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Boron stress response and accumulation potential of the extremely tolerant species *Puccinellia frigida*

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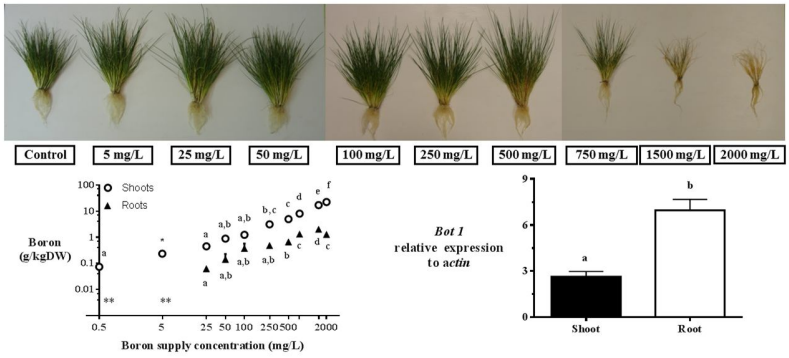
Graphical abstract



Highlights:

- *P. frigida* presents an extremely high boron toxicity threshold
- Restricting uptake and internal tolerance mechanisms could confer boron tolerance
- *P. frigida* is a boron hyperaccumulator over a wide range of concentrations
- The species has potential for phytoremediation purposes

Abstract

Phytoremediation is a promising technology to tackle boron toxicity, which restricts agricultural activities in many arid and semi-arid areas. *Puccinellia frigida* is a perennial grass that was reported to hyperaccumulate boron in extremely boron-contaminated sites. To further investigate its potential for phytoremediation, we determined its response to boron stress under controlled conditions (hydroponic culture). Also, as a first step towards understanding the mechanisms underlying its extreme tolerance, we evaluated the presence and expression of genes related with boron tolerance. We found that *P. frigida* grew normally even at highly toxic boron concentrations in the medium (500 mg/L), and within its tissues (>5000 mg/kg DW). We postulate that the strategies conferring this extreme tolerance involve both restricting boron accumulation and an internal tolerance mechanism; this is consistent with the identification of putative genes involved in both mechanisms, including the expression of a possible boron efflux transporter. We also found that *P. frigida* hyperaccumulated boron over a wide range of boron concentrations. We propose that *P. frigida* could be used for boron phytoremediation strategies in places with different soil characteristics and boron concentrations. Further studies should pave the way for the development of clean and low-cost solutions to boron toxicity problems.

Keywords: boron, hydroponic, hyperaccumulation, phytoremediation, *Puccinellia frigida*

Abbreviations:

B_{shoots}: Boron concentration in shoots

B_{supply} : Boron supply concentration

B_{roots}: Boron concentration in roots

BF: Bioconcentration factor

DW: Dry weight

EC_{50}: Effective concentration 50%

EST: Expressed sequence tag

FW: Fresh weight

LC_{50}: Lethal concentration 50%

PCR: Polymerase chain reaction

RGR: Relative growth rate

RRB: Relative rate of boron accumulation

RT-qPCR: quantitative real time PCR analysis

RWC: Relative water content

TF: Translocation factor

1. Introduction

High concentrations of boron in soils and irrigation water are toxic to plants [1] and restrict agricultural activities in polluted places [2]. This problem affects many arid and semi-arid regions worldwide, and has both natural and anthropic origins [3]. However, removal of boron from the environment is not a simple process and current technologies are ineffective, impractical or too expensive [4, 5].

An alternative approach to cleaning water and soils is phytoremediation, which has the advantage of being potentially cost-effective, efficient and environmentally-friendly [6]. During the last two decades, researchers have studied phytoremediation as a strategy to tackle the problems of boron toxicity [e.g. 7, 8-13]. One critical point for its success is the choice of plant species. Candidates for phytoremediation must tolerate high concentrations of boron in the medium, and either accumulate or exclude boron, depending on the phytoremediation strategy (phytoextraction or phytostabilization, respectively [6]).

It is known that boron tolerance and accumulation varies widely between different plant species and/or cultivars [14]. Nevertheless, the molecular basis of these processes is not well-understood [15]. Several studies have hypothesized that the main mechanism involved in tolerance to boron toxicity is the reduction of intracellular boron levels through efflux transporters [16-19]. In addition, the involvement of antioxidant protection and cellular homeostasis has also been proposed [20-22]. Finding the genes involved in boron tolerance is valuable for the future development of boron-tolerant crops or boron-accumulating plants for phytoremediation strategies.

Halophyte species are promising candidates for phytoremediation [23]. The *Puccinellia* genus (Poaceae) is known for its salt tolerance [24-27], although the phytoremediation potential of these species has not been fully explored [10, 28, 29]. *Puccinellia frigida* (Phil.) I.M. Johnst. (Poaceae)

is a little-studied Chilean native perennial grass that was recently found growing in a hydrothermal site with extremely high levels of boron (>4,000 mg/kg [30]). Soils in this site are also highly saline and alkaline, and present elevated arsenic concentrations [30]. These conditions are often found in boron contaminated places [3], which makes *P. frigida* a possible candidate for boron phytoremediation. In addition, *P. frigida* accumulates large amounts of boron in the shoots, which gives it potential for phytoextraction [30]. However, these field observations do not take into account that boron tolerance and uptake can be influenced by several environmental factors and soil properties, such as climatic conditions, soil pH, and soil salinity [31-33]. Therefore, the aim of this study was to determine the potential of *P. frigida* for phytoremediation strategies, by evaluating boron tolerance and accumulation under controlled conditions. To do so, the development of toxicity symptoms (changes in plant biomass, relative water content, and level of chlorosis), and the bioaccumulation and translocation factors (BF and TF) of *P. frigida* in response to different levels of boron stress in hydroponic culture, were studied. Moreover, the presence and expression of genes related with boron tolerance were evaluated.

2. Materials and Methods

2.1 Seed collection and hydroponic culture

P. frigida seeds were collected from a hydrothermal site in the Colpitas River sub-basin, Northern Chile (17° 57´S; 69° 25´W, at 4150 masl), and stored in paper bags, at room temperature (~20°C) in the dark until the experiments were performed (15 months later). Before germination, seed lemma and palea were removed by gently rubbing the seeds between two rubber blocks; and only firm, filled and smooth seeds were selected, according to Rámila et al. (Unpublished results). Selected seeds were germinated in Petri dishes at 10°C in the dark. Seedlings were transferred to a

perlite:peat mixture (1:1 v/v) and cultivated for six weeks until they reached a suitable size for transplanting (~ six leaves). Plants were transplanted to 13 cm diameter plastic pots (four plants per pot; 33 pots and 132 plants in total) containing perlite and an aerated modified half-strength Hoagland's solution [34]. After acclimation for ten weeks, plants from three pots were harvested (day 0), and the remainder were exposed to boron stress. Three pots (12 plants) were treated during five weeks with ten different concentrations of boron (added as H_3BO_3 (Merck, code 100165)) to the modified half-strength Hoagland's solution. Boron supply concentrations (B_{supply}) ranged from 0.5 mg/L (control concentration) to 2000 mg/L (0.5, 5, 25, 50, 100, 250, 500, 750, 1500 and 2000 mg/L) and solutions were brought to pH 5.8-6.0 prior to use (by adding KOH (Merck, code 105033)). Based on a preliminary test (data not shown), B_{supply} of 2000 mg/L was used as the highest concentration. B_{supply} of 0.5 mg/L was used as the control concentration in order to prevent boron deficiency, as low concentrations of boron are essential for plant growth [35]. Solutions were renewed twice a week, and the tanks were randomly rearranged in the greenhouse at each renewal. During the experiment, plants were maintained in a growth chamber with controlled climatic conditions (26 ± 2°C day/ 20 ± 2°C night, 14 h day/10 h night photoperiod).

At harvest, plants were rinsed three times in deionized water and separated into shoots and roots. Clean tissue was dried at 70°C for 48 h, ground and stored at 4°C in darkness until digestions were performed.

2.2 Determination of the growth and development of boron toxicity symptoms in *P. frigida*

To evaluate the response of *P. frigida* to boron stress, and determine the boron tolerance threshold, six indicators were used: (1) root elongation, (2) shoot elongation, (3) biomass production, (4) relative growth rate (*RGR*), (5) chlorosis, and (6) relative water content (*RWC*).

2.2.1 Root and shoot elongation, biomass production and *RGR* determination

Root and shoot elongation were determined at the time of harvest as the length of the longest root and shoot of each plant. Fresh weights (*FW*) of roots and shoots were determined by weighing the tissue after harvest, and the dry weight (*DW*) was determined after drying at 70°C for 48 h.

The *RGR* of the plants was calculated using the equation [36]:

$$RGR = \left[\frac{LnM_f - LnM_i}{D}\right]$$

where M_f = final *DW*, M_i = initial *DW* (average of the day 0 plants), and *D* = duration of the experiment (35 d).

2.2.2 Plant chlorosis and *RWC* determination

Plant chlorosis, the main visible symptom of boron toxicity [1], was estimated visually at harvest using a 0-4 scale, where 0 corresponded to 100% green tissue and 4 to 100% yellow tissue (Supplemental Fig. S1).

The *RWC* was calculated using the equation [37]:

$$RWC\ (\%) = 100 \cdot \left[\frac{FW - DW}{FW}\right]$$

where *FW* = final *FW* of the whole plant (shoots + roots) and *DW* = final *DW* of the whole plant.

2.3 Determination of boron concentration, accumulation and distribution in *P. frigida*

To determine the boron concentration in the tissues, boron was extracted using the high temperature oxidation:dry ashing method [38] with minor modifications. The boron concentration was determined using the azomethine-H method [39]. The standard plant reference material IPE 993 Black Poplar Hybrids leaf / *Populus* x *euramericana* (WEPAL, The Netherlands) was used to

verify the reliability of the measurements. The results were within ±15% of the certified value. Duplicates and reagent blanks were also utilized in the analysis as quality assurance controls.

The *RRB* was calculated using the equation [36]:

$$RRB = \left[\frac{LnB_f - LnB_i}{D}\right]$$

where B_f = boron in shoots at the end of the experiment, B_i = initial boron in shoots (average of the day 0 plants), and D = 35 d. The *RRB* was only determined for the shoots because there was insufficient root tissue for boron measurements in day 0 plants.

The *BF* and the *TF* were calculated as:

$$BF = \frac{B_{shoots}}{B_{Supply}}$$

and:

$$TF = \frac{B_{shoots}}{B_{roots}}$$

where: B_{shoots} = boron concentration in shoots (mg/kg DW) and B_{roots} = boron concentration in roots (mg/kg DW).

2.4 DNA and expression analyses

The presence of genes related with tolerance mechanisms was evaluated in the genome of *P. frigida*. Since it has yet to be sequenced, we performed the in silico DNA analysis using sequences described in *Puccinellia distans*, a highly boron tolerant plant, and very closely-related to *P. frigida* [41]. We selected four expressed sequence tags (ESTs) that were described as up-regulated in response to boron toxicity in *P. distans* [41]: a boron transporter (Bot1), an ABC-type transporter

from the C-family, an ascorbate peroxidase, and a chaperone ClpB1-like. In addition, we also examined the expression of the *Bot1* boron transporter in shoots and roots from *P. frigida*.

For DNA analysis, plant samples were collected from hydroponic experiments (supplemented with 25 and 250 mg/L of boron) and DNA was isolated from tissues as described previously [40]. Using primers designed in *P. distans* (300 nM; Supplemental Table S1 [41]), PCR was performed with 10 µl DNA to amplify partial gene sequences in *P. frigida*. The reactions were performed under the following conditions: 94°C for 10 min, 35 cycles of 94°C for 1 min, 54°C for 1.5 min, 72°C for 2 min, and a final extension at 72°C for 10 min. *Actin* was used as the internal control. The amplified PCR products were resolved on 1.5 % (w/v) agarose gels, isolated and sequenced (Macrogen DNA Sequencing Service). The identity of the PCR products was confirmed by sequence similarity searches against the NCBI data-base using the TBLASTX algorithm (http://www.ncbi. nlm.nih.gov) with a cut-off e value of $1e^{-5}$. Experiments were repeated four times with two replicates for each gene-specific primer.

For expression analysis, plants growing naturally under high boron concentrations were collected from the Colpitas River sub-basin (Section 2.1), placed in RNAlater® solution (Applied Biosystems), and stored at -20ºC. Total RNA from shoot and root tissues was isolated using RNeasy plant mini kit (Qiagen). One µg of total RNA was used for reverse transcription using the Improm-II reverse transcriptase (Promega) for first-strand cDNA synthesis. Gene-specific primers for the quantitative real time PCR (RT-qPCR) analysis were designed using primer3Plus (http://primer3plus.com/; Supplemental Table S2). RT-qPCR was performed using the Brilliant SYBR Green QPCR Reagents on a StepOnePlusTM Real-Time PCR System (Life Technologies). For mRNA level normalization, the actin transcript level was used (Supplemental Table S2). The absolute gene expression level was calculated using standard quantification curves with serial dilutions of PCR products for each gene. The total RT-qPCR reaction volume was 25 µl, containing

2.0 µl of cDNA template and 140 nM of each primer. The reactions were performed under the following conditions: 95ºC for 10 min, 40 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30s, followed by a melting curve analysis from 55 to 95ºC. Fluorescence values were acquired during the annealing period of the RT-qPCR procedure. All experiments were performed with three biological replicates, with their corresponding three technical replicates.

2.5 Statistical analysis

Data were tested for normality and homogeneity of variance by the Shapiro-Wilk and the Brown–Forsythe tests, respectively (α=0.05). Subsequently, one-way analysis of variance (ANOVA) was performed to analyse differences between treatments, and a Tukey multiple comparison test was used for comparison of the means. In the specific cases of chlorosis and plant biomass, the non-parametric test of Kruskal-Wallis was used to analyse differences between treatments, and the Dunn's post hoc test was employed for comparison of the means. To estimate boron toxicity thresholds, nonlinear regressions were used to fit sigmoidal dose–response curves. In the specific case of shoot biomass, data was log-transformed previous to nonlinear regression analyses. The normality of the residuals from these regressions was tested using the D'Agostino-Pearson omnibus K2 test. Differences in *Bot1* relative expression between shoots and roots of *P. frigida* were analysed by a Student's t-test. All statistical analyses were performed using the Graphpad Prism 6.0 software with a 95% confidence interval (α = 0.05).

3. Results

3.1 Growth and symptom development of *P. frigida* subjected to different levels of boron stress

3.1.1 Plant growth response to boron stress

The total plant, shoot and root biomass, and the *RGR* reached their highest values at 50 mg/L of B_{supply}. These parameters remained relatively constant between 100 and 500 mg/L, and tended to decrease at B_{supply} >500 mg/L (Figs. 1 and 2). Despite this trend, significant differences between treatments were observed only for root biomass and *RGR*, and at very high concentrations. The absence of significant differences between treatments may be explained by the high standard deviations (SD) observed in these parameters (Fig. 1 and Fig. 2a). High SDs are likely due to the substantial natural variability of non-domesticated plants grown from field collected seeds. High variability in biomass of plants grown from field collected seeds has been reported in the *Puccinelli*a genus elsewhere [34, 42]. Root biomass differed significantly between 50 mg/L of B_{supply} and $B_{supply} \geq$ 1500 mg/L (Fig. 1), and significant differences in *RGR* were found between 25 mg/L and 2000 mg/L, and between 50 mg/L and 2000 mg/L of B_{supply} (Fig. 2).

The length of the longest leaf and root tended to fall with increasing B_{supply} at concentrations >500 mg/L. However, significant differences compared to the control were found only for the longest leaf at 2000 mg/L (Fig. 2).

Taken together, these results highlight that a significant growth decrease was only observed at B_{supply} >1500 mg/L (Figs. 1 and 2).

3.1.2 Plant chlorosis and relative water content response to boron stress

The lowest chlorosis value (0 %) was reached at 50 mg/L, and then rose with increasing B_{supply}. Significant differences were observed between 50 mg/L and 2000 mg/L of B_{supply} , and chlorosis exceeded 50% of the leaf tissue for $B_{supply} \geq 1500$ mg/L (Figs. 3 and 4). The *RWC* of plants decreased significantly with $B_{supply} \geq 500$ mg/L.

3.1.3 Boron tolerance thresholds of *P. frigida*

Metal(loid) toxicity thresholds are useful parameters to estimate a plant's ability to establish and survive on a contaminated site. The effective concentration 50 (EC_{50}; concentration that produces a 50% reduction in biomass [43]) for each parameter was calculated (Table 1). The most sensitive parameters were the root biomass (810 mg/L) and chlorosis (800 mg/L). The lethal concentration LC_{50} was calculated as 1620 mg/L.

Another useful toxicity threshold value is the lowest concentration of the toxicant at which an additional dose causes a yield decrease [44]. According to our results, significant differences between the control and boron treatments were found only in the *RGR*, chlorosis and *RWC* (Table 1). Nevertheless, every growth parameter and boron toxicity symptom declined at B_{supply} above 500 mg/L. Therefore, this value would correspond to the boron toxicity threshold.

3.2 Boron concentration, accumulation and distribution in *P. frigida* tissues

The boron concentrations in shoots and in roots rose with increasing B_{supply}. B_{shoots} ranged from 74 to 22300 mg/kg DW, and reached 8000 mg/kg DW in plants without major symptoms of toxicity (at 750 mg/L). B_{roots} ranged from 65 to 2000 mg/kg DW (Fig. 5). The *RRB* increased significantly with increasing B_{supply} at concentrations ≥25 mg/L, reaching its peak (0.20 g/g day) at 1500 mg/L (Fig. 5).

Boron was stored mainly in shoots (> 97%) and the *TF* was >1 at every B_{supply} (4.1 to 18.3; Fig. 6). Only at 2000 mg/L was *TF* significantly greater than at other B_{supply}, which may be due to a reduction or loss of the mechanisms that regulate boron entrance to leaves, as demonstrated by the chlorosis that these plants exhibited (Fig. 3). The *BF* was >1 at every B_{supply} (10 to 148). At lower exposure concentrations (0.5 and 5 mg/L), the *BF* was extremely high, then decreased (25 – 500 mg/L), and finally remained relatively constant (500 – 2000 mg/L). This may be due to the boron requirements of the plant, such that at lower B_{supply}, plants would need to uptake a higher proportion of boron from the medium than at higher B_{supply}.

3.3 Molecular analysis of boron tolerance

Two putative genes encoding transport functions were identified in the genome of *P. frigida*, including the boron transporter, Bot1 (Fig. 7a). *Bot1* was expressed both in leaf and root tissues, and was strongly up-regulated in roots from plants grown in high boron concentrations (Fig. 7b). Other genes detected were a putative ascorbate peroxidase and a chaperone ClpB1-like (Supplemental Fig. S2).

4. Discussion

4.1 Chlorosis development and *RWC* were the best indicators of boron toxicity in *P. frigida*

The development of chlorosis and reductions in *RWC* are known symptoms of boron toxicity [45, 46]. In this study, these symptoms were better indicators of boron toxicity than the other parameters measured (plant, shoot and root biomass, *RGR*, leaf and root length). As such, the trend observed in the development of all toxicity symptoms in *P. frigida* in response to boron stress was better quantitatively reflected by *RWC* and chlorosis. For both symptoms, dose-response curves presented

the best fits (r^2> 0.9). *RWC* was significantly different from the control at lower B_{supply}, because of the smaller SDs that made differences more apparent. Chlorosis was the most sensitive parameter to boron stress, with the lowest EC_{50}. In consequence, because these parameters were the most reliable to follow boron toxicity in *P. frigida*, coupled with their inexpensive and easy-to-measure nature, we propose that they are most efficient indicators to monitor the establishment of *P. frigida* in a contaminated site.

4.2 Possible mechanisms of the extreme boron tolerance in *P. frigida*

P. frigida possesses extreme tolerance to boron stress, both in the medium and in its tissues. The toxic boron concentration depends on the species, but 3 mg/L in irrigation water is toxic to most plants [47]; *P. frigida* grew normally at boron concentrations exceeding 150 times this limit. In addition, the shoot growth EC_{50} was 1.7 times that of the most tolerant species found to date (1260 mg/L vs ~700 mg/L for *P. distans* [34]).

Boron in shoots largely exceeded what is considered toxic for most plants (250 mg/kg DW [48]) at almost every B_{supply}. Levels reached 8000 mg/kg DW in *P. frigida* shoots, which is the highest concentration reported for living plants, only comparable with the 6000 mg/kg DW measured in *P. distans* plants, which suffered a ~50% growth reduction in hydroponic culture [34]. Boron in roots was also high (up to 2000 mg/kg DW), indicating that both tissues possess internal tolerance mechanisms.

Plants can resist high boron concentrations in the medium through two main strategies: lowering net boron uptake and/or harboring internal tolerance mechanisms. In the case of *P. frigida*, we postulate that both mechanisms are at work.

P. frigida must possess an internal tolerance mechanism in order to grow normally, despite the extremely high shoot and root boron concentrations. Described mechanisms that could be driving

this are: (a) compartmentation of boron in vacuoles [49], (b) active efflux of boron from the intracellular phase into the apoplast [50], and (c) effective antioxidant responses [21, 51]. In an initial step to identify and characterize internal tolerance mechanisms, we found two ESTs for an ascorbate peroxidase and a chaperone ClpB1-like in *P. frigida* that are up-regulated under boron stress in *P. distans* [41] (Supplemental Fig. S2). Such genes related to antioxidant defense and protein disaggregation/degradation (to remove potentially harmful polypeptides), respectively, may help the plant to maintain cellular homeostasis, especially during stress [20, 21].

On the other hand, lowering net boron absorption may also be involved in a tolerance strategy employed by *P. frigida*. Although *BF* was >1 at every B_{supply}, *P. frigida* actually accumulates less boron than the levels reported in less tolerant species, such as: *Ocimum basilicum* [51], *Oryza sativa indica* IR36 and *Oryza sativa japonica* Nekken-1 [52], *Gypsophila arrostil* [34], and *Phalaris arundinacea* [53] (Supplemental Fig. S3). This indicates that mechanisms that restrict net boron uptake are also involved in the extreme boron tolerance of *P. frigida*. Two mechanisms are reported to decrease net boron uptake in plants: (a) lowering the expression of membrane channels involved in boron absorption [54], and (b) active efflux pumping of boron back into the medium [16, 17]. Consistent with this hypothesis, we found one EST similar to the barley transmembrane boron efflux transporter Bot1 (Fig. 7a). This transporter lowers internal boron concentrations, and plays a major role in enabling plants to withstand high external boron concentrations in tolerant landraces of other members of the Poaceae family, such as barley and wheat [16, 18]. In barley, Bot1 is expressed at similar levels in a tolerant landrace, irrespective of the external boron concentrations [18]. Thus it would be interesting to examine whether the Bot1 orthologue in *P. frigida* also serves as a constitutive means of eliminating boron from tissues under both toxic and non-toxic concentrations. Of note, and again in barley, the Bot1 transporter is highly-expressed in roots of boron-tolerant landrace [16, 18], facilitating the efflux of borate anions from root cells.

These reports are consistent with our results; the putative Bot1 in P. frigida was also up-regulated in roots compared to shoots (Fig. 7b). We also identified an EST for an ABC-type transporter from the C-family. This family of transporters is involved in the detoxification of metals and metalloids, such as cadmium, mercury and arsenic [55, 56], and also in controlling plant transpiration [57]. Arsenic concentrations are extremely high in soil samples in the Colpitas River sub-basin of Northern Chile (20,830 mg/kg) [30] where *P. frigida* samples were obtained, and this transporter may be one of the mechanisms employed by this species to tolerate such elevated arsenic levels. Further experiments, such as expression analysis and functional characterization of these and other genes, are necessary to understand the exact mechanisms underlying boron tolerance in *P. frigida*. Once discovered, these mechanisms could be transferred by genetic modification to high biomass plants, making them able to resist high amounts of boron in their tissues. These plants could then be used to phytoextract boron in contaminated sites. On the other hand, discovering the genes involved in the restriction of net boron uptake could be useful to generate tolerant crops. Once established, these species could increase agronomic activities in contaminated sites.

4.3 *P. frigida* is a potential candidate for phytoremediation of boron contaminated sites

Due to its extreme boron tolerance, *P. frigida* could be a good candidate to revegetate highly boron-contaminated places, where no other known species are able to establish. *P. frigida* grows in semi-arid areas in highly saline and alkaline soils that are rich in arsenic, conditions that are often found in boron contaminated places [30]. Revegetating boron contaminated sites would reduce soil erosion and increase organic matter [10], which would permit the subsequent cultivation of crops with higher market value or of other less tolerant phytoextractor species with higher boron removal rates [6].

P. frigida could also be used for boron phytoextraction at a wide range of boron concentrations. We found that *P. frigida* hyperaccumulates boron in the shoots, which could be harvested as a means of phytoextraction. Furthermore, *P. frigida* might be used to phytoextract boron in sites with different soil characteristics and climatic conditions, because it hyperaccumulates boron under different circumstances. In a previous study, it was established that *P. frigida* acted as a hyperaccumulator in a semi-arid area with alkaline and saline soils, that also had high concentrations of arsenic [30]. Boron tolerance and accumulation depend on climatic and soil conditions [31, 33]. In this study, however, we found that *P. frigida* also hyperaccumulated boron at a near neutral pH (6.0), under non-saline conditions, and at different temperature, light and humidity conditions, demonstrating the potential versatility of this species. The boron-rich biomass produced in the phytoextraction process could be used as fertilizer in boron deficient soils, which is a common problem that has been reported in more than 80 countries [58].

Phytoextraction efficiency is determined by both the metal concentration and biomass production in harvestable parts (shoots) [59]. In this study, the annual shoot biomass production was estimated by measuring the plant's growth rate in the 35 d of the experiment. The estimated biomass production was between 9.2 and 17.1 t/ha year. These values are in the range of those reported for other member of this genus (*P. ciliata* 5.6 t/ha year [60]; *P. stricta,* 4 - 10 t/ha year [61]). Although our calculated rate is based on hydroponic conditions, and is therefore probable an overestimate compared to the field, it corresponds to a first approach to quantifying the phytoextraction potential of *P. frigida*. Considering biomass production and the changes in shoot boron accumulation due to alterations in boron concentration in the medium as phytoextraction progresses, a preliminary estimation of phytoextraction potential was calculated. Phytoextraction of a soil (first 20 cm) with an initial boron concentration of 13 mg/L in the saturated extract (value reported for an actual contaminated soil in Northern Chile [62]), would be completed in approximately 2.3 years (final

concentration = 0.5 mg/L). This indicates that phytoextracting boron using *P. frigida* would be feasible, as the duration time is less than 10 years, the limit for considering phytoextraction as a viable technology [63]. Nevertheless, these results should be considered as a first orientation due to the many factors involved in phytoremediation that are not considered in hydroponic experiments (e.g. climate of the target site, management, microorganisms, boron interactions with soil surfaces, etc.). Also, the depth of soil contamination should be taken into account; because *P. frigida* doesn't have a deep root system (root depth is ~20 cm in the field).

Hydroponic and field experiments do not always correlate [64, 65]. Nevertheless, the results of this study support existing field evidence [30], and therefore should be considered as suitable indicators of the response of *P. frigida* to boron stress.

5. Conclusions

P. frigida was demonstrated to be extremely boron tolerant, and a boron-hyperaccumulator species under a wide range of boron concentrations. We preliminary estimated that it would phytoextract boron from a contaminated place in a reasonable period of time. In addition, we postulate with molecular evidence, that *P. frigida* resists boron through two mechanisms; lowering net boron uptake and possessing an internal tolerance mechanism in shoots.

Taking together the results from this study and previous field observations, we propose that *P. frigida* could be used for boron phytoremediation strategies such as phytorestoration and phytoextraction in places with varying characteristics (climate and substrate) and boron concentrations. Additionally, uncovering the genes that control boron tolerance and accumulation in this species would be useful to generate both hypertolerant crops able to grow in boron contaminated places, and hyperaccumulator plants for phytoextraction. Further studies concerning

these applications will open up the possibility of developing clean and low-cost solutions to boron toxicity problems.

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FIGURE CAPTIONS

Figure 1. Effect of different boron supply concentrations on: (a) total plant biomass, (b) shoot biomass and (c) root biomass of *P. frigida* after 35 d of exposure. Control concentration = 0.5 mg/L. Error bars show standard deviation. Different letters denote significant differences between the treatments ($p < 0.05$). *: insufficient data for comparisons. Nonlinear regressions are shown by dashed lines. The growth of *P. frigida* decreased significantly only at extremely high boron concentrations (> 1500 mg/L).

Figure 2. Effect of different boron supply concentrations on: (a) relative growth rate (*RGR*), (b) length of the longest leaf and (c) length of the longest root of *P. frigida* after 35 d of exposure. Error bars show standard deviation. Different letters denote significant differences between the treatments ($p < 0.05$). *: insufficient data for comparisons. Nonlinear regressions are shown by dashed lines.

Figure 3. Representative specimens of *Puccinellia frigida* exposed to different boron supply concentrations for 35 d. In general, concentrations over 3 mg/L in irrigation water are considered toxic to most plants (FAO, 1988).

Figure 4. Effect of different boron supply concentrations on: (a) chlorosis and (b) relative water content (*RWC*) in *P. frigida* after 35 d of exposure. Error bars show standard deviation. Different letters denote significant differences between the treatments ($p < 0.05$). *: insufficient data for comparisons. Nonlinear regressions are shown by dashed lines.

Figure 5. Effect of different boron supply concentrations on: (a) boron concentration in the tissue (shoots and roots) and (b) relative rate of boron accumulation (*RRB*) in the shoots of *P. frigida*. Error bars show standard deviation. Different letters denote significant differences between the treatments ($p < 0.05$). *: insufficient data for comparisons. **: not determined (insufficient root samples for analysis at 0.5 and 5 mg/L).

Figure 6. Effect of different boron supply concentrations on: (a) bioconcentration factor (*BF*) and (b) translocation factor (*TF*). Error bars show standard deviation. Different letters denote significant differences between the treatments ($p < 0.05$). *: insufficient data for comparisons. **: not determined (insufficient root samples for analysis at 0.5 and 5 mg/L).

Figure 7. Genetic and mRNA expression analysis of *Bot1*, a putative boron transporter of *P. frigida*. (a) PCR analysis of the genome of *P. frigida* for *Bot1* using DNA from plants grown hydroponically in 25 and 250 mg/L. (b) Expression analysis of *Bot1* in roots and shoots of *P. frigida* plants collected from the Colpitas River basin. *Actin* was used for normalization of gene expression. Error bars show standard deviation. Different letters denote significant differences between the treatments ($p < 0.05$).

Table 1. Boron supply concentration that produces a 50% reduction in each parameter, or 50% chlorosis (EC_{50}). $B_{Significant}$: boron supply concentration where significant differences with the control were observed.

Parameter	EC_{50} (mg/L)	$B_{Significant}$ (mg/L)
RGR	1170	*
Plant biomass	1190	*

Parameter	EC_{50} (mg/L)	$B_{Significant}$ (mg/L)
Shoot biomass	1260	*
Root biomass	810	*
Leaf length	> 2000	*
Root length	1720	*
Chlorosis	800	*
RWC	> 2000	≥500

* = no significant differences with the control at any concentration ($p>0.05$).