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# Aspirin regulates oxidative stress and physio-biochemical attributes in *Brassica juncea* under cadmium toxicity

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**ABSTRACT**

The current study aimed to evaluate the effects of aspirin (Asp) on growth, physio-biochemical variables, and oxidative stress in *Brassica juncea* subjected to cadmium toxicity. Cadmium (Cd) toxicity decreased the root and shoot development by 67.53 % and 64.4 % respectively, over the control. However, treatment with Asp showed improved root and shoot growth in Cd treated seedlings. This study demonstrates elevation in total soluble sugar (TSS), proline, and glycine betaine levels and suppressed total protein concentrations in Cd treated seedlings over control. On the treatment of Asp to Cd exposed plants, an enhanced level of the above said variables was reported. The activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and ascorbate (ASC) increased in plants with Cd stress over control, followed by enhanced elevation of the same on supplementation of Asp. Supplementation of Asp reduces the accumulation of malondialdehyde (MDA) and $H_2O_2$, confirming the plant metals' stress protection properties of Asp. Thus studies confirm aspirin's involvement in protecting plant growth and development against cadmium toxicity.

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## 1 Introduction

Plants being immobile are exposed to a wide range of abiotic and biotic stress. Among abiotic stress, metal-induced toxicity is the most prevailing cause of extensive water and soil pollution (Ahmed et al. 2016). Few metals are important for the regular functioning of plants, but, many more are deleterious and impede normal plant growth and development (Ahmed et al. 2012). Cadmium is an environmental pollutant and biologically toxic metal (Godt et al. 2006). Cadmium tends to build up in the soil of anthropogenic and natural activities (Vitória et al. 2001). Elevated levels of heavy metals in the environment, particularly soil result in the generation of reactive oxygen species (ROS) (Liu et al. 2010), this impacts the germination of seeds, growth, and development of plants (Jonak et al. 2004) and disrupts the transport of electrons in the chloroplast (Cui and Wang 2006). Heavy metals also impede photosystems I, and II and interfere with the transfer of $K^{2+}$, $Ca^{2+,}$ and abscisic acid in the guard cells of the plants. Further, these metals also disturb the $Ca^{2+}$, $Zn^{2+,}$ and $Fe^{2+}$ in proteins which results in the release of free radicals (Minglin et al. 2005). Metals cause the oxidation of biomolecules causing oxidative stress and thus cell damage (Romero et al. 2002). Oxidative stress results in lipid peroxidation, leading to cell membrane disruption (Nouairi et al. 2006). Plants prevent the harmful effect of free radicals by enhancing the generation of antioxidant enzymes during heavy metal stress. Plants have inbuilt enzymatic and nonenzymatic systems like catalase, ascorbate peroxidase, and superoxide dismutase to scavenge free radicals generated from oxidative stress due to phytotoxicity (Sharath Chandra and Sukumaran 2020).

Exposure to cadmium toxicity changes the enzyme activity of superoxide dismutase, catalase, and ascorbate peroxidase, which are essential to preserving normal cellular hydrogen peroxide levels to shield the cell from oxidative stress-induced cellular and tissue damage. Antioxidant enzymes like catalase, SOD, and peroxidase are elevated during stress (El-Beltagi et al. 2010). Catalase and peroxisomes are found in the cytosol and peroxisomes of plants respectively. Cadmium-associated deprivation of glutathione causes intracellular hydrogen peroxide accumulation which results in cell death (Schutzendubel and Polle, 2002). Similarly, the accumulation of proline during heavy metal stress facilitates protecting the biomolecules from denaturation, thus increasing the plant tolerance to abiotic stress (Lesko and Simon-Sarkadi 2002).

Salicylic acid (SA) is not only a plant growth regulator but also an important non-enzymatic oxidant, playing a significant part in numerous physiological mechanisms in plants (Fariduddin et al. 2003). Acetylsalicylic acid or aspirin (Asp) is one among the many derivatives of salicylic acid. It elicits plants' defense mechanisms against diseases and protects plants from viral, bacterial, and fungal infections. Aspirin mimics the role of plant growth hormone and is involved in the promotion of plant growth. Thus, aspirin functions similarly to salicylic acid as a plant hormone (Pallag et al. 2014). *Brassica juncea* (Indian mustard) is extensively used as a model plant for phytoremediation, because of its higher biomass and capacity to accumulate high concentrations of heavy metals, like cadmium up to 400 $\mu$g/g DW in shoots (Haag-Kerwer et al. 1999). *B. juncea* possesses ten times more biomass generation capacity as compared to other heavy metal accumulators. It also shows a fast growth rate and collects other toxic heavy metals available in the soil. Thus *B. juncea* is selected as a suitable plant system for phytoremediation studies (Salt et al. 1998). The main purpose of the present study is to understand the role of aspirin (Asp) in tolerating cadmium-induced toxicity in *B. juncea* by studying growth and development, physio-biochemical variations, and oxidative stress.

## 2 Materials and Methods

### 2.1 Seed collection and Experimental setup

Certified and viable seeds of *B. juncea* were surface sterilized for 10 min with a 5 % sodium hypochlorite (NaOCl) solution. Further, priming of seeds was performed with 0.5mM of aspirin (Asp), for 10 h. Aspirin exposed and non-exposed seeds were grown in Petri dishes covered with Whatman filter paper which is set aside in a growth chamber with a photoperiod of 24 hrs and incubated for 8 days. Eight days old germinated seedlings were further transferred to trays containing perlite: sand: peat (1:1:1 v/v/v) added with 200 µM of cadmium solution (cadmium chloride). The control plants were subjected to only distilled water. Each exposure is the mean of three replications and each replicate contains five plants. The samples were obtained for experimentation 12 days after treatment. Determination of cadmium and aspirin concentrations was made based on earlier reports (Senaratna et al. 2000; Shanmmugaraj et al. 2013).

### 2.2 Plant growth analysis and biomass accumulation

The amount of germinated seed was counted and the ratio of germination was calculated using the formula

$$\text{Germination (\%)} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds inoculated}} \text{ x } 100$$

The length of the root and shoot were measured manually using a scale. Dry weight (W) of the root, shoot, and leaves of the mustard plant was dried for 60 h at 65ºC in the oven and then evaluated.

### 2.3 Estimation of Glycine betaine and proline levels

Glycine betaine levels were evaluated based on Grieve and Grattan (1983) method. The results were determined at 365 nm by spectrophotometer. The dosage for glycine betaine was taken at

50-200 mgml$^{-1}$ which was dissolved in 1N $H_2SO_4$. Levels of proline were evaluated by Bates et al. (1973) method. Absorbance was analyzed at 520 nm in a spectrophotometer, with toluene as blank.

**2.4 Estimation of total soluble sugars (TSS) and total protein**

Total soluble sugars (TSS) were determined by Dev (1999) method. The absorbance was determined at 485nm using a spectrophotometer. Total protein content was assessed by Lowry et al. (1951) method. The absorbance was recorded at 595 nm by spectrophotometer with BSA as control.

**2.5 Determination of Lipid peroxidation (MDA), Hydrogen peroxide ($H_2O_2$), and Ascorbate**

Lipid peroxidation (accumulation of malondialdehyde "MDA") was determined by the method of Heath and Packer (1968). Optical density was recorded at 600 nm with 20 % TCA (trichloroacetic acid) and 1% TBA (thiobarbituric acid) as blank. Hydrogen peroxide level was determined by the procedure of Velikova et al. (2000). Hydrogen peroxide concentration was expressed as µM g$^{-1}$ FW. Ascorbate was measured by the method of Foyer et al. (1983). The absorbance was recorded at 265 nm and expressed as µM g$^{-1}$ FW.

**2.6 Antioxidant enzyme assays**

Plant material (2g) was homogenized at pH 7.5 in 100 mM Tris HCl in the presence of 10 mM magnesium chloride, 5 m Mdithiothreitol, 1 mM EDTA, 1.5% polyvinyl pyrolidone, 5 mM magnesium acetate and 1 mM phenylmethanesulfonyl. The sample was filtered, and the homogenate was centrifuged for 15 min at 10,000 rpm. Subsequently, after the centrifugation, the supernatant was used as a source of enzyme. For determination of APX activity, tissues were homogenized separately with 2 mM Ascorbate.

Superoxide dismutase (SOD) activity estimation was performed according to Kono (1978), which resulted in the photo-reduction of nitroblue tetrazolium (NBT). The readings were taken at 540 nm in a spectrophotometer. SOD unit indicates the enzyme quantity that impedes 50 % photo-reduction of nitroblue tetrazolium. SOD levels were expressed as U g$^{-1}$ FW.

Catalase activity was determined by the procedure of Aebi (1984). The absorbance was read at 240 nm in a spectrophotometer and reported as mmol g$^{-1}$ FW. APX activity was assessed by the method of Nakano and Asada (1981). The absorbance was measured at 265 nm, and the activity was reported as mmol min$^{-1}$ g$^{-1}$ FW.

**2.7 Statistical analysis**

Statistical analysis was performed by one-way analysis of variance (ANOVA) and Duncan`s multiple range test (DMRT) was employed to determine the significant difference between the samples. The values indicate the mean ± SE (n=3). $P \leq 0.05$ significantly differs.

**3 Results**

**3.1 Effect of Aspirin on germination and growth under cadmium stress**

The observations regarding the influence of cadmium and aspirin on the germination and growth of *B. juncea* are shown in Table 1. The toxicity of cadmium reduces the percentage of germination by 75.86% in comparison to the control. But the application of aspirin to cadmium exposed plants exhibited only a 3.44 % decrease in germination over the control. Further, cadmium stress led to a decrease in the length of both root and shoot (Table 1). Root length declined by 67.53 % with cadmium application, however, plants exposed to cadmium in presence of aspirin demonstrated enhanced root length and were almost similar to the control. Shoot length was reduced by 64.4 % under cadmium influence against control (Table 1). Supplementation of aspirin improved the shoot length by 71 % when compared to cadmium-applied plants alone. Dry weight (DW) was reduced by 57 % in cadmium-treated plants over the control. Aspirin co-application with cadmium exhibited enhanced dry weight by 56 % over the plants exposed to only cadmium.

**3.2 Aspirin betters Glycine betaine and proline levels under cadmium toxicity**

Cadmium toxicity increased glycine betaine content by 24.23 % in treated plants over the control (Table 2). However, aspirin co-

Table 1 Effect of Cd (200 µM) and Aspirin (0.5 mM) individually and in combination on various growth parameters of *Brassica juncea*

| Treatment | Germination | Root length (cm) | Shoot length (cm) | Dry weight (mg) |
|---|---|---|---|---|
| Control | 87 ± 0.57$^{d}$ | 6.53 ± 0.21$^{d}$ | 13.12 ± 0.44$^{d}$ | 63.52 ± 0.25$^{d}$ |
| Aspirin | 85 ± 0.71$^{d}$ | 6.66 ± 0.43$^{d}$ | 13.85 ± 0.52$^{d}$ | 61.65 ± 0.14$^{d}$ |
| Cadmium | 21 ± 0.14$^{b}$ | 2.12 ± 0.29$^{b}$ | 4.67 ± 0.06$^{b}$ | 27.53 ± 0.19$^{b}$ |
| Cd+Asp | 84 ± 0.32$^{a}$ | 6.48 ± 0.41$^{a}$ | 13.55 ± 0.26$^{a}$ | 63.37 ± 0.07$^{a}$ |

Date values are the means of three replicates ± SE. All the values represent a significant difference ($P \leq 0.05$). Similar superscript denotes no significant difference between the means of the treatment in the columns.

Table 2 Effect of individual and combined application of Cd (200 µM) and Aspirin (0.5 mM) on Protein, Total soluble sugar (TSS), proline, and glycine betaine level in *Brassica juncea*

| Treatment | Protein mg $g^{-1}$ FW | TSS µg$g^{-1}$ FW | Proline µg$g^{-1}$ FW | Glycine betaine µmol$g^{-1}$FW |
|---|---|---|---|---|
| Control | 7.65 ± 0.24[d] | 4.66 ± 0.18[d] | 9.62 ± 0.04[d] | 3.72 ± 0.12[d] |
| Aspirin | 7.81 ± 0.43[d] | 4.95 ± 0.16[d] | 14.19 ± 0.25[c] | 3.79 ± 0.55[d] |
| Cadmium | 3.77 ± 0.51[b] | 6.22 ± 0.37[b] | 17.33 ± 0.51[b] | 4.91 ± 0.02[b] |
| Cd+Asp | 6.53 ± 0.15[a] | 8.16 ± 0.12[a] | 26.48 ± 0.28[a] | 5.53 ± 0.19[a] |

Date values are the means of three replicates ± SE. All the values represent a significant difference (P ≤0.05). Similar superscript denotes no significant difference between the means of the treatment in the columns

Table 3 Effect of an individual or combined application of Cd (200 µM) and aspirin (0.5 mM) on various enzymes and oxidative stress markers

| Treatments | Antioxidant enzymes | | | Non-enzymatic oxidants | Oxidative stress markers | |
|---|---|---|---|---|---|---|
| | SOD (U g-1 FW) | CAT (mmolg-1 FW) | APX (mmolmin-1 g-1 FW) | ASC (µmg-1FW) | MDA (µMg-1FW) | $H_2O_2$ (nmg-1 FW) |
| Control | 45 ± 0.12[d] | 0.58 ± 0.06[d] | 2.54 ± 0.17[d] | 253 ± 1.2[d] | 2.19 ± 0.04[d] | 336 ± 1.6[d] |
| Aspirin | 52 ± 0.55[c] | 1.02 ± 0.13[d] | 2.42 ± 0.23[d] | 295 ± 1.7[c] | 2.21 ± 0.16[d] | 323 ± 2.6[d] |
| Cadmium | 69 ± 1.2[b] | 1.72 ± 0.55[b] | 4.17 ± 0.44[b] | 342 ± 0.9[b] | 7.05 ± 0.32[b] | 697 ± 3.4[b] |
| Cd+Asp | 76 ± 0.73[a] | 2.11 ± 0.09[a] | 6.09 ± 0.36[a] | 422 ± 1.5[a] | 4.16 ± 0.07[a] | 431 ± 1.5[a] |

Date values are the means of three replicates ± SE. All the values represent a significant difference (P ≤0.05). Similar superscript denotes no significant difference between the means of the treatment in the columns

application to cadmium exposed plants demonstrated a further increase of 12 % over cadmium treated plants alone. Proline levels were also increased under cadmium stress by 45 % when compared to the control (Table 2). Application of aspirin along with cadmium exhibited further accumulation of proline levels by 35 % over cadmium alone treated plants.

### 3.3 Aspirin increases total soluble sugar and total protein under cadmium toxicity

Plants exposed to cadmium stress demonstrated an elevation of 25 % in total soluble sugar as compared to the control (Table 2). However, the co-application of aspirin to Cd-induced stressed plants further elevated the total soluble sugars by 24 % over cadmium exposed plants. While in the case of total protein levels, it diminished by 50 % in cadmium stress-induced plants as compared to the control (Table 2). Supplementation of aspirin to cadmium stressed plants exhibited a 53 % increase in protein levels over cadmium alone treated plants.

### 3.4 Aspirin preserves MDA and hydrogen peroxide content under cadmium toxicity

Accumulation of malondialdehyde (MDA) was 70 % under cadmium toxicity as compared to the control. Aspirin co-application with cadmium demonstrated a reduction in MDA accumulation by 41 % in comparison to cadmium treated plants (Table 3). Hydrogen peroxide levels in plants increased by 52 % under cadmium stress over the control (Table 3). However, the addition of aspirin to cadmium exposed plants lowered the accumulation of hydrogen peroxide by 38 % in comparison to only cadmium treated plants. Plants treated with only aspirin displayed no significant changes in MDA and hydrogen peroxide levels.

### 3.5 Impact of aspirin and cadmium on antioxidant enzyme activity

The observations of antioxidant enzymes and non-enzymatic responses on individual or combined application of cadmium and aspirin treated *B.juncea* plants are presented in table 3. Further, superoxide dismutase activity increased by 35 % in cadmium treated plants; further elevated by 41 % upon co-application of aspirin to cadmium exposed plants. Catalase and APX elevated by 66 % and 39 % respectively in cadmium stresses plants in comparison to the control (Table 3). However, aspirin supplementation to cadmium treated plants further elevated by 73 % and 59 % over cadmium stressed plants alone. The Ascorbate (ASC) levels also increased by 26 % in cadmium stressed plants over control; further, the levels of ascorbate enhanced by 40 % on co-application of aspirin to only cadmium treated plants.

## 4 Discussion

Abiotic stress due to heavy metal pollution is a globally frequently seen phenomenon. At higher concentrations, heavy metals can aggregate in plants that cause toxic effects leading to undesirable

variations in morphological, biochemical, and physiological mechanisms (Chandra et al. 2017; Mahadimane and Chandra 2020;). In this study, cadmium treated plants demonstrated significant variation in the germination of seeds, biomass content, root length and shoot length over the control plants. The seeds treated with cadmium exhibited underdeveloped growth and limited root formation. Inhibition of plant growth and decrease in biomass content is the primary phenomenon that takes place in response to abiotic stress due to heavy metal toxicity (Shekhawat et al. 2010; Rashmi et al. 2019). The reduction in the root length and shoot length observed may be associated with the buildup of cadmium in the plant roots that diminishes the uptake of minerals and water, which will eventually influence the plant's biochemical mechanisms and physiology. Elevated concentration of cadmium in the soil leads to root tip damage, a decrease in the transportation of water to multiple tissues that in due course results in declined transpiration rate and inhibition of photosynthesis by disturbing the enzymes catalyzing the Calvin cycle, finally leading to stunted growth (Baudhha and Singh 2011; Shanmmugaraj et al. 2013; Ranjitha and Sharath Chandra 2020). During metal induced stress third of the root growth decreased and it matched with the underdeveloped shoot system. The observations were in correlation with Baudhh and Singh (2011) and Shanmmugaraj et al. (2013).

Cadmium toxicity elevates proline levels in the current study (Table 2) and results are in correlation with the findings of Sirhindi et al. (2016) who also described the elevation of proline levels in *T. aestivum* under heavy metal toxicity. Proline buildup under heavy metal toxicity has been identified as a potential marker of abiotic stress tolerance (Ashraf and Foolad 2007). Proline helps in rebuilding chlorophyll, activates the citric acid cycle, and amounts to the energy source (Ramon et al. 2003). Proline also plays a major role in osmotic regulation and stabilized biomolecules. Proline possesses the ability to scavenge free radicals and protect cells and tissues from oxidative damage (Ahmad et al. 2015). Glycine betaine (GB) also increases with cadmium toxicity (Table 3) and is reported as an important solute under heavy metal stress (Munns 2005). Similarly, glycine betaine also plays multiple roles in maintaining membrane integrity, stabilizing the PS II complex, osmotic regulation, preserving RUBISCO activity, and detoxification of reactive oxygen species (Ashraf and Foolad 2007). Glycine betaine is also involved in protecting the protein's structural integrity from stress due to heavy metals (Sakamoto and Murata 2002). Under metal toxicity, glycine betaine and proline have been shown to regulate gene expression by activation of transcription and replication (Rajendrakumar et al. 1997). Aspirin, a derivative of natural plant growth regulator salicylic acid, was found to be playing a potentially similar biological role to that of salicylic acid in the present study. Proline with free radical scavenging capacity might be triggered by aspirin to defend the cell from the oxidative burst. Glycine betaine content elevates aspirin exposure and the results correlate with reports on plant growth regulators by Gao et al (2004). External application of aspirin enhances the GB levels due to the up-regulation of betaine aldehyde dehydrogenase (BADH) expression (Gao et al. 2004).

Soluble proteins are known to reduce with an increase in heavy metal toxicity (Perva et al. 2020). The reduction in protein levels in response to cadmium toxicity and similar heavy metal stress increases protease activity, which causes protein degradation (Palma et al. 2002). Cadmium caused protein content decline, which may be due to the production of free radicals and binding of heavy metals to protein –SH groups that denature protein structure and further diminish the activity of –SH-containing enzymes (Seregin and Kozhevnikova 2006). In the present study, aspirin increases the total protein levels which corroborates with reports on similar plant growth regulators. Several reports suggest that many proteins synthesized during abiotic stress may be due to growth regulators like salicylic acid, and jasmonic acid (Thaler 1999). Plant stress regulators have been known to increase the expression of various proteins during abiotic stress. The elevation in total soluble sugar levels under cadmium toxicity may be attributed to over resistance of photosynthetic organelle (Prokopiev 1978) and depleted transport of starch to cells of the mesophyll. Increased accumulation of toxic heavy metals disrupts the metabolism of carbon because of the undesirable interaction of the ribulose-bisphosphate carboxylase enzyme (Stiborova et al. 1987). Elevated sugar may also be associated with the degradation of starch. Sugar build-up results in the plants absorbing extra water from the neighboring environment (Hajar et al. 1996)

Hydrogen peroxide is a lethal reactive oxygen species (ROS) and increases with cadmium stress, and the findings (Table 3) correlate with Hao et al. (2006). Heavy metal toxicity is also known to facilitate the accumulation of hydrogen peroxide in wheat leaves (Gajewska et al 2006). Malondialdehyde (MDA) is a result of lipid peroxidation and is an indicator of oxidative stress (Chandra and Sukumaran 2020). Heavy metal stress has been reported to increase hydrogen peroxide and lipoxygenase activity, which causes lipid peroxidation. Increased MDA content is also reported in eggplant during heavy metal stress (Pandey and Rajeev 2010). Additionally, several heavy metals are reported to cause an elevation in MDA content in *Bruguiera gymnorrhiza* thus considered an important biomarker in the identification of abiotic stress (Zhang et al. 2007). In the present study co-application of aspirin curtails the generation of hydrogen peroxide, which can preserve the impact on membrane lipids. Reduction in MDA levels during cadmium toxicity with supplementation of growth regulator was also reported in *Kandelia obovata* (Chen et al. 2014). Aspirin might play a similar role to other plant regulators in scavenging free radicals thus avoiding the accumulation of hydrogen peroxide, avoid lipid peroxidation, and accumulation of MDA.

Ascorbate, which is found in most flora and fauna, is an important non-enzymatic antioxidant synthesized in the mitochondria. Ascorbate levels were increased in cadmium induced toxicity in the present study. The oxidative outburst caused by cadmium is negated by the ascorbate cycle thus defending cellular damage (Singh et al. 2006), however, supplementation of aspirin further increased the concentration of ascorbate, confirming the antioxidant system response in plants for the growth regulator.

The elevation in antioxidant enzyme activities can be seen in Table 3 and agrees with the study of Awasthi and Sinha (2013) conducted on *Luffa cylindrical* during heavy metal toxicity. Superoxide dismutase (SOD) which is considered the primary defense enzyme system in living beings increases under metal toxicity in eggplant (Pandey and Rajeev 2010). Heavy metal stress is found to increase ascorbate peroxidase activity in several plant models as wheat (Gajewska et al. 2006) and rice (Maheshwari and Dubey, 2009). Increased catalase and ascorbate peroxidase activity has been reported in *Wolfia arrhiza* under abiotic stress due to heavy metals (Piotrowska et al. 2009). Aspirin increases the activity of antioxidant enzymes in the present study (Table 3), which coincides with the findings of Chen et al. (2014).

*B. juncea* is shown to increase the expression of the catalase 3 gene (CAT3) under cadmium toxicity (Minglin et al. 2005). Gene expression of SOD, CAT, and APX is also been reported to increase in Chickpea under saline conditions (Rasool et al. 2013).

**Conclusion**

Elevated levels of cadmium in the soil are harmful to plant growth, development, and productivity. Biochemical and physiological stress induced by cadmium toxicity is irreversible and leads to adverse effects on root and shoot growth, biochemical variables, and oxidative stress in the present study. However, the co-application of aspirin alleviates the toxic effects of cadmium through the regulation of osmotic and biochemical mechanisms. Application of aspirin in soil known to be affected with cadmium content may be a sustainable way to protect the plants and thus enhance their productivity.

**Conflict of interest**

None declared

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