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Sodium and boron exclusion in two *Brassica juncea* cultivars exposed to the combined treatments of salinity and boron at moderate alkalinity

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Abstract: Salinity and high boron (B) concentrations are important co-limitations to crop production on naturally occurring alkaline soils in low rainfall regions of the world. Although the interactive effects of salinity and B toxicity on *Brassica juncea* growth have been reported in slightly acidic soils, very little is known about the interactive effects in alkaline soils. In the current study, a moderately tolerant (Vaibhav) and sensitive (Xinyou5) variety, were grown hydroponically for four weeks to assess mild salinity (50 mM NaCl) with or without high B (1 mM B) at moderate alkalinity (pH 8.5/5 mM $NaHCO_3$). The growth of the two varieties was more affected under the combined treatment than either salinity or high B alone. Although growth rate reduction was similar among the varieties, Vaibhav maintained a lower sodium (Na) and B and a higher potassium (K) concentration in the leaves than Xinyou5. In response to salinity, Vaibhav demonstrated essential tolerance mechanisms of partial exclusion and presumably compartmentalization of Na, leading to greater biomass than Xinyou5. Despite being able to better exclude B, Xinyou5 suffered a greater growth penalty, indicating higher B sensitivity than Vaibhav. In conclusion, screening for individual stresses is not necessarily the best strategy because plant responses to a single stress either salinity or high B may not always be the same as observed when both stresses are present together. Therefore, *Brassica* germplasm screening is essential for stresses in combination but not separately.

Key words: alkalinity; high boron; juncea canola; oilseed rape; salinity; stress combination; tolerance

Introduction

Some soils in low rainfall regions are naturally prone to multiple subsoil constraints such as salinity, sodicity, alkalinity and high boron (B) concentration (Rengasamy 2006). In Australia, more than 80% of sodic soils are dense clays with pH > 8.5 which affects ~30% of the land area (Rengasamy 2002 & 2006). Due to spatial variability across landforms and complex interactions among these constraints, management options are limited to growers (Dang et al. 2006; Nuttall et al. 2003). Therefore, crops with combined tolerance to these abiotic stresses are required to maintain crop productivity in these regions.

Plant growth responds to salinity in two phases: a rapid, osmotic phase and a slower, ionic phase (Munns & Tester 2008). Whole plant tolerance to salinity is achieved through ion exclusion (avoidance), compartmentalization of sodium (Na) and chloride (Cl; tolerance) and osmotic adjustment (Blumwald et al. 2004; Munns & Tester 2008).

Boron is an essential micronutrient for normal growth of higher plants, but high soil B concentration causes significant damage to many crops including cereals and oilseeds (Nuttall et al. 2006). Boron concentrations in the range of 1–5 mM in the soil solution inhibit plant growth (Reid et al. 2004). On the basis of B chemistry, Reid et al. (2004) described possible effects of high tissue B in higher plants as disruption of cell wall development, metabolic disruption by binding to the ribose moieties of adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH), and disruption of cell wall division and development by biding to ribose, either as a free sugar or within ribonucleic acid (RNA; Stangoulis & Reid 2002). Similar to salinity, plants tolerant to high B accumulate less B in their tissues due to a reduced net B-uptake into both roots and shoots compared to sensitive genotypes (Reid & Fitzpatrick 2009). Similarly, Hayes & Reid (2004) found B tolerance in barley was achieved by active B efflux from roots that reduced B accumulation in the plant.

High salinity and B often co-occur in natural and agricultural environments (Nuttall et al. 2006) and there are reports of the combined effects of salinity and high B concentration on growth and mineral nutrition in major crops (Grievea et al. 2010; Nuttall & Armstrong 2010; Wimmer & Goldbach 2012). How-

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ever, the nature of the interaction of salinity and B in plants is not yet clearly understood, as different plant species have different responses to these stresses alone and when combined. For example, adverse effect of the combined salinity and high B treatment on growth and yield of bell pepper (*Capsicum annuum* L.) was less severe compared to wheat (Yermiyahu et al. 2008). Similarly, in broccoli (*Brassica oleracea* ssp. *italica* L.), the combined stresses of low salinity (2 dS m^{-1}) and high B (2.2 mM) greatly increased B concentration (>100 mM B kg^{-1} dry weight) in leaf margins.

Brassica juncea L. is considered a moderately salt tolerant crop (Ashraf & McNeilly 2004). However, its adaptability to low rainfall areas, where salinity boron and alkalinity occur, is poorly understood, even though this region is the target environment for developing this crop (Burton et al. 2004).

The purpose of this research was to measure the individual and interactive effects of mild salinity and high B under alkaline conditions on *B. juncea* growth, B-uptake and sodium accumulation within two contrasting varieties. Understanding this interaction will assist breeders in selecting environments to evaluated cross-breds best adapted to low rainfall farming systems.

Material and methods

Plant material and growth conditions

Seed of two *B. juncea* varieties (Vaibhav and Xinyou5) were obtained through the Australian Centre for International Agricultural Research (ACIAR) project for 'Oilseed *Brassica* improvement in China, India and Australia' (project ID: CIM/1999/072). The two varieties used were Vaibhav which originates from India and Xinyou5 from China. The varieties were selected on their contrasting responses to the effects of high salinity (100 mM NaCl) under alkaline conditions, Vaibhav being moderately tolerant and Xinyou5 highly sensitive (Javid 2012).

Seed were surface-sterilized in 70% ethanol and 0.5% sodium hypochlorite for two minutes before germination on wet Whatman number 1 filter paper (90 mm) in Petri dishes for 48 h in the dark (Campbell et al. 1998). Germinated seedlings were maintained for 72 h under a 16/8 h photoperiod period. Five uniformly sized seedlings were selected for each treatment combination and each was placed into a hole-cut into a plastic lid and secured with foam wrapped around the stem. Lids were then placed onto 2 liter (L) black plastic buckets within which the roots were suspended in 1.75 L of modified Hoagland solution). This was made up macronutrients stock solution "A" 2 mL/L (as $Ca(NO_3)_2.4H_2O$: 0.5 mM and KNO_3: 1.0 mM); stock solution "B" 1 mL/L (as $MgSO_4 \cdot 7H_2O$: 4.25 mM, Na_2SO_4: 0.5 mM and $(NH_4)_2SO_4$: 0.25 mM); stock solution "C" 1mL/L (as KH_2PO_4: 0.02 mM) and stock solution "D" 1.25 mL/L (KOH: 341.0 μM, DTPA: 113.6 μM, $Fe(NO_3)_3 \cdot 9H_2O$: 32 μM, $MnCl_2 \cdot 4H_2O$: 8 μM, $ZnSO_4 \cdot 7H_2O$: 16 μM, $CuSO_4 \cdot 5H_2O$: 8 μM, $B(OH)_3$: 50 μM, $Na_2MoO_4 \cdot 2H_2O$: 0.16 μM, $Na_2SiO_3 \cdot 5H_2O$: 0.5 mM). The mixtures were buffered with 5 mM bicarbonate (HCO_3^-) to maintain pH 8.5 (Javid et al. 2012).

Salinity and boron treatments

Salinity and B treatments were selected based on typical levels found in soils in the low rainfall areas in the Mallee region of southeastern Australia (Nuttall et al. 2003). For the salinity treatment, a low NaCl concentration of 50 mM was applied in two split doses of 25 mM NaCl on two successive days to avoid osmotic shock to the seedlings. For the high B treatment, supplementary B was added as boric acid at a concentration of 1 mM. A combination of salt (50 mM) and B (1 mM) was also used for assessing the interactive effect of these treatments on plant growth. An alkaline nutrient solution (pH 8.5) without any treatment (salinity or B) was used as the control.

All nutrient solutions were aerated continuously for the duration of the experiment (4 weeks) and changed regularly on every third day to avoid nutrient deficiency and pH fluctuation. The pH of the nutrient solutions was also monitored daily. The experiment was conducted in an evaporatively cooled growth chamber with an average photosynthetic photon flux density (PPFD) of 300 μmol m^{-2} s^{-1}, relative humidity of 85% and a temperature of approximately 22/16 °C under a 16/8 h day/night cycle at The University of Melbourne, Australia.

Plant growth and dry matter measurement

At harvest, plants were separated into leaf, stem and root tissues. The length of the tap root of individual plants was measured and fresh weights were recorded for all plant parts. For dry matter measurements, samples were oven dried at 70 °C for 72 h and weights were recorded from three replications of each variety.

Chemical analysis of tissues

For chemical analysis, leaf and root samples were separated and dried at 70 °C overnight before being ground to a fine powder at 8,000 rpm with the Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany). Following Javid et al. (2012), the ground dry samples (~100 mg each) were digested in 70% nitric acid (trace metal grade) overnight and incubated at 110 °C for four hours to ensure complete digestion. Digested samples were then diluted with deionized water to a final concentration of 5% nitric acid. Analyses for Na, K and B were carried out using Inductively Coupled Plasma Spectrometry (ICP-AES) in the School of Botany, The University of Melbourne.

Statistical analyses

Two-way analyses of variance were performed with GENSTAT for Windows (10th Edition, VSN International, UK). The analyses were performed on treatments of variety, salinity, boron, salinity/boron interactions, and variety/treatment interactions using three replicates for each. Statistical analyses for tissue elemental composition used data from two replicates and within each replicate (container) five seedlings of each variety were grown. Where interactions were significant, Fisher's protected least significant differences test was calculated at $p < 0.05$.

Results

Effects of salinity, high boron and the combined stress on B. juncea growth

Shoot dry weight (DW) of both varieties was substantially ($p < 0.001$) reduced under the combined treatment (high B and salinity) compared to mild salinity (50 mM NaCl) or high B (1 mM) treatment alone (Table 1). In response to salinity, shoot DW of Xinyou5 was significantly ($p < 0.001$) reduced by 50% but in

Table 1. Tolerance (%) of *B. juncea* to salinity (50 mM NaCl), high boron (1 mM) and the combined stress of salinity and boron at pH 8.5 after 4 weeks of treatment. Values are means ($n = 15$), different letters (a–d) indicate significant differences for variety × treatment (Fisher's protected LSD test, $p < 0.05$).

Cultivars	Treatment	Dry shoot wt%	Dry Root wt%	Root length%
Vaibhav	Salinity	10b	8a	28a
Xinyou5		50c	79bc	55c
Vaibhav	Boron	27a	2a	37ab
Xinyou5		11b	53b	48bc
Vaibhav	Salinity.Boron	79d	83c	50bc
Xinyou5		67d	89c	61c

Table 2. Effect of salinity (50 mM NaCl), high B (1 mM) and the combined treatment (50 mM NaCl and 1 mM B) on root Na, K and B concentrations of *B. juncea* varieties Vaibhav and Xinyou5. Different letters (a–g) indicate significant differences for variety × treatment (Fisher's protected LSD test, $p < 0.05$).

Treatment	Sodium (Na) %		Potassium (K) %		Boron (B; mg kg^{-1})	
	Vaibhav	Xinyou5	Vaibhav	Xinyou5	Vaibhav	Xinyou5
Control	0.43a	0.72cd	96.0c	21.0a	1.98b	3.07a
Salinity	1.88f	4.18g	53.8b	24.2a	0.80cd	0.59d
Boron	0.65bc	0.55ab	248.6e	210.1d	1.88b	1.86b
Salinity.Boron	1.70e	0.86d	213.7d	269.3f	0.96cd	0.60d

Vaibhav the reduction was just 10% relative to the alkaline control (Table 1). High B treatment significantly ($p < 0.001$) increased shoot DW in Vaibhav but it was reduced in Xinyou5 relative to the alkaline control (Table 1). Similar to shoot DW, root DW under the combined treatment was reduced ($p < 0.001$) by about 85% in the two varieties (Table 1). In response to salinity and high B treatment alone, root DW of Vaibhav did not change but was reduced by 79% and 53% in Xinyou5, respectively (Table 1).

The combined effect of salinity and high B was more severe than either of the stresses alone on root length of both varieties. The root length was significantly ($p < 0.020$) reduced by 50% in Vaibhav and 61% in Xinyou5 (Table 1). In response to salinity, root length was significantly ($p < 0.040$) reduced in both varieties but reduction was less for Vaibhav (28%) than in Xinyou5 (55%; Table 1). Under the high B treatment, root length was reduced significantly ($p < 0.020$) by 37% and 48% in Vaibhav and Xinyou5, respectively (Table 1).

Effects of salinity, high B and the combined stress on concentrations of Na, K, K/Na ratio and total plant Na uptake

Leaf Na concentration increased 16-fold in Vaibhav and 26-fold in Xinyou5 ($p < 0.001$) after 4 weeks of salinity treatment (Fig. 1A). However, total plant Na uptake significantly ($p < 0.001$) increased 9-fold in Vaibhav compared to 2.8-fold in Xinyou5 (Fig. 2). In response to the high B treatment alone, leaf Na concentration as well as total plant Na uptake did not significantly increase in either variety (Fig. 1A, 2). Under the combined treatment with additional high B, leaf Na concentration increased 12- and 20-fold in Vaibhav and Xinyou5, respectively (Fig. 1A). Similarly, total plant Na uptake significantly ($p < 0.001$) increased in both varieties by nearly 66% relative to the control (Fig. 2). Root Na concentration under salinity increased 4-fold in Vaibhav and 6-fold in Xinyou5 but only slightly under high B alone (Table 2). Under the combined treatment with additional high B, root Na concentration increased 4-fold in Vaibhav but remained unchanged in Xinyou5 (Table 2).

Salinity significantly ($p < 0.003$) reduced leaf K concentration by 15% and 30% in Vaibhav and Xinyou5, respectively (Fig. 1B). In the high B treatment, leaf K concentration significantly ($p < 0.003$) increased by 111% in Vaibhav and 81% in Xinyou5. Under the combined treatment, leaf K concentration was not affected in Vaibhav but was significantly ($p < 0.003$) reduced (39%) in Xinyou5 (Fig. 1B). In response to salinity treatment, root K concentration was significantly ($p < 0.001$) reduced by 44% in Vaibhav but was unchanged in Xinyou5 (Table 2). Root K concentrations were increased under high B and the combined treatment in both varieties (Table 2).

Compared to the control, the leaf K/Na ratio was substantially ($p < 0.001$) reduced (95%) under salinity and there was a similar reduction in response to the combined treatment in both varieties after 4 weeks of treatment (Fig. 2C). Leaf K/Na ratio under high B treatment was either significantly increased by 78% in Vaibhav or reduced by 33% in Xinyou5 (Fig. 2C).

Effects of salinity, high boron and the combined stress on concentrations of boron

Leaf B concentration under salinity was decreased by 44% in Vaibhav and 29% in Xinyou5 (both $p < 0.001$; Fig. 1D). Leaf B concentration under high B treatment was substantially increased by 2.7-fold in Vaibhav and 4.7-fold in Xinyou5. Similarly, leaf B-content

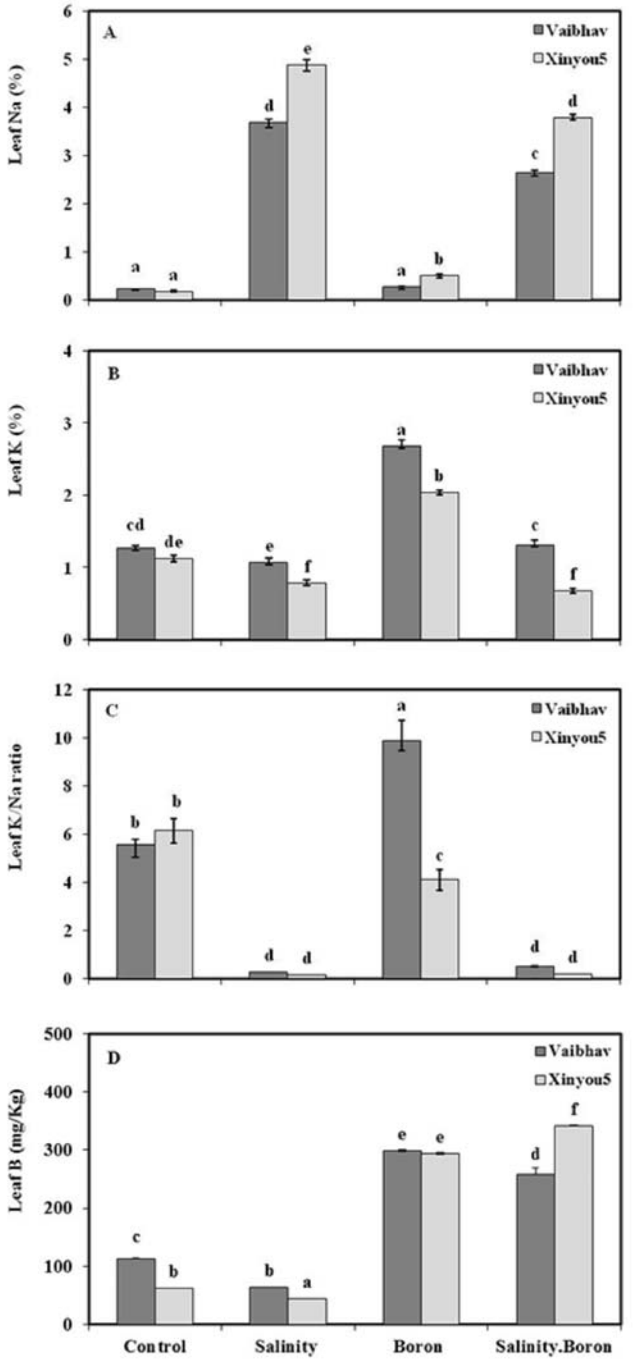

Fig. 1 Effect of salinity (50 mM NaCl), high B (1 mM) and the combined treatment (50 mM NaCl and 1 mM B) on (A) leaf Na, (B) leaf K and (C) K/Na ratio and (D) leaf B of *B. juncea* varieties Vaibhav and Xinyou5. Values are means ($n = 10$), error bars are standard errors of mean, different letters (a–f) indicate significant differences for variety × treatment (Fisher's protected LSD test, $p < 0.05$).

was increased by 2.4-fold and 6-fold in Vaibhav and Xinyou5, respectively (both $p < 0.001$; Fig. 3). Leaf B concentration under the combined treatment was significantly increased by 1.3-fold and 4.6-fold in Vaibhav and Xinyou5, respectively (Fig. 1D). However, leaf B-content under the combined treatment was either significantly decreased (by 53% in Vaibhav) or increased (by 77% in Xinyou5) (Fig. 3). Root B concentration was either reduced (by 44% in Vaibhav) or increased (by 15% in Xinyou5) under salinity treatment (Table 2). Root B concentration under high B treatment increased by 1.6-fold and 9-fold in Vaibhav and Xinyou5, respectively. Root B concentration under the combined treatment was increased 120% in Vaibhav and 1180% in Xinyou5 (Table 2). Root B-content of Xinyou5 was double that of Vaibhav under the high B treatment alone. Whereas B-content was reduced by 65% in Vaibhav but increased by 34% in Xinyou5 under the combined treatment (Fig. 3)

Discussion

Abiotic stress tolerance relevant to field conditions is best assessed through prolonged exposure of plants to moderate stress (Munns & Tester 2008; Yamaguchi et al. 2005). In the current study, we investigated *B. juncea* responses to prolonged (4 weeks) treatment of mild salinity (50 mM NaCl), high B (1 mM B) and the combination of these stresses under alkaline conditions, which is common in low rainfall areas (Javid et al. 2011 & 2012; Nuttall et al. 2006).

Salinity effects on B. juncea

Shoot and root growth of Vaibhav under prolonged mild salinity showed small reduction while Xinyou5 was more affected (Table 1). Salt tolerant *B. juncea* varieties generally accumulate more biomass than sensitive varieties when grown under mild saline conditions (Ashraf 2001; Javid et al. 2012). In the present experiment, 50 mM NaCl salinity only reduced the osmotic potential by –0.25 MPa and the biomass reduction, in particular in Xinyou5, was probably mainly caused by increased leaf Na concentration (Fig. 1A). Conversely, the better biomass of Vaibhav under salinity (despite high leaf Na concentration) suggested that Vaibhav was able to tolerate Na compared to Xinyou5. Salt-sensitive *Brassica* species commonly show higher leaf Na concentration under saline conditions (Javid et al. 2012; Kumar et al. 2009), which presumably entered through the transpiration stream and accumulated to high levels.

Plant roots can adapt their architecture in response to a variety of external stimuli to maintain optimal growth patterns (Malamy 2005) but root length under high salinity is usually reduced (Sun et al. 2008). In our experiment, mild salinity significantly reduced the root length of both varieties, but more so for Xinyou5 than Vaibhav (Table 1), potentially causing a greater effect on reduced supply of nutrient from roots to shoots.

Under saline conditions, increased Na influx can block high-affinity K-transporters and thus reduce K concentrations (Amtmann & Sanders 1998). Thus higher leaf Na concentration caused significant reductions in leaf K and K/Na ratio and eventually reduced growth. In response to salinity, higher K concentrations in Vaibhav compared to Xinyou5 may have contributed to more efficient photosynthesis and biomass (Munns et al. 2006), because a strong positive correlation exist

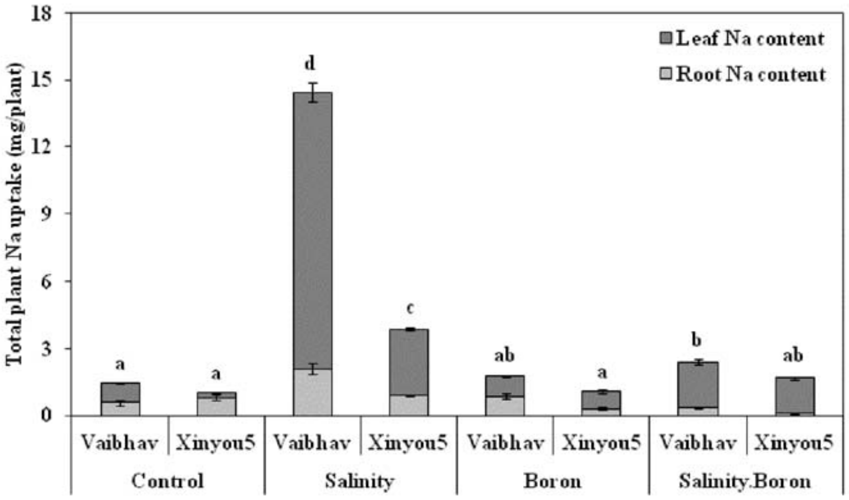

Fig. 2 Effect of salinity (50 mM NaCl), high B (1 mM) and the combined treatment (50 mM NaCl and 1 mM B) on plant Na uptake of *B. juncea* varieties Vaibhav and Xinyou5. Grey (▨) and black (■) bars represent root and leaf Na content, respectively. Values are means ($n = 10$), error bars are standard errors of mean, different letters (a–d) indicate significant differences for variety × treatment (Fisher's protected LSD test, $p < 0.05$).

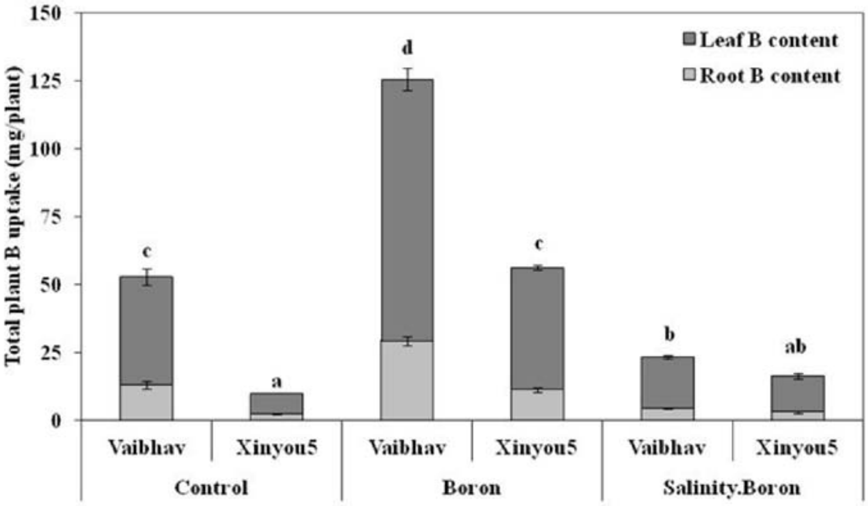

Fig. 3 Effect of high B (1 mM) and the combined treatment (1 mM B and 50 mM NaCl) on plant B uptake of *B. juncea* varieties Vaibhav and Xinyou5. Grey (▨) and black (■) bars represent root and leaf B content, respectively. Values are means ($n = 10$), error bars are standard errors of mean, different letters (a–d) indicate significant differences for variety × treatment (Fisher's protected LSD test, $p < 0.05$).

between plants' ability to retain K in salt treated leaves and their salinity tolerance (Wu et al. 2013). Under our experimental conditions, the higher K/Na ratio is considered important under saline conditions for tolerance (Shabala & Cuin 2008). The leaf K/Na ratio in both varieties was substantially reduced, therefore, may not have contributed to salinity tolerance.

Total plant Na uptake in Vaibhav was six times greater than in Xinyou5, which suggested salinity tolerance in Vaibhav was due to both partial exclusion and subsequent compartmentalization of Na. Higher plant Na uptake in Vaibhav could be explained by the need of the plant to maintain intracellular water potential below the soil water potential, to enable the cells to take up the water required for continued growth (Møller & Tester 2007). This was evident by the higher biomass response of Vaibhav compared to Xinyou5.

Boron toxicity effects on B. juncea

Reduced growth of shoots and roots is common in plants exposed to high B levels (Nable et al. 1990). Under high B, shoot biomass of Xinyou5 was halved compared to Vaibhav (Table 1), which could be due to disruption of root cell division, lower leaf chlorophyll contents and photosynthetic rates, and decreased lignin and suberin levels (Reid 2007 & 2013). Similarly, root growth (root biomass and length) was more affected by high B in Xinyou5 than in Vaibhav (Table 1). Root growth was previously reported to reduce in solution culture experiments in sensitive *Brassica* cultivars (Kaur et al. 2006).

Tolerance to B toxicity in plant is achieved by reduced uptake of B into the root, which then leads to reduced uptake into the shoot (Reid & Fitzpatrick 2009). In our experiment, leaf B concentrations and the total plant B uptake in Vaibhav was more than Xinyou5, however, its shoot and root growth (except root length) was not affected by high B treatment and (Table 1), which suggested B may have been redistributed. According to Reid & Fitzpatrick (2009) in tolerant cultivars, B is redistributed from the intracellular phase where it is toxic, into the apoplast, where B is less toxic (compartmentalization) to plant. Therefore, redistribution of B could be important for B tolerance and should be further investigated in tolerant *B. juncea* varieties. In contrast, it can be hypothesized that Xinyou5 does not possess these important stress avoiding/tolerance mechanisms, which caused biomass reductions even at lower B-uptake levels.

Interactive effects of salinity and high boron on B. juncea

Both Vaibhav and Xinyou5 were most affected by the combined treatment (50 mM NaCl and 1 mM B) compared to either salinity or high B alone. Shoot and root growth was substantially decreased, and leaf symptoms of chlorosis and necrosis were most severe under the combined treatment (data not shown). Growth reductions under the combined treatment were clearly associated with increased Na and B concentrations in leaves (Fig. 1). The leaf Na concentration was lower under the combined treatment when compared to salinity alone but still increased by many fold relative to the control. The effect of salinity, boron and alkalinity interaction likely to have affected the various transport mechanisms in the plant including the uptake of Na. Therefore, our results suggested that under salt stress, the activity of specific membrane components might be influenced directly by boric acid, regulating the functions of certain aquaporin isoforms and ATPase as possible components of the salinity tolerance mechanism (Martinez-Ballesta et al. 2008). Reduced Na under the combined treatment could also be because of reduced evapotranspiration as previously reported in broccoli (*Brassica oleracea* L.) by (Smith et al. 2013). The increased accumulation of leaf Na in Xinyou5 reduced leaf K thus, decreased K may have disrupted enzymatic activities mediated by K including the photosynthetic rate (Marschner 1995; Munns et al. 2006), which caused severe growth reduction in Xinyou5.

Under the combined treatment, leaf and root B-content in both varieties was substantially lower than observed under high B treatment alone (Fig. 3), which is similar to responses found in wheat (Masood et al. 2012: Wimmer & Goldbach (2012). This may be due to stomatal closure and reduced transpiration by the presence of both salinity and high B together. Thus, it can be postulated that a decline in the transpiration rate caused by salinity may reduce the passive absorption and diffusion of B (Alpaslan & Gunes 2001). Thus, the reduction of B uptake found in our study suggests that an exclusion mechanism was operating.

In summary, the interactive effect of salinity and high B treatment was more severe on plant growth of both *B. juncea* varieties than when either stress was experienced alone. Vaibhav was found more tolerant to both salinity and high B stress in isolation. However, both varieties responded similarly to the combined stresses. In terms of a breeding strategy, the selection of crossbreds for adaptation to the conditions in the low rainfall regions should be done under conditions where salinity, boron and alkalinity are present. It is beyond the scope of this experiment to compare the response under neutral or acidic conditions, but the combined effect is more important than the response to the individual effects. So that parallel selection rather than sequential or tandem selection are proposed for screening of the *Brassica* germplasm for the current target environment.

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