
Extremely high boron tolerance in *Puccinellia distans* (Jacq.) Parl. related to root boron exclusion and a well-regulated antioxidant system

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**Abstract:** Recent studies indicate an extremely high level of tolerance to boron (B) toxicity in *Puccinellia distans* (Jacq.) Parl. but the mechanistic basis is not known. *Puccinellia distans* was exposed to B concentrations of up to 1000 mg B L⁻¹ and root B uptake, growth parameters, B and N contents, H₂O₂ accumulation and -OH-scavenging activity were measured. Antioxidant enzyme activities including superoxide dismutase (SOD), ascorbate peroxidase, catalase, peroxidase and glutathione reductase, and lipid peroxidation products were determined. B appears to be actively excluded from roots. Excess B supply caused structural deformations in roots and leaves, H₂O₂ accumulation and simultaneous up-regulation of the antioxidative system, which prevented lipid peroxidation even at the highest B concentrations. Thus, *P. distans* has an efficient root B-exclusion capability and, in addition, B tolerance in shoots is achieved by a well-regulated antioxidant defense system.

**Keywords:** antioxidant enzymes; boron toxicity; *Puccinellia*; reactive oxygen species; root and leaf anatomy.

1 Introduction

Boron (B) toxicity is a major constraint to crop production in areas where underground water for irrigation contains excessive B concentrations (e.g. arid or semiarid states of India, western desert of Egypt, Spain, Arizona, northern Greece, Philippines and southern California) [1] or soil profiles are inherently high in B as in California, South Australia, Jordan, Syria, Iraq, Egypt, Morocco, Chile and Turkey [2, 3]. While the exact process by which B toxicity impairs plant function remains unclear, progress has been made in defining mechanisms of tolerance. Boron efflux transporters in the roots appear to explain higher B tolerance in barley cultivars [4] which enable the tolerant cultivars to maintain lower root and shoot B concentrations. However, other evidence suggests that in some plants, B tolerance is also associated with the ability to tolerate high shoot B concentrations [5]. The mechanisms by which this occurs are not clear. Reid and Fitzpatrick [6] suggest that B transporters in barley leaves partition excess leaf B into necrotic tissue, and that this results in sections of the leaf containing excessively high B concentrations, while the rest of the leaf maintains a moderate level of B. However, earlier studies indicate that *P. distans* does not produce typical leaf necrosis symptoms even at high shoot B concentrations. This suggests that other mechanisms are used by this species to tolerate high internal B.

*Puccinellia distans* (Poaceae) grows naturally around borax mines in Turkey on soils containing 10–15 times higher B concentrations than the concentrations that wheat can tolerate [5, 7, 8]. Moreover, the shoots of this species contain very high B concentrations when grown in high B soils. Tolerance of *P. distans* to high B in growth media was reported previously [5, 7, 8]. The genotype used in this study has the extraordinary ability to survive around 277 mg B kg⁻¹ of soil at a borax mining site in Central Turkey. Considering that most crops are unable to tolerate soil B concentrations above 5 mg kg⁻¹ [9], and that some exceptional pea varieties tolerate only up to 100 mg B kg⁻¹
of soil [10], the tolerance of *P. distans* is truly remarkable. The fact that *P. distans* is a grass species, distantly related to wheat, adds relevance to the study of B tolerance mechanisms in this species. Hence, it has the potential to serve as a model for understanding how plants cope with high internal B concentrations, an aspect of utmost importance in cereals. Previous studies suggest that B efflux transporters may exclude B from *P. distans* shoots at moderate external B concentrations [11]. However, at 500–1000 mg B L⁻¹, shoot B concentrations increased markedly while plant growth was only moderately depressed [8]. This suggests that *P. distans* has the capacity to tolerate high internal B. A transcriptomic study with *P. distans* [11] suggests that the antioxidant defense system is upregulated under high external B. However, there has been no further exploration of the role of the antioxidant systems in *P. distans* B tolerance.

It is widely acknowledged that B toxicity causes oxidative stress because of the formation of reactive oxygen species (ROS) such as superoxide (O²⁻), hydrogen peroxide (H₂O₂) and hydroxyl (·OH) radicals [3]. If the accumulated levels of O²⁻ and H₂O₂ exceed the scavenging ability of the defense system, excess ROS cause lipid peroxidation and membrane damage. To prevent the damaging effects of these reactive molecules, plants have evolved a scavenging system comprising a range of antioxidant molecules (e.g. phenols, ascorbic acid (AsA), glutathione (GSH), and anthocyanins) and antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX) and glutathione reductase (GR) [12]. The enzymatic action of SOD dismutates O²⁻ into H₂O₂ and O₂. The H₂O₂ generated is then scavenged by CAT and several classes of peroxidases [APX and glutathione peroxidase (GPX)]. Intracellular levels of H₂O₂ are regulated by a number of enzymes in plants, but CAT and APX are considered the most important, and certain concentrations of H₂O₂ can enhance the activities of SOD, CAT and APXs as well as upregulate SOD and GR gene expression [12]. Besides APX, GR, monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) are also involved in the Halliwell–Asada pathway, contributing to the regeneration of reduced ascorbate while scavenging H₂O₂ [12]. Enhanced activities of antioxidant enzymes under B toxicity have been reported by several previous studies [13–15]. However, the contribution of antioxidants to scavenging of B-induced ROS and their involvement in the high level of B tolerance in *P. distans* requires more research.

This study aimed to analyze the tolerance mechanism of *P. distans* under toxic B conditions, and to obtain insight into the roles of the B exclusion mechanism and antioxidative defense system in providing B tolerance. Since this grass can accumulate very high internal B concentrations, while maintaining satisfactory growth (Figure 1), we predict that it has a mechanism to tolerate B toxicity and may therefore be a useful model for related cereal species. Novel information on the tolerance mechanism may advance breeding of cultivars suitable to grow in problematic areas with high B in soil or irrigation water. Thus, the objectives of this study were to investigate the effects of high B on the growth of *P. distans*, B uptake by roots, B and N concentrations in planta, H₂O₂ accumulation, ·OH scavenging activity, lipid peroxidation, and antioxidants enzyme activities, as well as on the anatomy of roots and leaves.

## 2 Materials and methods

### 2.1 Plant material and B stress applications

Seeds were obtained from *P. distans* (Jacq.) Parl. plants growing in the Eskisehir Kirkpa Borax Mine deposit in Anatolia. The seeds were surface-sterilized by 5% sodium hypochlorite treatment for 10 min and then washed with de-ionized water (DI-H₂O) three times. After germination, seedlings were transferred to hydroponic culture in constantly aerated 1/5 Hoagland solution (pH 6.0) containing 3 μM H₃BO₃ (corresponding to 0.033 mg B L⁻¹) [16] and grown in a plant growth room at a relative humidity of 45–55%, a photoperiod of 16 h light and 8 h dark at 22±1 °C and PAR of 350–400 μmol m⁻² s⁻¹. Plants at the three-leaf stage were treated with 1/5 Hoagland solution containing 0 (B-deficient), 0.033 (control), 2.5, 25, 250, 500, and 1000 mg B L⁻¹ in the form of boric acid. Each group consisted of 40 plants in four pots (10 plants per pot). The study was conducted in a completely randomized design with four replicates. The experiments were repeated three times with similar results. The plants were harvested before (Day 0) and 30 days after B treatment, when the first signs of B toxicity symptoms became visible. Harvested roots and leaves were rinsed three times in distilled water and then blotted dry on filter paper, all within 1 min, and then immediately frozen in liquid nitrogen and stored at −80 °C until use.

### 2.2 Growth parameters

After 0 and 30 days of B treatment, 20 plants from each group were taken at random and divided into separate shoot and root fractions. Length and fresh weight (FW)
of shoots and roots were measured and their dry weight (DW) determined after drying them at 70°C in a forced-draft oven to constant weight.

### 2.3 Shoot and root B and N content

For boron determination, dried plant material was digested with HNO₃ in a microwave system (Mars 5, CEM Corp., Matthews, NC, USA) according to Huang et al. [17]. B in the filtrate was analyzed by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES, Vista AX, Varian, Sydney, Australia) and the content calculated per DW and per plant.

To determine the nitrogen content, 0.2 g of dried plant material was ground and combusted with oxygen; helium was used as carrier. An aliquot of combustion gas containing nitrogen oxides was reduced to molecular nitrogen and then passed through a tube containing magnesium perchlorate and sodium hydroxide on a silicate carrier to remove water and carbon dioxide. Nitrogen was measured with a thermal conductivity detector using helium as a reference in a CNS-2000 Analyzer (Leco, St. Joseph, MI, USA) according to the manufacturer’s instructions [18]. All determinations were made in triplicate.

### 2.4 Short-term root B uptake experiment

Four plants each of either 12-day-old barley cultivars (Hamidlye [B-sensitive] and Tokak [B-tolerant] [19]) or of 20-day-old *P. distans* were placed in black pots containing
2.8 L of 1/5 Hoagland solution (only the roots of the plants were immersed) with 0, 0.033, 2.5, 25, 250, 500 and 1000 mg B L⁻¹, and all pots were aerated continuously (pH 6.0). After 24 h, a 1-mL aliquot from the solution of each pot was removed for determination of B. A control was run without plants. Because of the high permeability of B, roots were not rinsed prior to analysis but just blotted. B concentrations in both the solutions and the roots were determined by ICP-AES (see Section 2.3). Evaporation was also recorded. B uptake by the plants was calculated based on the loss of B from the external solutions.

2.5 H₂O₂ content

Hydrogen peroxide (H₂O₂) contents in mature and fully expanded leaves were estimated using the method of Velikova et al. [20]. The H₂O₂ content was determined from a standard curve, and the values were expressed as μmol g⁻¹ fresh weight.

2.6 Hydroxyl-scavenging activity

Hydroxyl-scavenging activity was assayed in leaves according to the 2-deoxyribose oxidation method [21]. The absorbance was measured at 520 nm. Inhibition of 2-deoxyribose oxidation by hydroxyl radicals was calculated as the percentage of inhibition according to the formula:

\[
\text{Hydroxyl radical scavenging activity} = \left( \frac{(A_0 - A_1)}{A_0} \right) \times 100
\]

where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of leaf extracts.

2.7 Lipid peroxidation level

The level of lipid peroxidation products in leaves was determined in terms of malondialdehyde (MDA) content according to the method of Rao and Sresty [22]. MDA concentration was calculated from the absorbance at 532 nm, using an extinction coefficient of 155 mM⁻¹ cm⁻¹, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm.

2.8 Antioxidant enzyme activities

All procedures were performed at 4 °C. For protein extraction, 0.5 g of mature leaf samples with no chlorotic or necrotic lesions were homogenized with mortar and pestle with 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA-Na₂ and 2% (w/v) polyvinylpolypyrrolidone (PVPP). For APX extraction, 2 mM ascorbate was included in the homogenization buffer. Samples were centrifuged at 14,000 g for 40 min, and supernatants were used for determination of protein content and enzyme activity assays. Total soluble protein was measured according to Bradford [23] using bovine serum albumin as standard. All spectrophotometric analyses were conducted on a Shimadzu (UV 1600) spectrophotometer.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by the ability of SOD to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm [24]. One unit of SOD activity was defined as the quantity of SOD required to produce 50% inhibition of NBT reduction, and the specific enzyme activity was expressed as unit mg⁻¹ protein.

Catalase (CAT; EC 1.11.1.6) activity was estimated according to [25] by measuring the initial rate of disappearance of H₂O₂ at 240 nm. The decrease in the absorbance was followed for 3 min and 1 μmol H₂O₂ destroyed per min was defined as unit of CAT.

Peroxidase (POX; EC 1.11.1.7) activity measurement was based on the method described by Herzog and Fahimi [26]. The increase in the absorbance at 465 nm was followed for 3 min. A unit of POX activity was defined as μmol mL⁻¹ H₂O₂ decomposed per min.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was measured according to Nakano and Asada [27]. The concentration of oxidized ascorbate was calculated by using an extinction coefficient of 2.8 mM⁻¹ cm⁻¹. A unit of APX activity was defined as μmol mL⁻¹ oxidized ascorbate per min.

Glutathione reductase (GR; EC 1.6.4.2) activity was measured according to Foyer and Halliwell [28]. GR activity was based on the rate of decrease in NADPH using an extinction coefficient of ε = 6.2 mM⁻¹ cm⁻¹. One unit of GR was defined as the amount of enzyme oxidizing 1 μmol of NADPH min⁻¹.

The specific activities of all enzymes were expressed as units mg⁻¹ protein.

2.9 Microscopy

For observation of anatomical changes in roots and leaves of *P. distans*, plants were harvested before (Day 0) and 30 days after B treatments. Root and leaf sections of harvested plants were excised into small blocks (30 × 30 × 30 μm³) and then fixed in 70% (v/v) ethanol. Paraffin-embedded plant material was sectioned using a
micrometre [29] and then viewed under a Leica DM3000 microscope.

2.10 Statistical analysis

All phenotypic data obtained were subjected to one-way analyses of variance (ANOVA), and significantly different groups were determined by the least significant difference (LSD) test at \( P < 0.05 \).

3 Results

3.1 Growth parameters

Shoot lengths of *P. distans* plants increased \( 10\% \), \( 29\% \), \( 19\% \), \( 13\% \) and \( 18\% \), respectively, with a B supply of \( 0.033 \), \( 2.5 \), \( 25 \), \( 250 \) and \( 500 \) mg L\(^{-1} \), when compared to \( 0 \) mg B L\(^{-1} \), but there was no significant change at \( 1000 \) mg B L\(^{-1} \) \( (P > 0.05) \) (Figures 1 and 2A). By contrast, there was no significant effect of B on root length (Figure 2A). Fresh and dry weights of shoots were increased \( 24\% \) at \( 0.033 \) mg B L\(^{-1} \) in comparison with no B, while the highest increases (\( 41\% \) and \( 126\% \), respectively) were observed at \( 2.5 \) mg B L\(^{-1} \), representing a dosage close to the toxic level for B-sensitive plants. The increases of the shoot fresh and dry weights at \( 25 \) and \( 250 \) mg B L\(^{-1} \) were interestingly higher than those at \( 0.033 \) mg B L\(^{-1} \), which is the typical range of B optimal for most plants (Figure 2B and C). Shoot fresh weight significantly decreased at \( 500 \) and \( 1000 \) mg of B L\(^{-1} \) (\( 18\% \) and \( 26\% \), respectively) while shoot dry weight was decreased by \( 44\% \) at \( 1000 \) mg of B L\(^{-1} \), compared with that at \( 0.033 \) mg of B L\(^{-1} \).

Like for the shoot, root fresh and dry weights were highest with increases by \( 70\% \) and \( 42\% \), respectively, at

![Diagram](image.png)

*Figure 2: Puccinellia distans* plants grown as in Figure 1 were analyzed for length (A), fresh weight (B), dry weight (C), and boron concentration (D) of shoots and roots. Total B content per plant is shown in (E). The values are means of \( n = 20 \pm SE \) for (A), (B) and (C); \( n = 4 \pm SE \) for (D) and (E). Bars with different letters indicate significant differences at \( P < 0.05 \) based on the LSD test.
2.5 mg B L\(^{-1}\) in comparison with no B L\(^{-1}\) (Figure 2B and C). Thus, the growth performance of \(P. \text{distans}\) was best at 2.5 mg B L\(^{-1}\).

3.2 Shoot and root B and N concentrations

Shoot B concentrations of \(P. \text{distans}\) plants increased \((P < 0.01)\) continuously with increasing B in the nutrient solution and reached the highest value at the dose of 1000 mg B L\(^{-1}\) (4103 mg B kg\(^{-1}\); Figure 2D), which was 79-fold the value in the absence of B. On the other hand, B concentration in roots did not increase significantly up to 250 mg B L\(^{-1}\) \((P > 0.05)\), but reached 2010 mg B kg\(^{-1}\) at 1000 mg B L\(^{-1}\) \((P < 0.001)\). The total B content per plant after the 30-day treatment (Figure 2E) also increased in parallel to internal B concentrations (Figure 2D). This was preferentially due to B accumulation in the shoot, because the total B content per root was significantly increased only at 1000 mg B L\(^{-1}\) \((P > 0.05)\).

The N content of shoots and roots (% of g\(^{-1}\) DW) of \(P. \text{distans}\) plants changed significantly in response to B treatments (Figure 3). Although the boron-deficient condition (0 mg B L\(^{-1}\)) caused a 17% decrease in the N content of shoots, it caused a 21% increase in the roots. At all B concentrations, the shoot N content varied only slightly, while at 500 and 1000 mg B L\(^{-1}\), the N content of the roots was reduced by about 25%.

3.3 Short-term root B uptake

The low degree of growth inhibition observed at very high B concentrations in the growth medium (Figures 1, 2A, B and C) and the ability of \(P. \text{distans}\) plants to maintain low tissue B concentrations in media containing 1000 mg B L\(^{-1}\) (Figure 2D), as well as a low B content in roots and shoots per plant (Figure 2E), prompted us to explore a possible exclusion mechanism in this plant. We performed a separate short-term (up to 24 h) root B-uptake experiment to establish whether exclusion does occur, and included two Turkish barley (\(Hordeum \text{vulgare}\) L.) cultivars, i.e. B-sensitive Hamidiye and B-tolerant Tokak [19], in addition to \(P. \text{distans}\), for comparison. Thus, while in the previous experiment B content was determined at the end of a 30-day incubation of \(P. \text{distans}\) in solutions of different B concentrations, in this experiment B taken up from the solution was determined after 24 h. The B-sensitive barley cv. Hamidiye took up more B than the B-tolerant cv. Tokak at 500 and 1000 mg B L\(^{-1}\) B (Figure 4A), and root B concentrations were considerably (up to

Figure 3: Nitrogen content (% of dry weight) in shoots and roots of \(P. \text{distans}\) plants grown for 30 days at the indicated B concentrations. The values are means of four replicates ± SE \((n = 4)\). Bars with different letters indicate significant differences at \(P < 0.05\) level based on the LSD test.

Figure 4: B-uptake by roots (A) and B concentrations in roots (B) of \(P. \text{distans}\) and barley cvs. B-sensitive Hamidiye and B-tolerant Tokak. Twelve-day-old barley and 20-day-old \(P. \text{distans}\) seedlings were exposed to the indicated B concentrations for 24 h, and B uptake was calculated from disappearance of B from the medium. The inserts are enlarged sections of the graphs. FW, root fresh weight. Error bars represent ± SE \((n = 4)\). Bars with different letters are significantly different at \(P < 0.05\) based on the LSD test.
three-fold) higher in cv. Hamidiye as compared to Tokak (Figure 4B). B uptake, and even more so root B concentration, of *P. distans* were much lower than in either of the two barley cultivars (Figure 4A and B). Root B-uptake was not significantly different among the three species up to a B concentration of 25 mg L⁻¹, while at higher B concentrations, B uptake was about two-fold higher by cv. Hamidiye, and slightly less by cv. Tokak, as compared to *P. distans* (Figure 4A).

### 3.4 Reactive oxygen species

Compared to the control (0.033 mg B L⁻¹), H₂O₂ concentrations in leaves of *P. distans* were significantly increased both in the B-deficient condition as well as at B concentrations in excess of 25 mg L⁻¹, up to more than 200% at 1000 mg B L⁻¹ (Figure 5A). -OH radical-scavenging activity was increased both in the absence and in the presence of excess B in the medium, with a maximum at 250 mg B ml⁻¹ (Figure 5B).

#### 3.5 Lipid peroxidation

The effect of B on MDA levels was less than in the case of H₂O₂ and the -OH radical, with a slight maximum at 25 mg B ml⁻¹ (Figure 5C).

#### 3.6 Antioxidant enzyme activities

Superoxide dismutase (SOD) activity: Compared with the control (0.033 mg B L⁻¹) treatment, SOD activities in leaves of *P. distans* were decreased 13%, 14% and 15%, respectively, by 2.5, 25 and 250 mg B L⁻¹. However, an 18% increase was observed in SOD activity at the dose of 500 mg B L⁻¹ compared to the control group, while the dose of 1000 mg B L⁻¹ did not decrease SOD activity (Figure 6A).

Peroxidase (POX) activity: Boron-deficient condition (0 mg B L⁻¹) enhanced POX activity by 23% as compared to the control treatment (0.033 mg B L⁻¹). Furthermore, 500 and 1000 mg B L⁻¹ caused 52% and 10% increases in POX activity, respectively. Nevertheless, POX activity was

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*Figure 5*: Levels of H₂O₂ (A), hydroxyl radical scavenging activity (B), and malondialdehyde (MDA) (C) in leaves of *P. distans* plants on days 0 and 30 of exposure to indicated B concentrations. Values of columns with *, **, *** are significantly different at *P < 0.05, 0.01, 0.001*, respectively.
Figure 6: Activities of SOD (A), POX (B), CAT (C), GR (D) and APX (E) in leaves of *Puccinellia distans* plants on days 0 and 30 of exposure to indicated B concentrations. Values of columns with *, **, *** are significantly different at *P* < 0.05, 0.01, 0.001, respectively.

decreased 10%, 29% and 25%, respectively, at the doses of 2.5, 25 and 250 mg B L⁻¹ in comparison to the control (Figure 6B). POX activity was strongly correlated with SOD activity but negatively with shoot and root dry and fresh weights and MDA content (see Supplementary Material, Table S1).

Catalase (CAT) activity: Compared to the control (0.033 mg B L⁻¹), CAT activity in leaves decreased by 15% and 18% in 25 and 1000 mg B L⁻¹, respectively, while it was increased by 17% in 250 mg B L⁻¹. The differences in CAT activity in leaves between control (0.033 mg L⁻¹ of B) and 0, 2.5, and 500 mg B L⁻¹ treatments were not statistically significant (Figure 6C). Activity of CAT was positively correlated with -OH-scavenging activity, but negatively with root dry weight and MDA. CAT activity was not correlated with other anti-oxidants (see Supplementary Material, Table S1).

Glutathione reductase (GR) activity: Glutathione reductase activity in leaves decreased depending on the dosage of B. Compared to control (0.033 mg B L⁻¹) treatment, GR activity was decreased at all doses of B (2.5, 25, 250, 500 and 1000 mg B L⁻¹), and the greatest decreases (42% and 48%, respectively) were observed at the doses of 25 and 1000 mg B L⁻¹ (Figure 6D).

Ascorbate peroxidase (APX) activity: Boron-deficient condition (0 mg B L⁻¹) caused 38% enhancement in APX activity in leaves and similarly, 25, 250, 500 and 1000 mg B L⁻¹ treatments resulted in 13%, 7%, 32% and 51% increases
In APX activity, respectively, when compared to the control group (Figure 6E). APX activity was strongly correlated with the H₂O₂ level. APX levels were also strongly correlated with SOD and POX (see Supplementary Material, Table S1).

3.7 Microscopy

Root and leaf cross-sections of *P. distans* plants exposed to different B concentrations for 30 days are illustrated in Figures 7 and 8, respectively. Observation of root cross sections of plants exposed to 25 and 250 mg B L⁻¹ revealed a reduced number of xylem arms (Figure 7D and E). Increased lacunar spaces were observed in leaf cross sections of 25, 250 and 500 mg B L⁻¹ treatments (Figure 8D, E and F) and lamina thickness was decreased at 250 mg B L⁻¹ (see Supplementary Material, Table S3). Significant structural deformations in roots were observed at 500 mg B L⁻¹ treatment involving changes in shape and loss of cell boundaries (Figure 7F). Root cortical cells were partially fragmented and the number of trachea decreased. A prominent increase in the number of trachea was observed in

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**Figure 7**: Root anatomy of *P. distans* plants grown for 30 days at different B concentrations. Right panel contains magnified micrographs of the root cross-sections in the left panel; bar = 50 μm. Day 0, 0.033 mg B L⁻¹ (control) (a, a'); Day 30, 0 mg B L⁻¹ (b, b'); Day 30, 2.5 mg B L⁻¹ (c, c'); Day 30, 25 mg B L⁻¹ (d, d'); Day 30, 250 mg B L⁻¹ (e, e'); Day 30, 500 mg B L⁻¹ (f, f'); Day 30, 1000 mg B L⁻¹ (g, g'). ep, Epidermis; m, metaxylem; en, endodermis; p, pericycle.

(At day 30, plants exposed to 0.033 mg B L⁻¹ did not differ significantly from those exposed to 2.5 mg B L⁻¹, and image is not shown.)
root cross sections at 25 and 500 mg B L⁻¹ (Figure 7D and F; see Supplementary Material, Table S2).

Cuticles of leaves of *P. distans* plants treated with 2.5 and 250 mg B L⁻¹ were thinner (Figure 8C and E). On the contrary, the cuticle of plants treated with 1000 mg B L⁻¹ was thicker than in the other treatments (Figure 8G). Epidermis cells were flat and smooth in control groups, but began to lose their regular arrangement at increasing B concentrations (Figure 8 and Supplementary Material, Table S3). Vascular areas generally became narrower with increasing doses of B. Diminution was observed in the size of bundle cells of leaf cross sections in 250 mg B L⁻¹ treatment groups on day 30 (Figure 8E and Supplementary Material, Table S3).

4 Discussion

The extremely high level of B in the natural soils where *P. distans* grows suggests an unusually high level of B tolerance. Some studies have drawn attention to the exceptional B tolerance of this species [5, 7]. The high tolerance to external B is confirmed in the present study, where plant growth continued at 1000 mg B L⁻¹ (Figures 1 and 2), and is in agreement with a previous study in which *P. distans* was reported to grow well at 1250 mg B L⁻¹ [8]. By contrast, B toxicity becomes apparent in agricultural crops at concentrations higher than 5-20 mg B kg⁻¹ of soil [30] or 1-2 mg B L⁻¹ in irrigation water [9]. The exceptionally high level of B tolerance in this grass makes it a species of considerable interest since there is a need to identify more B-tolerant germplasm of wheat and barley for cultivation in high B soils [31].

Previous studies showed that in shoots of wheat growing at the toxic level of 25 mg B kg⁻¹, B concentrations in the shoots reached 1500 mg kg⁻¹ DW [32]. At 12 mg of B kg⁻¹ soil, soybean plants accumulated 520 mg B kg⁻¹ [33]. In the area of the Kirka Borax Mine (277 mg B kg⁻¹ soil), we identified another plant species, i.e., *Gypsophila sphaerocephala* Fenzl ex Tchihat. var. *sphaerocephala*
( Caryophyllaceae), besides P. distans [5]. While B concentrations of roots, culms, leaves, and seeds of G. sphaeroccephala were as high as 51, 232, 3245 and 2093 mg kg⁻¹, respectively, B concentrations in the same organs of P. distans on the same soil were only 261, 117, 802 and 501 mg kg⁻¹ [5]. Therefore, P. distans plants appear to have effective mechanism(s) to exclude B from their tissues in growth media with extreme B concentrations in such a way that they can escape B toxicity. Boron tolerance may arise either from highly effective B exclusion or high internal tolerance of B, or both. The crucial importance of B efflux transporters in excluding B from the cytoplasm has been reported by many researchers for many different plant species, including cereals such as wheat and rice besides the model plant Arabidopsis thaliana [11, 34–37]. Genes encoding B-efflux transporters have been cloned from wheat and barley [34]. P. distans was found to be capable of maintaining root B concentrations even at a much lower level than the B-tolerant barley cv. Tokak (Figure 4A and B). Furthermore, a transcriptomic study by Padmanabhan et al. [11] demonstrated upregulation of B efflux transporters in P. distans under B toxicity. Therefore, a highly effective B exclusion mechanism is likely the primary mechanism of the B tolerance of P. distans. Further work is now underway to identify boron transporter genes in P. distans.

Even though there was evidence of highly effective B exclusion at up to 250 mg B L⁻¹, we found extremely high internal B concentrations (up to 4103 mg kg⁻¹ DW, Figure 2D) in shoots of P. distans at 500 and 1000 mg B L⁻¹ in the present study. A highly regulated antioxidant defense may represent an additional B toxicity tolerance mechanism for this species [11]. We therefore examined the levels of components of the antioxidative system.

Previous studies reported that both B deficiency and toxicity in plants caused oxidative stress [38]. Plants suffering from B toxicity have also been shown to exhibit increases in the contents of MDA and H₂O₂, suggesting excessive oxidative stress and peroxidation of membrane lipids [13, 14], which was also observed in plants suffering from B deficiency. In the present study, the accumulation of H₂O₂ in leaves of P. distans was lowest at 0.033 mg B L⁻¹, which is the adequate level for most plants in hydroponic systems, and was also low at 2.5–25 mg B L⁻¹ which is in the toxic range for most plants. Accumulation of H₂O₂ in leaves of P. distans plants increased remarkably at 500 and 1000 mg B L⁻¹, which are extremely toxic concentrations for all plants. Damage of membrane lipids is generally related to the H₂O₂ level, because high concentrations of H₂O₂ would accelerate the generation of highly toxic •OH radicals in the Haber–Weiss reaction and thereby cause lipid peroxidation [39]. Although the H₂O₂ content continued to increase from 250 to 1000 mg B L⁻¹ (Figure 5A), •OH-scavenging activity decreased in these treatment groups, which might have prevented the peroxidation of membrane lipids. A negative correlation between the accumulation of H₂O₂ and the growth of the plants (see Supplementary Material, Table S1), but a positive correlation between the B concentration in the tissues and the accumulation of H₂O₂, suggest that toxic effects of B were related to the accumulation of H₂O₂, but that the anti-oxidant defense system prevented the elevated H₂O₂ from increasing lipid peroxidation in leaves. MDA levels varied only little with increasing B concentrations and actually were lower at high B concentrations (Figure 5C), indicating a highly effective mechanism for the avoidance of oxidative damage to membranes.

To control the production of ROS, plants have developed many enzymatic and non-enzymatic components of anti-oxidant defense. Tolerance to B stress is expected to depend largely on increases in the levels of components of the antioxidant defense system which contains both antioxidant compounds and antioxidant enzymes [13, 14, 40, 41]. In the present study, variations in SOD and CAT activities in leaves of P. distans were negligible, whereas Ardic et al. [14] found enhanced SOD and CAT activities in the shoots of a B-tolerant chickpea cultivar (Gökçe) at both 1.6 mM and 6.4 mM B treatments. Although Ardic et al. [13] demonstrated enhanced activities of POX, SOD and CAT in roots of this chickpea cultivar and hence alleviation of boron-toxicity induced oxidative stress, POX activity in leaves of P. distans did not change, except for an increase at 500 mg B L⁻¹. APX activity of P. distans reached a maximum level, both with no added B and at the highest doses of B (500 and 1000 mg B L⁻¹), whereas GR activity decreased with increased B doses. Similarly, enhanced APX activity was observed in the leaves of the B-tolerant chickpea cv. Gökçe at 1.6 mM and 6.4 mM B treatments [14], and decreased GR activity was reported for leaves of the B-tolerant sunflower cv. Özdemirci at a soil concentration higher than 40 mg B kg⁻¹ [42]. In the present study, high constitutive activity of the antioxidant defense system may explain avoidance of oxidative stress in P. distans at high external B. Higher constitutive levels of the specific activities of CAT, GR and APX in P. distans plants observed in the present study as compared to previous studies [13–15, 40] might indicate that adaptation to extremely high B concentrations is constitutive rather than stress-induced. There are several pieces of evidence that plants with high levels of antioxidants (either constitutive or induced) exhibit greater tolerance to the oxidative damage caused by ROS [43]. Therefore, we suggest...
that constitutively high levels of CAT, GR and APX allow constant preparedness for oxidative stress induced by toxic B concentrations in *P. distans* plants.

There are a limited number of studies on the effects of boron on the anatomy of plants. Comparison of leaf cross-sections of control (0.033 mg B L$^{-1}$) and of excess B-treated plants revealed that the cuticle of *P. distans* treated with 2.5 mg B L$^{-1}$ was thinner, while it was thicker at 1000 mg B L$^{-1}$ (Figure 8). Papadakis et al. [44] also reported a thicker cuticle on the leaves of ‘Clementine’ mandarin plants irrigated with 2.5 mg B L$^{-1}$. Besides a thicker cuticle, deformations in cell shape and a decreased number of xylem arms in root cross-sections, as well as a decreased size of bundle cells and increased lacunar spaces in leaf cross-sections were evidence of B-induced damage at tissue level.

## 5 Conclusions

The tolerance of the grass *P. distans* to B toxicity far exceeds that of cereal cultivars and most dicotyledonous species as well. Even though *P. distans* exhibited symptoms of B stress under B-deficient conditions and at levels at which crop cultivars would fail to survive (250–1000 mg of B L$^{-1}$), its ability to survive under the latter conditions was very remarkable. *Puccinellia distans* appears to exclude B at concentrations of up to 250 mg L$^{-1}$, but exclusion was less effective at 500 and 1000 mg B L$^{-1}$ and plants thus tolerated high internal B concentrations. The root B-uptake experiment also confirmed efficient B exclusion from roots of *P. distans* plants upon exposure to toxic B concentrations. On the other hand, increases in -OH-scavenging activity and SOD, POX and APX activities under toxic B stress conditions, as well as exceptionally high constitutive levels of CAT, GR and APX, imply the existence of a well-regulated antioxidative defense system that suppresses lipid peroxidation despite enhanced accumulation of H$_2$O$_2$.

The next challenge will be to understand the mechanism of this extreme tolerance against B toxicity at the molecular level through characterization of the B transporters of *P. distans*. Then it might eventually be possible to breed crops for improved B tolerance in arid and semiarid agricultural regions with high soil B. McDonald et al. [31] have pointed out that B toxicity commonly co-occurs with salinity and sodicity. Thus, we will consider addressing joint tolerance to all these stresses rather than focusing on B tolerance alone.

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