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researchsubmission@hotmail.com

Research Paper

OXIDATIVE DAMAGE AND OSMOTIC STRESS IN PERIWINKLE (*Catharanthus roseus* L.) PLANTS SUBJECTED TO B NUTRITION

Archana[1] and Nalini Pandey[2]

[1, 2] Plant Nutrition and Stress Physiology Laboratory,
Department of Botany,
University of Lucknow, Lucknow-226007, India.

Abstract

A sand culture experiment was conducted to evaluate the effects of B stress on periwinkle (*Catharanthus. roseus* L var. Nirmal), an economically important medicinal plant. The plants were grown at 0.033, 0.33 and 3.3 mg B L^{-1} supply and optimum growth was observed in plants receiving 0.33 mg B L^{-1}. Periwinkle was found to be sensitive to B deficient (0.033 mg B L^{-1}) and toxic (3.3 mg B L^{-1}) supply and exhibited oxidative stress. Boron deficiency and toxicity increased activity of polyphenoloxidase (PPO) due to accumulation of phenolics. Boron stress caused increased activities of SOD, APX, POD and GR and decreased activity of CAT. A decrease in ascorbate and increase in total non-protein thiols was also observed in B deficient and toxic plants. The increased concentration of MDA, electrical conductivity, H_2O_2, proline and water uptake capacity under B stress in young leaves, suggests membrane damage and osmotic stress in periwinkle plants. To our best knowledge no investigation has been conducted on antioxidative responses of *C. roseus* L. (family- Apocynaceae) under B deficiency as well as toxicity.

Key words: Antioxidative components; Boron deficiency and toxicity; Lipid peroxidation; Periwinkle; Proline.

INTRODUCTION

Boron is an essential micronutrient for higher plants. Although its requirement in plants is very low, it has been assigned important roles in many metabolic processes [1]. Boron is important in crop production both from view point of its effects due to deficiency and toxicity. The roles of B as a structural element of plant cell walls and its implications for plant growth have been well established. However, increasing evidence suggests one or more functions of boron beyond cell wall structure [2].

During oxidative stress, the excess production of reactive oxygen species (ROS) causes membrane damage that eventually leads to cell death. For protection against ROS, plants contain antioxidants enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR), as well as non enzymatic antioxidants such as ascorbate (Asc), dehydroascorbate (DHA), monodehydroascorbate (MDA), and glutathione (GSH) [3]. The extent of oxidative stress in a cell is largely determined by the amounts of superoxide ($O_2^{\cdot-}$), Hydrogen peroxide (H_2O_2), and hydroxyl ($^{\cdot}OH$) radicals. Therefore, the balance of SOD, APX, and CAT activities are crucial for suppressing toxic ROS levels in a cell. Under environmental stresses such as drought, salinity, mineral nutrient disorders etc., plants

along with producing antioxidants also accumulate compatible solutes (or compatible osmolytes) such as proline, sugar alcohols (sorbitol, mannitol), glycine betaine, that perform osmotic adjustment in plants. It has been reported by Gunes et al. [4] under boron toxicity that rather than performing osmotic adjustment proline an indicator of water stress in plants is also involved in scavenging ROS.

In view of the above, the present study was designed to investigate the oxidative damage, enzymic and non-enzymic antioxidant system together with proline concentration in periwinkle (*Catharanthus roseus* L. var Nirmal) subjected to B deficiency and toxicity.

MATERIALS AND METHODS

Plant material and growth conditions

Plants of *Catharanthus roseus* L. var Nirmal were raised, from seeds sown in polyethylene pots containing purified silica sand [5]. The composition of nutrient solution used for growing the plants was: 4 mM KNO_3, 4 mM Ca $(NO_3)_2$, 2 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 0.1 mM Fe EDTA, 10 µM $MnSO_4$, 1 µM $CuSO_4$, 1 µM $ZnSO_4$, 0.1 µM Na_2MoO_4, 0.1 µM NaCl, 0.1 µM $CoSO_4$ and 0.1 µM $NiSO_4$ with variable levels of B supply- 0.033, 0.33 and 3.3 mg B L^{-1}.

To study the effect of B on oxidative metabolism and antioxidative responses in *C. roseus* L. the concentration of malondialdehyde (lipid peroxidation) and hydrogen peroxide (H_2O_2), certain components of non-enzymatic and of enzymatic antioxidative system such as ascorbate, total non-protein thiols (NPT), SOD, CAT, POD, APX and GR were determined. In addition the activity of PPO and phenolic components were also observed. To evaluate the osmotic stress in plants the concentration of proline was determined. All the biochemical analysis was carried out in replicates (n =3) with a completely randomized design at 36 and 48 DAS (days after supply). Entire study was conducted in glasshouse under control conditions of light, humidity and temperature.

Chloroplastic pigments, MDA, and H_2O_2

Chloroplastic pigments were extracted and assayed in 80 % acetone [6]. Lipid peroxidation was measured in terms of malondialdehyde (MDA) formation and H_2O_2 was determined as a titanium chloride complex [6].

Electrical conductivity (E.C.), Proline, Water uptake capacity (WUC) and Total phenols

Electrical conductivity in leaves was determined according to the method described by Sullivan and Ross [7]. Proline was estimated colorimetrically as ninhydrin complex in toluene [6]. The standard calibration curve was prepared using α-proline. Water uptake capacity (WUC) of leaf was determined by using formula- [turgid wt. - fresh wt.]/ dry wt. Total phenols were determined in alcohol soluble fractions [6]. The standard curve was prepared using different concentrations (10-100 µg) of phenol.

Ascorbate, dehydroascorbate and Non-protein thiols

Ascorbate (Asc) was assayed according by extracting fresh leaf tissue in 10 % TCA [8]. Dehydroascorbate (DHA) was determined by reduction of DHA and estimating total Asc. Amount of ascorbate was determined by preparing a standard curve with L-ascorbic acid (Sigma). Non-protein thiols (NPT) were estimated by a reaction carried out with the use of 10 mM DTNB and 0.1mM GSH (glutathione reduced). The colour intensity of extract was measured in a spectrophotometer at 412 nm with in 15 min [9].

Enzyme assay

The activity of catalase (CAT) (EC 1.11.1.6) and pPeroxidase (POD) (EC 1.11.1.7) were assayed in fresh leaf tissue extracts prepared by homogenizing fresh tissue samples in ice cold glass distilled water (1:10) in a cold mortar and pestle at 4^0C [10].

Polyphenol oxidase (PPO) (EC 1.14.18.1) (DOPA oxidase) was assayed in an assay mixture containing 0.1 M phosphate buffer pH 6.5 and suitably diluted enzyme extract [11]. The reaction was initiated by the addition of 0.01 M DL-DOPA (3, 4 –dihydroxy 1- phenol alanine). The reaction was allowed to proceed for 30 minutes at 30 ^{o}C and 0.25 M lead acetate was added to stop the reaction. Optical density of the supernatant was measured at 470 nm. The enzyme activity was expressed as difference in optical density min^{-1} mg $protein^{-1}$.

For assay of superoxide dismutase (SOD) (EC 1.15.1.1), ascorbate peroxidase (APX) (EC 1.11.1.11) and glutathione reductase (GR) (EC 1.6.4.2) leaf samples were homogenized with 150 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA and 2% PVP. For APX assay 1 mM ascorbate was also included in the extracting medium. The homogenates were centrifuged at 15,000 g for 10 min and the supernatant were used for assay of enzyme activities.

SOD activity was determined by measuring the ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) in a reaction mixture containing 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 0 to 50 µl enzyme extract [12]. One unit of SOD represents the amount that inhibits the NBT reduction by 50 %. Activity of APX was determined by oxidation of ascorbate which was followed as fall in absorbance per min. at 290 nm after adding hydrogen peroxide [13]. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.5 mM ascorbate and 0.1mM hydrogen peroxide. The amount of ascorbate oxidized was calculated by using the extinction coefficient of 2.8 mM^{-1} cm^{-1}. GR assay was performed in a 3 ml reaction mixture containing 100 mM phosphate buffer pH 7.0, 1 mM GSSG, 1 mM EDTA, 0.1 mM NADPH and 25 to 50 µl of the enzyme extract. The oxidation of NADPH was followed by monitoring the decrease in absorbance per min. at 340 nm. And the amount of NADPH oxidized was calculated using the extinction coefficient of 6.22 mM^{-1} cm^{-1} [14].

Native PAGE and localization of enzymes on polyacrylamide gels

SOD was localized on 10 % native gel using photochemical method of Beauchamp and Fridovich [12]. SOD activity was localized by soaking the gels in 2.45 mM NBT for 20 min followed by immersion in a solution containing 28.0 mM TEMED, 0.028 mM riboflavin, and 36 mM potassium phosphate buffer (pH 7.8). Identification of individual isozymes of Mn SOD and Cu/Zn SOD was done by soaking the gels in 5 mM H_2O_2 or 2 mM KCN prior to activity staining. The Mn SOD is resistant to both KCN and H_2O_2, Cu/Zn SOD is sensitive to both the reagents.

GR isoforms were separated on 15% native PAGE and visualized on treatment with 50mM Tris buffer pH 7.5 containing 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide, 2,6-dichlorophenol indophenol, 3.44 GSSG and 0.4 mM NADPH [14] .

Statistical analysis

Standard analyses of variance (ANOVA) were used to assess the significance of treatment means. The data are presented as mean values ± standard error (SE, n=3). Differences between treatments means were compared using LSD at the 0.05 probability level.

RESULTS AND DISCUSSION

Growth differences were shown by plants after 35 days of variable boron supply. Plants receiving supply of 0.33 mg B L^{-1} showed maximum growth and those treated with 0.033 and 3.3 mg B L^{-1} supply showed depression in growth. The appearance of deficiency symptoms such as reduction in leaf area, chlorosis, curling of leaf lamina, downward cupping of young emerging leaves and toxicity such as apical and marginal chlorosis in the older leaves suggested the immobile nature of B in *C. roseus* plants and have been reported earlier [15].

Chlorophyll concentration decreased in the leaves of plants subjected to deficiency as well as toxicity of B (Fig. 1). Decrease in Chl *a/b* ratio in leaves was observed in the present study and would inhibit photosynthetic processes as Chl *a* is the main site of photosynthetic reduction. Metwally et al. [16] reported a decreased photosynthetic pigments concentration (Chl *a*, *b* and Car) in wheat cultivar leaves grown under B toxicity. It has been reported [17] that reduction of photosynthesis in the young leaves of tea plants subjected to B deficiency occurred mostly due to stomatal limitation whereas in the older leaves it occurs mainly through reduction of leaf Chl a/b ratio.

Similar to chlorophyll, carotenoid concentration also decreased under low as well as toxic supply of boron at both 36 and 48 days. The decrease was more marked under deficient B supply (Fig.1). Carotenoids are membrane bound antioxidants and protect membranes against ROS induced lipid peroxidation by quenching the singlet oxygen. Carotenoids not only quench singlet oxygen but also prevent the formation of singlet oxygen by quenching the triplet excited state of chlorophylls [16]. The decrease in Chl/Car ratio under B stress observed in present

study may be an adaptive response to increased ROS generation in plants facing stress. But under severe stress conditions (48d), increased Chl/Car ratio was observed, indicating more damage to carotenoids which has been suggested to be more destructive than its reverse because carotenoids protect chloroplast from photooxidative damage [18].

The lipid peroxidation, measured as MDA concentration showed marked increase in periwinkle under B stress. The lipid peroxidation at deficient as well as toxic level of B, resulting in the formation of hydroxyl radical (OH^-) by high H_2O_2 concentration might be due to accelerated Haber weiss reaction as earlier reported [3]. In accordance with the present results, others have recently found that excess B increased both MDA and H_2O_2 concentrations in apple root stock [19] grape [20] and tomato [21]. Contrary to our results Karabal et al., [22] found no relationship between the H_2O_2 concentration and lipid peroxidation in barley cultivars under B toxicity. Hajiboland et al. [17] also reported increased MDA content in tea (*Camellia sinensis* L.) leaves with no significant changes in H_2O_2 concentration under boron deficiency.

Membrane damage under boron stress was determined by evaluating solute leakage from cells. It was found that the amount of electrolyte leakage was more under B deficiency and toxicity than control plants (Fig. 2). Enhanced electrolyte leakage under boron stress indicated the direct involvement of B in cell integrity. Earlier it was demonstrated that increased membrane permeability was associated with increased MDA contents together with a decrease in unsaturated fatty acid contents, indicating enhanced lipid peroxidation reactions [23]. In our experiments boron stress also resulted in increased membrane permeability reflected in terms of increased electrolyte leakage and MDA contents. Under similar conditions there was also accumulation of H_2O_2 contents which suggest the involvement of oxidative stress in these processes. This observation is contradictory to the findings of Karabal et al. [22] and Pfeffer et al. [24] who reported that membrane damage due to B toxicity and B deficiency respectively were not correlated with active oxygen species.

Deficient as well as toxic supply of boron showed accumulation of proline (Fig. 2). Water uptake capacity (WUC) of a leaf was higher under deficiency and excess of boron than control and this increase was more marked in deficient leaves (Fig. 2). An increased proline level is a common response of plants to B stress treatment. Plants under B stress not only showed oxidative damage but also experiences water stress. The observed higher water uptake capacity (WUC) in leaves of boron stressed plants suggested that plants experience water stress because of having low water content in these tissues. Water uptake capacity of a plant indicates the amount of water required by the tissues to get turgid. We have earlier observed that under B stress condition there was accumulation of proline with decreased water potential in leaves of periwinkle, a feature common to water stress in plants [15]. In the present experiment also under B stress accumulation of both proline and H_2O_2 contents together with increased WUC reiterates that B stress is accompanied with osmotic stress which contradicts the finding of Karabal et al. [22] and Gunes et al. [4] who reported that B toxicity was not accompanied with osmotic stress. Proline by forming a complex with ROS molecule is able to detoxify them and hence in this way it helpful in protection of plants against oxidative damage [25]. An increased proline level together with enhanced H_2O_2 contents is a common response of plant cells upon osmotic stress treatments.

Compared with control, the increased concentration of phenols (Fig. 2) with increased activity of PPO (Fig. 4) was observed under B deficiency and excess in periwinkle at both stages. There is evidence that boron is one of the nutrients responsible for the changes in the concentration and metabolism of phenolic compounds in vascular plants. There is accumulation of phenolic compounds particularly caffeic acid and quinones under boron stress, which are highly reactive and lead to enhanced generation of superoxide ions ($O_{\cdot 2}^-$), which cause peroxidative damage to cellular membranes [26]. Reports suggested that boron stress induced qualitative as well as quantitative changes in the phenolic pool in plants [27, 28]. Boron deprivation of plants also increases the activities of polyphenol oxidase (PPO) [27, 28]. PPO is not a component of ROS, in spite of this; it plays an important role in the oxidative processes by oxidizing the phenolic compounds accumulated under stress conditions, into quinones. Recently, Cervilla et al. [29] also reported accumulation of phenolic compounds with increased PPO activity in tomato leaves

grown under boron toxicity. Cakmak and Römheld, [26] suggested that the loss of membrane integrity under boron stress may be due to accumulation of phenols and their oxidation products.

Accumulation of phenols together with increase in PPO activity in leaves of B stressed plants may lead to the production of quinones which in turn are responsible for the production of toxic O_2 species. This enhanced generation of toxic O_2 species are known to induce increase in activity of SOD in B stressed plants. Boron deficient and toxic periwinkle plants showed enhanced SOD activity (Fig. 4) which is in accordance with many other workers [17, 20, 21,]. Our report on the enhanced activity of SOD in B toxic plants fails to find support from earlier reports on barley [22] and citrus [30] in which almost no change in leaf SOD activity was observed under B toxicity. PAGE separation and activity staining revealed 4 isoforms of SOD, the upper most was Mn SOD and three of Cu/Zn SOD. All the isoforms of Cu/Zn SOD showed increase in activity under B deficiency and toxicity compared to control as reflected by the intensity of bands formed and revealed the up regulation mechanism of plants against oxidative damage (Fig. 5).

A H_2O_2 scavenging enzyme, CAT with relatively low affinity for H_2O_2 showed decreased activity in periwinkle under B deficiency and toxicity (Fig. 4) which probably led to excessive accumulation of H_2O_2 in the plants. Peroxidases in plant cell are present in free and/or bound form. Under stressful conditions the bound peroxidase is released to its free state and increases the proportion of free peroxidases. This may be also occurring under B deficiency and toxicity which caused the increased activation of peroxidase (Fig. 4).

APX is another important antioxidant enzyme involved in scavenging of H_2O_2. In the ascorbate-glutathione cycle, APX reduces H_2O_2 using ascorbate as an electron donor. The specific activity of APX increased in periwinkle under deficiency and toxicity of B (Fig. 4); however, it was not sufficient to detoxify the H_2O_2 which was appreciably accumulated in the plants.

Glutathione reductase (GR) is concerned with reduction of oxidized glutathione in the chloroplast and cytosol and regeneration of ascorbic acid. The GR activity was significantly enhanced under B deficiency and toxicity (Fig. 4). All isoforms of GR also showed enhanced activity as indicated by higher expression of bands formed (Fig. 5). This increased activity of GR in leaves also suggested being a protective adaptation against enhanced ROS.

Total non-protein thiol contents (particularly GSH) was found to be increased under B stress (i.e. deficiency a well as toxicity, Fig. 3), which is in consonance with the observations in citrus [32] and tomato [21], but contrary to the observation in sunflower which showed decrease in GSH contents under excess B supply [32]. GSH acts as a cell redox regulator and may act as an AOS scavenger. Over expression of this antioxidant under B stress suggests its important role in protecting against oxidative stress triggered by B deficiency and toxicity.

Ascorbic acid, is an important antioxidant molecule and quenches ROS including O_2^-, OH^- and $^1\Delta g$ O_2 directly, apart from effectively scavenging H_2O_2 [33]. In perwinkle leaves there was decrease in concentration of ascorbate both under deficient and toxic boron supply at both the stages (Fig. 3). This decrease might be due to the reduced biosynthesis of ascorbic acid and/or stimulated consumption (oxidation) of ascorbic acid during B stress in leaves. There was increase in the concentration of DHA both under deficiency and toxicity. The ratio of DHA to Asc was found to be increased under B stress compared to control at both days of treatment (Fig. 3). Decreased ascorbate concentration with increased ratio of DHA to ascorbate indicates a disturbed redox status under boron stress. The results showed that non-enzymatic antioxidant such as ascorbate and glutathione along with antioxidative enzyme activity could give a protective function against oxidative stress under boron stress.

The results of present study showed that B deficiency as well as toxicity appears to result in oxidative damage and induce a general response of oxidative stress. The decrease in the concentration of carotenoids, ascorbate and accumulation of phenols led to the induction of oxidative stress by causing the excess production of ROS, leading to oxidative damage. Kobayashi et al. [34] proposed that a quick signal transfer from the cell wall to the cytoplasm could be involved in gene induction after the cellular redox imbalance imposed by boron stress. This suggests that boron is possibly involved in the oxidative stress induced expression of genes encoding for the enzyme of the antioxidative defense system [34]. This induction of oxidative

stress triggered an increase in the activities of antioxidative enzymes (SOD, APX, GR and POD) to overcome the oxidative damage due to accumulation of highly reactive phenolic compounds such as quinones, semiquinones, hydroquinones etc in boron stressed plants. Accumulation of MDA content together with phenols and H_2O_2 and also the accumulation of proline with increased WUC under B deficiency and toxicity, suggest the correlation of membrane permeability (reflected in terms of electrolyte leakage) with ROS and osmotic stress. From the present study it was also concluded that besides having up regulatory mechanism of enzymic and non-enzymic antioxidants under boron deficiency and toxicity plants experience oxidative damage more so in former due to higher accumulation of H_2O_2, phenols and excessive lipid peroxidation and membrane leakage. Osmotic stress due to accumulation of proline and low water content probably enhances the oxidative damage in boron stressed plants.

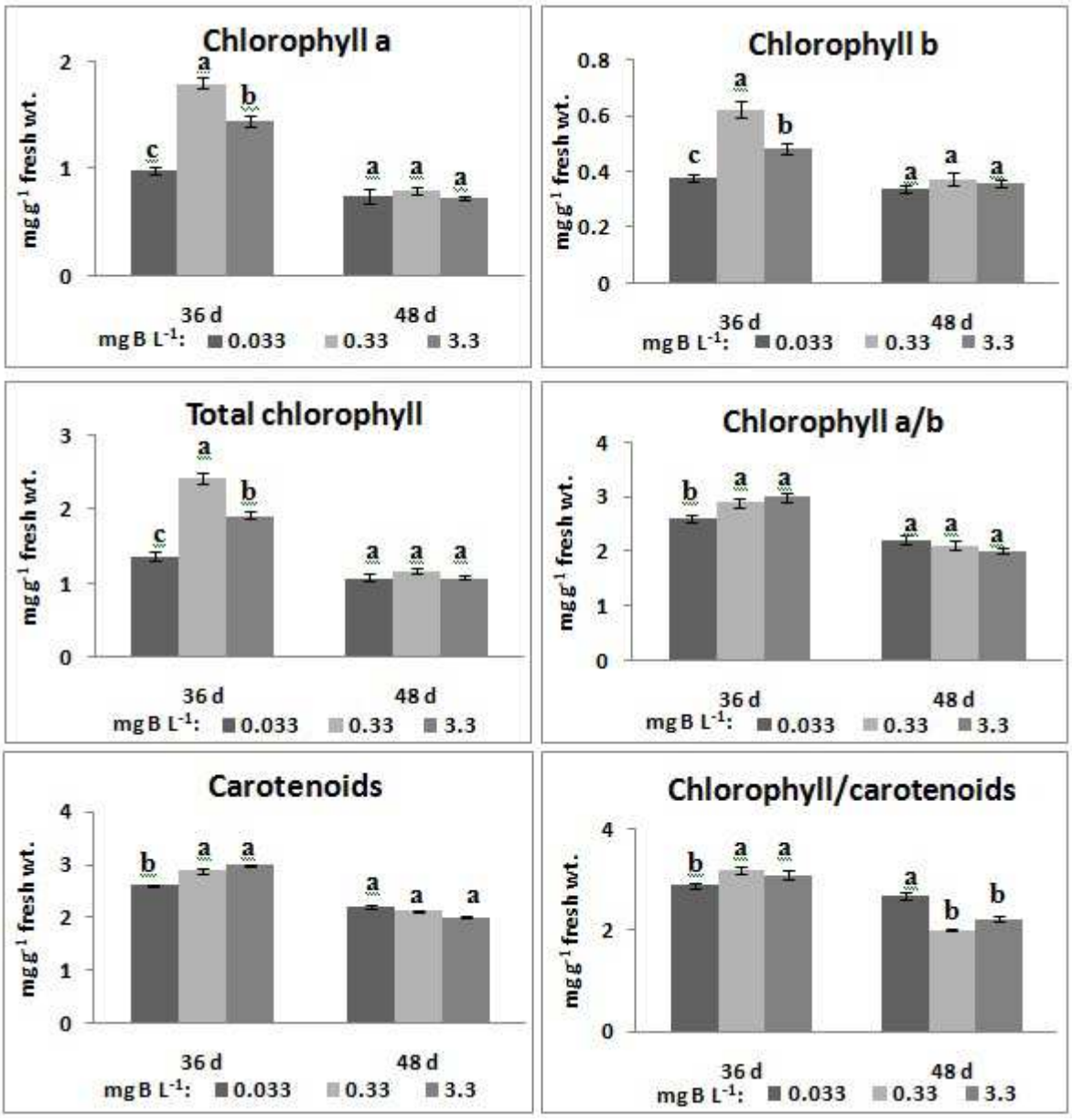

Figure 1. Boron deficiency and toxicity effect on the concentration of chloroplastic pigments in leaves of *Catharanthus roseus* L. var. Nirmal. Different letters indicate significant difference at P=0.05 (control=0.33 mg B L^{-1} supply).

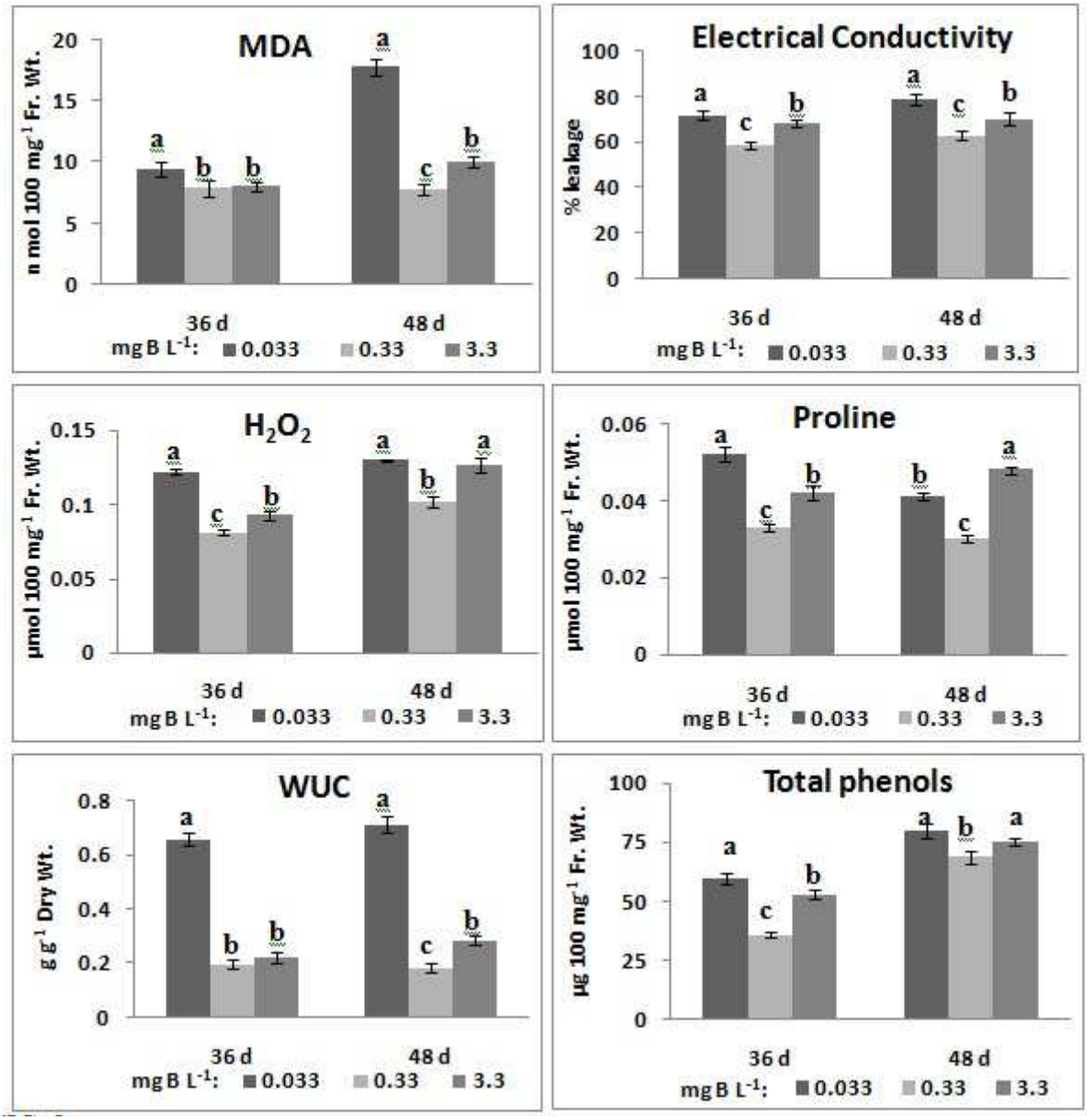

Figure 2. Boron deficiency and toxicity effect on the concentration of MDA, H_2O_2, electrical conductivity, proline, WUC and phenols in leaves of *Catharanthus roseus* L. var. Nirmal. Different letters indicate significant difference at P=0.05 (control=0.33 mg B L^{-1} supply).

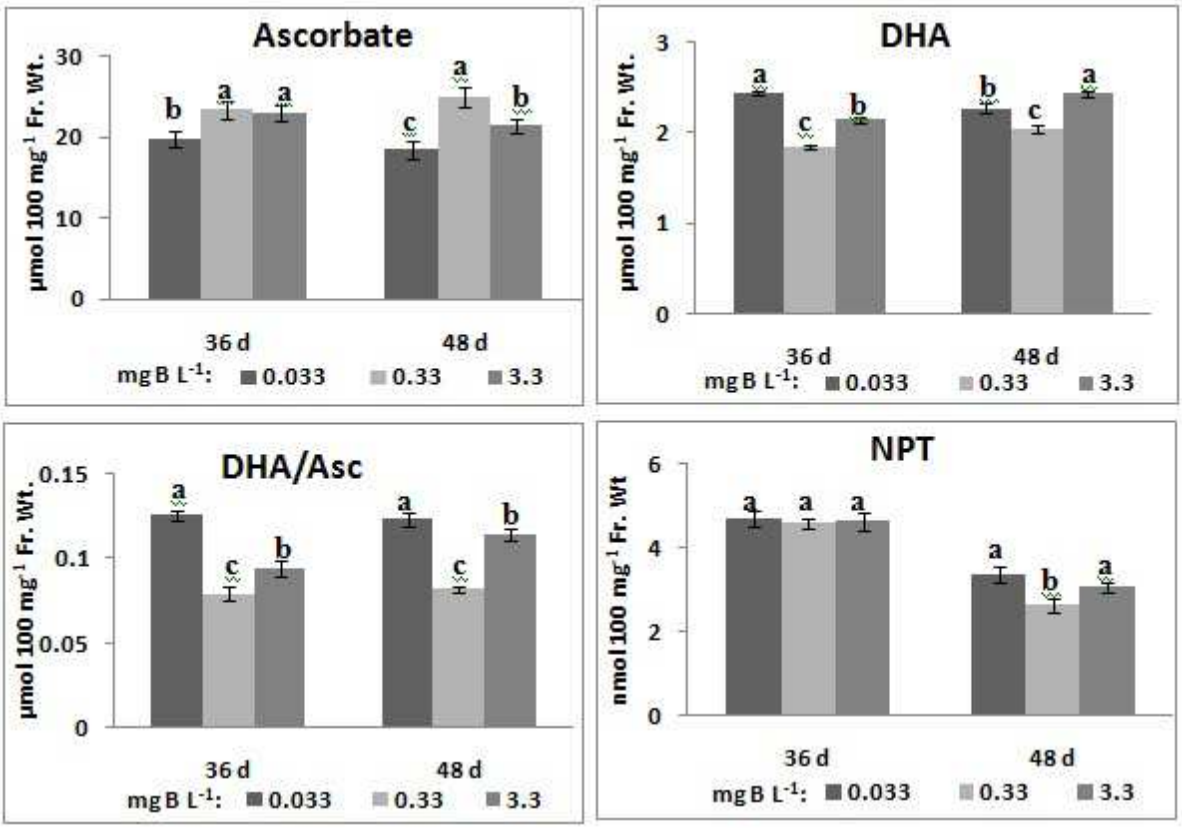

Figure 3. Boron deficiency and toxicity effect on the concentration of Asc, DHA and NPT in leaves of *Catharanthus roseus* L. var. Nirmal. Different letters indicate significant difference at P=0.05 (control=0.33 mg B L^{-1} supply).

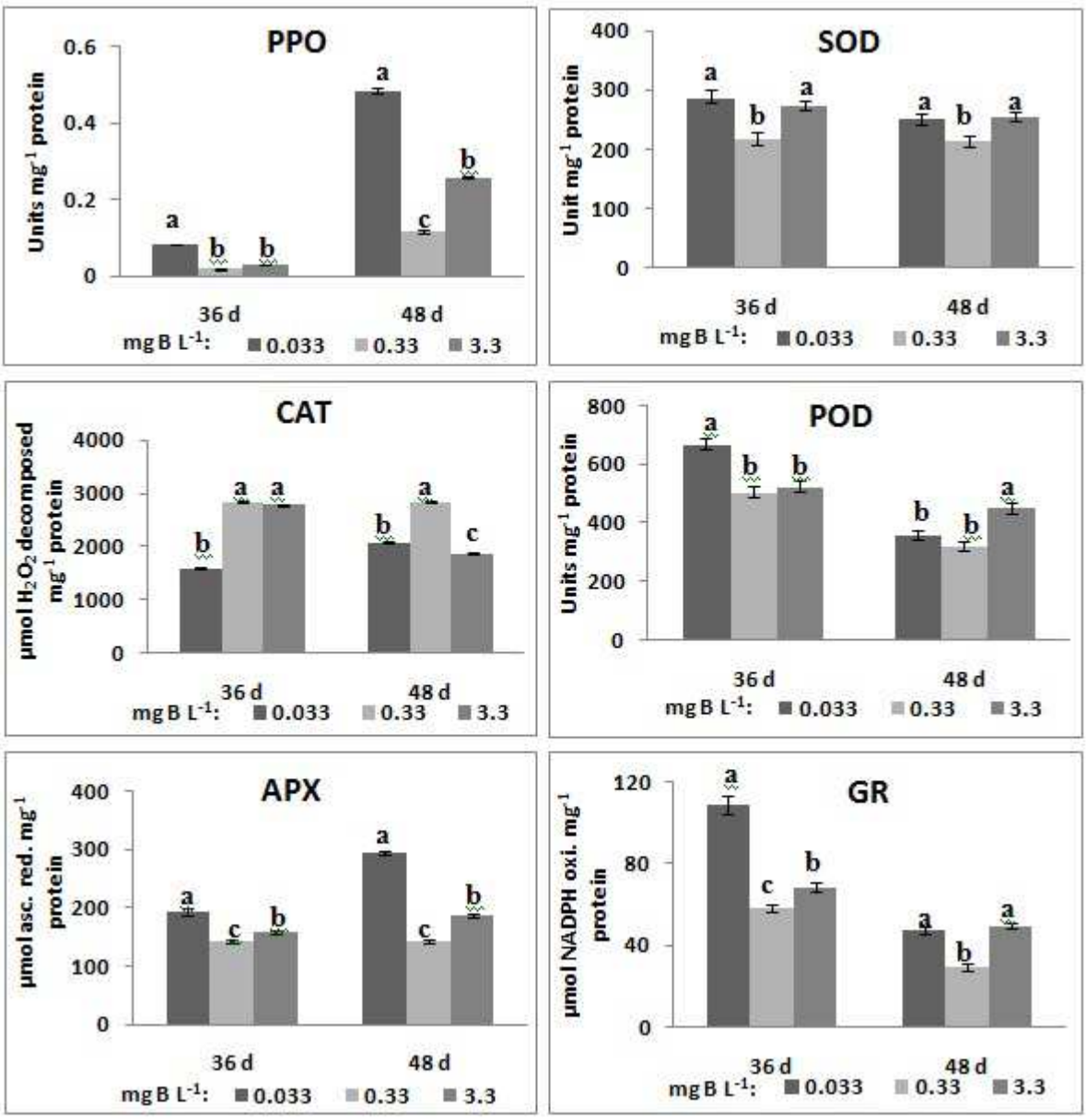

Figure 4. Boron deficiency and toxicity effect on the activity of PPO, SOD, CAT, POD, APX and GR in leaves of *Catharanthus roseus* L. var. Nirmal. Different letters indicate significant difference at P=0.05 (control=0.33 mg B L^{-1} supply).

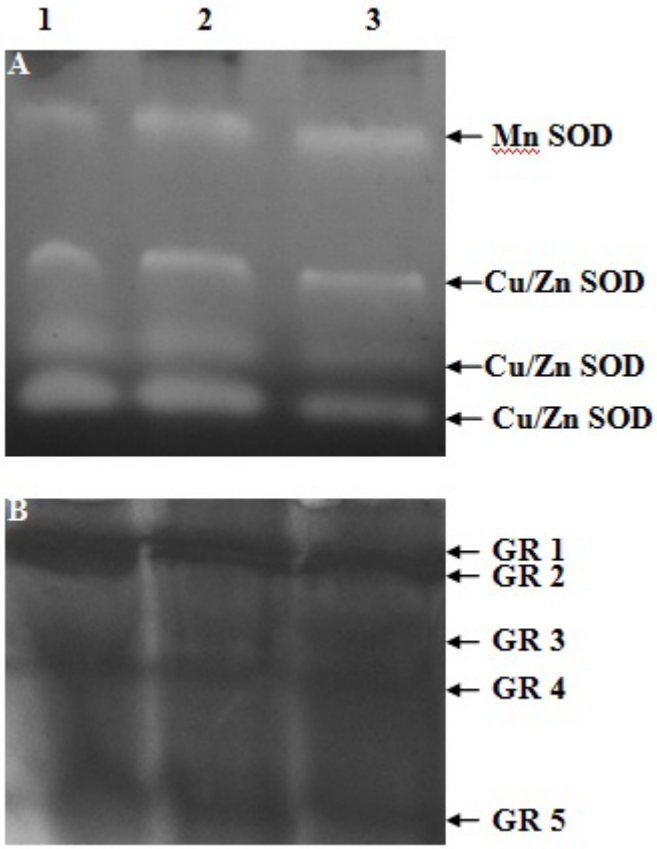

Figure 5. Native gels stained for superoxide dismutase (A) and glutathione reductase (B) isoforms in leaves of *Catharanthus roseus* L. var. Nirmal. Arrows indicate the different isoforms in the leaves. Lane 1-3 represents the isozyme profiles in the leaves of plant grown with 1- 0.033 mg B L^{-1}; 2- 0.33 mg B L^{-1}; 3- 3.3 mg B L^{-1}.

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