standardization of the content of HMs and oil in the southern chernozems of the Taman according to the degree of disruption of ecological functions

<table>
<thead>
<tr>
<th>Soils</th>
<th>Non-contaminated</th>
<th>Weakly contaminated</th>
<th>Medium contaminated</th>
<th>Strongly contaminated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of decrease in the integral indicator</td>
<td>&lt; 5 %</td>
<td>5 – 10 %</td>
<td>10 – 25 %</td>
<td>&gt; 25 %</td>
</tr>
<tr>
<td>Disturbed environmental functions</td>
<td>–</td>
<td>Informational</td>
<td>Chemical, physicochemical, biochemical, holistic</td>
<td>Physical</td>
</tr>
<tr>
<td>Element</td>
<td>HM content in soil, mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>&lt;105</td>
<td>105-115</td>
<td>115-145</td>
<td>&gt; 145</td>
</tr>
<tr>
<td>Cu</td>
<td>&lt;60</td>
<td>60-120</td>
<td>120-350</td>
<td>&gt;350</td>
</tr>
<tr>
<td>Ni</td>
<td>&lt;65</td>
<td>65-120</td>
<td>120-350</td>
<td>&gt;350</td>
</tr>
<tr>
<td>Pb</td>
<td>&lt;60</td>
<td>60-120</td>
<td>120-350</td>
<td>&gt;350</td>
</tr>
<tr>
<td>Substance</td>
<td>Oil content in soil, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oil</td>
<td>&lt; 0.25</td>
<td>0.25-0.50</td>
<td>0.50-1.5</td>
<td>&gt;1.50</td>
</tr>
</tbody>
</table>

Note: 1. Definition of the integral indicator by (Kazeev et al., 2016). 2. Classification of environmental functions by (Dobrovolsky & Nikitin 1990).
The conducted study confirmed the feasibility of the use of microbiological indicators, enzyme activity and phytotoxicity to assess soil conditions in the context of chemical contamination. Regional norms for the MPC of Cr, Cu, Ni, Pb, and oil in southern chernozems of the Taman based on the disturbance of the ecological functions of soils are proposed.

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

References


PHYTOCHEMICAL CHARACTERIZATION OF *Spondias* SP AND *Spondias tuberosa* ARRUDA CÂMERA EXTRACTS OF OCCURRENCE IN PARAIBA SEMIARID

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KEYWORDS
*Spondias*
Secondary metabolites
Drugs
Bioactive compounds

ABSTRACT

Present study was aimed to characterize the phytochemical profile of the leaf extracts of *Spondias* sp and *Spondias tuberosa*. During study total five trees of *Spondias* sp (Cajarana do sertão) and *Spondias tuberosa* (Umbu) with good phytosanitary was used for phytochemical analysis. From these selected trees total 5kg leaves per tree were collected and air dried at 40°C. These leaves samples were ground and pulverized; from this 200g of powder sample were used to prepare ethanolic crude extract and phytochemical analysis was performed at the Laboratory of Pharmaceutical Technology, Federal University of Paraíba. The phytochemical screening was carried out by preliminary scouting method for identify the presence of alkaloids, steroids, tannins, flavonoids, terpenoids, and saponins. Results of study revealed that the presence of steroids, tannins, flavonoids and terpenoids from the leaf extracts of *Spondias* sp and *Spondias tuberosa*, these phytochemicals are important constitute of herbal medicines and pharmaceutical industry and worked as antioxidant, anti-infective, anti-allergic and anti-inflammatory agents.

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1 Introduction

Fruit and vegetables extract have various active ingredient and substances which are responsible for applicability in food and health. This has been stimulating the development of the study on many plants within the scope of organic chemistry in order to predict the structure and chemistry of these compounds is extremely broad and diverse (Silva et al., 2014). The phytochemical researches allow researchers to estimate the chemical constituents of the plant species or valuing their presence in various plant species (Matos, 1997; Silva et al., 2007).

The use of medicinal plants in the Northeast Brazilian population is the result of their historical heritage. They have great potential in identification, utilization, and commercialization of biologically active natural products, and have great diversity in the structure and physicochemical properties. Approximately 75% of the 121 most commonly used Brazilian drugs are derived from empirical knowledge.

The caatinga biome is very rich in medicinal plants diversity, due to their morphological characteristics, such as xilopodes and shells that accumulate reserves; they are also possessed of pharmacologically active substances. The Northeastern floras have vast biodiversity of medicinal plants which did not existing elsewhere in the world and have great pharmaceutical potential. Most communities in this region live in precarious socio-economic situations, so there is an incentive for the use of medicinal plants.

The family Anacardiaceae has valuable pharmacological importance, where 25% of the genera of this family are known as severe contact dermatitis-causing. Species of this family became very important in search of bioactive substances. The most widely studied members of this family are Spondias, Lannea, Semecarpus, Schinus, Pistacia, Lithraea, Tapirira and Melanorrhoea (Correia et al., 2006).

The Spondias are the most fruitful trees which group several important fruit species, such as cajarana do sertão (Spondias sp.), umbu (Spondias tuberosa) cashew (Anacardium occidentale L.), mango (Mangifera indica L.) and pistachio (Pistacia vera L.), which are economically exploited in many tropical and subtropical areas of the world (Asuquo et al., 2013). They contain various groups of chemicals such as tannis, terpenoids (sesquiterpenes and monoterpenes), flavonoids and some of these are already detected and isolated (Dantas et al., 2014).

The main groups of compounds having pharmaceutical properties derived from plants include terpenoids, alkaloids, lectins, polypeptides, phenolic substances and polyphenols (simple phenols, phenolic acids, quinones, flavones, flavonols, and flavanols), tannins and coumarins (Haida et al., 2007).

Researches on Spondias species are in scarcity and there are gaps related to scientific knowledge of bioactive compounds produced by these species in this biome. Impacting in a Brazilian reality where, although it has the greatest plant diversity of the world and many medicinal plants are broad popular knowledge, the amount of information on these plants has grown little now (Corrêa & Salgado, 2011).

This aims of this study was to determine the presence of secondary metabolites with pharmacological active ingredients in crude extract of Spondias sp (Cajarana do Sertão) and Spondias tuberosa Arruda Camera (Umbu) species presenting a new alternative to the research of new drugs, through natural sources, mainly presenting as a source of medicines for the low-income population.

2 Material and Methods

Present study was carried out in Paraíba central semiarid region. Samples of Spondias sp (Cajarana do Sertão) (Figure 1A) were collected from the rural area of municipality Santa Terezinha, Paraíba, Brazil, in the farm Lajedo while the sample of Spondias tuberosa Arruda Câmara (Umbu) (Figure 1B) were collected from the rural areas of the municipality of Matureia- PB in the farm Santo Antonio. Various plant parts such as leaves, flowers and fruits are collected for making herbarium specimens for their identification, by using the usual techniques for herborization recommended by Forman & Bridson (1989), and recorded in the Brahms program, being deposited in the Herbarium of the Caatinga of the Health and Rural Technology Center (CSTR) at the Federal University of Campina Grande (UFCG) under the number 494 and 495 respectively. Morphological analysis for the identification, descriptions of species were carried out as according to Mobot (2012) and Forzza (2012).

Further, 5 Kg of leaves per tree were collected and transported to the STPF/UAEF/CSTR Campus of Patos-PB of the UFCG for extracts preparation. The collected samples were air dried in an oven with circulating air at 40°C, it was followed by the grinding these leaves in a Wiley mill and pulverized, resulting in 3.0 Kg per species powder were obtained. Obtained powder mixed with 96% EtOH for 72 hours and repeated in every three days to obtain approximately 200 g of crude ethanolic extract per species. Then this material was filtered and concentrated on rota-evaporator to yield approximately 80 g per species.

The phytochemical analyses were performed in the Laboratory of Pharmaceutical Technology (LTF), of the Federal University of
Paraíba (UFPB) by preliminary scouting technique, by the preliminary scouting method.

Hydroalcoholic extract were used for the identification of available phytochemicals such as heterosides saponosides (determination of foam), tannins (reaction with gelatin and ferric chloride), heterosides flavonics/terpenoids (magnesium ribbon and fluorescence), steroids (reaction Liebermann- Burchard), alkaloids (reactions Reactive: Dragendorff, Mayer, Burchard and silico tungstic acid) according to Matos (1997) and Costa (2000).

3 Results and Discussion

Preliminary phytochemical screening of secondary metabolites present in the leaf extracts of *Spondias sp* and *Spondias tuberosa* Arruda Câmara have been presented in Table 1. Results of study verify the presence of steroids, tannins, flavonoids, terpenoids and the absence of alkaloids and saponins. A positive correlation was reported in the formation of precipitates and the appearance of color and foam; accordingly available phytochemicals are classified as weakly positive, moderate positive, positive and strong positive.

In relation to steroidal compounds, the results were positive, as compared with the crude extract, was noted the appearance of a green or blue coloration after Liehrman-Burchard reaction. Steroids are actively involved in the development and control of the human reproductive system, functioning as cardiotonic, vitamin D precursors, anti-inflammatory agents and anabolic agents, analgesics.

Similarly, the tests conducted for the presence of tannins were found positive by forming a green or blue coloration and also by precipitate formation. This secondary metabolite has vast therapeutic applications such as prevention of lipids peroxidation and nucleotide degradation; it also accelerates the healing process (Macedo et al., 2007).

For finding out the presence of flavonoids, the conducted tests were showing color ranging from pink to a deep red. Several biological activities such as antioxidant, anti-inflammatory and anticancer activities are associated with the flavonoids (Verdi et al, 2005)

The fluorescence UV reactivity indicated the presence of terpenoids. This metabolite is of therapeutic interest, constitute a large group of chemical substances biosynthesized extracted from natural products. Terpenoids have several well defined biological activities including antifungal, anti-inflammatory and analgesic (Silva et al., 2007).

The reactive Bouchardat, Mayer, Dragendorff, and Bertrand presented negative result as in the alkaloids identification, by lack of precipitate formation floculose or clouding of the solution. Similarly, the test conducted for saponins were also found negative in the absence of permanent foam or the collar after the solution was stirred.

Several authors who have worked on various *Spondias* species such as Caja (*Spondias mombin*) Umbu (*Spondias tuberosa* Arr Cam.), *Spondias pinnata*, umbu-cajazeira (*Spondias sp*), and with other Anacardeacea as Aroeira (*Myracrodruon urundeuva*) also

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*Figure 1* Sample of the selected species (a) *Spondias* sp. (Cajarana do sertão) and (b) *Spondias tuberosa* Arruda. Câmara (Umbu)
Phytochemical characterization of *Spondias* sps

confirmed the presence of constituents reported in this study (Gupta & Moreira, 2010; Asuquo et al., 2013; Dantas et al., 2014).

Some species of Anacardiaceae which are of the same family as *Spondias*, *Mangifera indica* (common mango) and *Anacardium occidentale* L. (cashew), did not have some of these chemicals such as tannins, flavonoids, and steroids (Correia et al., 2006; Silva et al., 2007).

Absence of alkaloid in some members of the group of *Spondias* was also reported by Gupta & Moreira (2010) and Correia et al., (2006) when they are working with *Spondias pinnata* and *M. indica*. While, Silva et al. (2008), Asuquo et al. (2013) and Dantas et al. (2014) detected the presence of alkaloids, using the same methods of analysis when they were working with Umbu (*Spondias tuberosa* Arr. Cam.) and Caja (*Spondias mombin*). This shows that the secondary metabolism of various phytochemicals can differ from environmental differences inherent in the ecosystem, for the same family (Matos, 1997); this issue is relevant through standardization needs of medicinal plant raw materials aimed at the validation of medicinal plants used locally, control of existing herbal medicines.

In present study, presence of saponins was not observed and this fact was confirmed by the various researcher when they were working on the other species such as *Spondias tuberosa* Arr Cam (umbu), *A. occidentale* (Cashew) and *M. indica* (mango) of the same family (Silva et al., 2008; Bessa et al., 2013; Silva & Almeida, 2013), although work done with *Spondias mombin* (Asuquo et al., 2013), *Myracrodruon urundeuva* and *Spondias pinnata* revealed the presence of saponins (Dantas et al., 2008).

Observed chemical compounds have hemolytic, anti-inflammatory, antifungal, antibacterial, antimicrobial, antiparasitic, cytotoxic, antitumor and antiviral activities. The discrepancies in the results for alkaloids and saponins qualitative comparisons may be due to the variation in various factors such as soil, climate, the collection of material, temperature and chemicals (Cechinil & Yunes, 1998).

The chemical constituents present in the extracts of *Spondias* studied may respond mainly by the biological activity which confirms a pharmacological value to this family, such as antioxidant, anti-infection activities, antibacterial, antifungal and anti-protozoan, anti-inflammatory and analgesic agents (Rocha et al., 2011).

**Conclusion**

The phytochemical analysis provides relevant information about the presence of secondary metabolites in plants so that it can reach the isolation of active principles in production of new herbal medicines. The data obtained can be concluded that the species showed good results for the tannins, flavonoids, steroids, terpenoids and not showing the presence of alkaloids and saponins, these analyzes indicated that the studied plant species has compounds that can be potentially active in biological models and pharmacological. Tests are required for the fractionation of crude extracts obtained from leaves to identify the

### Table 1 Phytochemical screening by preliminary scouting in species *Spondias* sp. and *Spondias tuberosa* Arruda Câmara

<table>
<thead>
<tr>
<th>Chemical Group</th>
<th>Method for Identification</th>
<th><em>Spondias sp</em></th>
<th><em>Spondias tuberosa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>ALKALOIDS</td>
<td>Bouchardat</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mayer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Dragendorff</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Acid silico-tungstic</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>STEROIDS</td>
<td>Reagent 0.12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reagent 0.25</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Reagent 0.50</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>TANNINS</td>
<td>Gelatin 0.5% - 0.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gelatin 0.5% - 1.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Gelatin 0.5% - 2.0</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>FeCl$_2$ 2.0% - 0.5</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>FeCl$_2$ 2.0% - 1.0</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>FeCl$_2$ 2.0% - 2.0</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>FLAVONOIDS</td>
<td>TAPE - MAGNESIUM</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>TERPENOIDS</td>
<td>FLUORESCENCE</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SAPONINS</td>
<td>FOAM</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Negative reaction (-); Weakly positive reaction (+); Moderately positive reaction (++); Positive reaction (+++)
active ingredients and perform bioassays to prove possible biological activity.

References


