Genotypic Variation in the Morphological and Physiological Response to Boron Toxicity in Barley (*Hordeum vulgare* L.) and Weed Species

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Declaration

I HEREBY DECLARE that the work presented in this thesis does not incorporate any material previously submitted for another degree in any university. To the best of my knowledge and belief, it does not contain any material previously written or published by another person, except where due reference is made in the text. I am willing to make the thesis available for photocopy and loan if it is accepted for the award of ph D.

E.Y. Choi

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Abstract

Boron (B) is one of the seventeen essential elements for the growth and development of vascular plants, however, excess B causes toxicity, and is a significant problem for cereal production in many areas of southern Australia that have B toxic sub-soils. Recent research has identified a range of genotypic variation in response to B toxicity with mechanisms including B-exclusion and an inherent ability to tolerate excess B in plant tissues. The physiological mechanism of both B toxicity and tolerance are still not well understood and the aim of this thesis was to study the mechanism underlying the morphological responses of tolerant plants.

Root and shoot responses to varied levels of subsoil boron were observed in two barley varieties and three weed species common to agricultural areas of South Australia. When B was applied to the subsoil, both roots and shoots were severely impacted on in the B-intolerant Clipper and lincoln weed, whereas in the tolerant plants VB9953 and Barley grass, the affect was not so severe and good shoot growth was maintained. Tap-rooted and fibrous-rooted species redistributed more fine roots to the topsoil at high subsoil B when compared to the control. This reflects a tolerance mechanism linked to avoidance of B-toxicity. As expected, the root avoidance mechanism was not evident when plants were grown in a solution culture environment.

The mechanism of B-tolerance in the barley variety Sahara was related to a reduction of B uptake first at the site of xylem loading and them within the rest of root. The reduced B transport from root to shoot in Sahara may be due to a physical barrier between the root cortex and xylem, or due to a slowly developing active efflux mechanism. In contrast, VB9953 grown at the same B supply accumulated B in the shoot tissue without a reduction in plant growth, which indicates its mechanism of B-tolerance is in part due to its ability to tolerate toxic levels of B in its plant tissue. The high tolerance of within the tissue is most likely a result of expression of the 2H QTL for B tolerance, and is associated with a capacity to regulate carbohydrate

metabolism in the source and sink organs enabling the maintenance of root growth. This may also be associated with osmotic regulation.

Variety variation in the outer and intercellular structure of the root tips are observed with a two-dimensional root scanner and a confocal microscope to determine whether poor root growth caused by B toxicity is associated with inhibition of cell division. The B-tolerant variety, Sahara increased the length of its meristematic zone, increased cell density, and root elongation when grown at high B supply, whereas the opposite was observed for the B-intolerant Clipper that decrease in cell division under high external B in Clipper. When one looks at the concentration of reducing sugars in the dividing zone of Clipper, there is a significant reduction in roots grown at high B supply, whereas in Sahara and VB9983 reducing sugar levels increase, which can be used to explain how B-tolerant genotypes are able to maintain root elongation under B toxicity.

On further examination of sugar profiles in source and sink tissues, B toxicity led to a reduced sucrose content in the shoot and root tip of the B-sensitive Clipper, and this result was accompanied by an increased invertase activity in the shoot and a decrease in the root. In contrast, the B-tolerant VB9953 had enhanced sucrose contents within the leaf tissue that corresponded to increased sugar deposition in the root growing zone. Interestingly, the content of sucrose in the remains of the root (excluding root tip) were not affected by B toxicity in both varieties that may lead one to believe that B toxicity does not lead to the blocking of the phloem, but has an effect on the phloem unloading process in the root tip. VB9953 may act as a detoxification mechanism of high B concentration in the leaf tissue, and help to regulate the osmotic potential in plant cells.

From this thesis, one may hypothesize that four mechanisms of B-tolerance exist; 1) the B-exclusion mechanism in Sahara is most likely associated with a physical barrier at xylem loading that also develops within in the whole root, and 2) this may also include an efflux system. In VB9953, the mechanism appears not entirely associated with B-exclusion because B appears to build up in both root and shoot. 3) This internal detoxification of excess B in VB9953 may be linked to enhanced sugar levels in the shoot and root that may also have a role in help osmoregulation under B toxicity. The trait appears to be associated with the B-tolerance mechanism on chromosome 2 H of the barley genome as VB9953 has only the 2H QTL from the donor parent Sahara (Jeffries *et al.*, 2000). Some species were clearly able to compensate for root sensitivity to subsoil B by maintaining or increasing root biomass in the upper soil depths where the concentrations of B were not toxic, thus they can continue to maintain shoot and root growth. 4) This avoidance strategy operates in response to non-uniform distribution of high B concentration in the rooting medium.

CHAPTER 1

General Introduction

Boron (B) is an essential trace element required for the normal growth of higher plants (Dugger, 1983; Loomis and Durst, 1992) but when present in the plant at toxic concentrations it becomes a limitation for plant growth, and consequently reduces crop yield. Boron occurs at concentrations potentially toxic to plant growth in soils of mainly arid and semiarid areas where there is little leaching by rainfall (Stangoulis and Reid, 2002 and references therein). B toxicity in the subsoil (depth > 20 cm) is considered a particular problem across a large proportion of the agricultural regions of southern Australia where a Mediterranean-type climate exists (Cartwright *et al.*, 1986). One strategy used to sustain crop yields where B-toxicity exists, is to grow B tolerant varieties. Breeding for B tolerance has been a strategy implemented by wheat and barley programs in southern Australia and the strategy has been highly successful (Paull *et al.*, 1988 (a, b), 1991; Jefferies *et al.*, 1999, 2000; Rathjen, *et al.*, 1995; Moody, *et al.*, 1993; Hollamby, *et al.*, 1994 a, 1994 b). However, the gene pool from weed species endemic to B-toxic soils has been suggested, anecdotally, as a further source of B-tolerance genes, although actual data to substantiate this is lacking.

The majority of the plant breeding effort, such as for barley and wheat, has been conducted using phenotypic effects as selection criteria, including the severity of B-toxicity symptoms in the shoot and general biomass production. More recently, B-tolerance screening work has reported root length as an indicator (Chantachume, *et al.*, 1995; Jefferies, *et al.*, 2000) since B-toxicity generally results in reduced root growth with shorter root axes and less lateral roots (Huang and Graham, 1990), but this approach has not yet been widely used. The rapid effect of B toxicity on root morphology and growth may occur due to B interfering with cell division in the root tip; for example, excess B alters chromosome fragments, chromosome stickiness and micronuclei in the root tip of broad bean.

(*Vicia* faba L.) (Liu, *et al.* 2000). In an earlier study, B supplied at a concentration of 10 mM abolished miotic activity in pea (*Pisum sativum* L. cv. Alaska) root tips over a 24 hr period (Klein and Brown, 1981). Interestingly, genotypes exist that are able to tolerate B supplied in excess of normal requirement and still maintain root elongation, however, few studies in this area have been undertaken to understand the physiological mechanisms of B toxicity (or tolerance) in relation to the inhibitory effect of B on root growth. Such studies necessitate observing the effects of B toxicity on morphological development and ultra-structural responses of root systems as well as measuring physiological parameters.

In addition, most B tolerance screening work is carried out in solution culture with entire root systems exposed to the B concentrations. There is no moisture limitation to any part of the root system and the shoot is grown under relatively ideal conditions. Features of root growth may be quite different when the plants are grown in soil yet there are relatively few reports of controlled environment screening and assessing for B tolerance in this medium (Riley, 1987; Paull *et al.*, 1990; Grieve and Poss, 2000). Furthermore, apart from the work of Holloway and Alston (1992) and Yau (2001, 2002), there are no published research studies describing root system morphological responses to B in soil under simulated field conditions where high B is not uniformly distributed in the 'entire' profile, but occurs at depths below 20 cm or more. Clearly there is a need to observe the response of the root system in soil and also effects on shoot growth, and to compare this with those results already documented. However, there are acknowledged limitations in extracting roots from soil for studies of a physiological and ultra structural nature. Thus, there is certainly value in using solution culture to easily simulate a wide range of B concentrations to investigate physiological and ultra-structural responses.

Recent evidence suggests a range of genotypic variation in response to toxic levels of B with tolerance mechanisms associated with reduced accumulation of B in plant tissues, and/or tolerance of high B accumulation in the shoot (Nable *et al.*, 1997).

Tolerance mechanisms may be due to subtle differences in membrane permeability that affect B uptake (Nuttall, 2000; Dordas *et al.*, 2000; Stangoulis *et al.,* 2001), to active efflux from the root (Pfeffer *et al.,* 1999; Dannel *et al.,* 1998), or to protein synthesis and specific enzymes' activity that might have a function in the membrane or cell wall integrity and unknown internal detoxification (Mahboobi *et al.,* 2000; Wimmer *et al.,* 2003; Karabal *et al.,* 2003; Fawzia *et al.,* 1994). Molecular approaches have been successfully conducted to identify genetic markers providing evidence for the involvement of specific genes in B tolerance (Jefferies *et al.,* 1999, 2000). These chromosomal regions with loci for B tolerance are associated with reduced leaf symptom of B toxicity, reduced B uptake, and increased relative root length density. The physiology related to these mechanisms is still not well understood.

In general, the growth of a root system depends on the metabolic utilization of sucrose, the main carbon and energy source in root metabolism (Gasparikova, 1992). It is well established that carbon deficiency causes a reduction in the cell division rate and decreased root elongation (Van't Horf, 1968; Muller *et al.,* 1998), symptoms often associated with B-toxicity. This concept suggests that the change of root morphology and growth may be due to a toxic effect of B on carbohydrate metabolism in the root. There is evidence for a specific effect of B on sugar metabolism. Under B toxicity, the reducing sugar, glucose, declines in both the leaf sap and root of sugar beet, and in addition, N (measured as NO_3^-) accumulates in the leaf sap commensurate with a reduction of nitrate reductase activity under excess B supply (Bonilla, 1980). B toxicity also leads to reduced levels of the reducing sugar, α-amylase, during barley seed germination (Jimenez and Barea, 1979). The repression of protein synthesis in germinating seeds at high B (Haba, *et al.*, 1985) may account for such an effect. Lower protein content was also observed in the root tips of sugarcane in excess B with a simultaneous decrease in enzymes, which involved in carbohydrate metabolism, specifically aldolase and glyceraldehydes 3-phosphate dehydrogenase. These observations imply both physiological and

biochemical basis for B toxicity (Bowen, 1972). Wimmer *et al.* (2003) also proposed that the metabolic disruption caused by B toxicity results from a loss of function or an alternation of enzyme activities with structural changes due to binding of B to pyridine nucleotide coenzymes (NAD^+), ATP, RNA, and several sugar moieties in the symplast. However, it is still unclear whether the mechanism of B toxicity and/or tolerance in root growth is caused by reduced translocation of sucrose in the phloem, or simply an alteration of the sugar status between source and sink regions of the plant. This will be investigated further in this thesis to understand the relationship between physiological responses of plants and B tolerance and toxicity. More information on the physiological mechanisms of B toxicity may assist in developing new molecular approaches by improving our understanding of tolerant traits.

In summary, this thesis attempts to integrate observations on morphological responses of whole root systems of both crop and weed species to toxic B concentrations in the subsoil, with measurements of specific ultra-structural changes in root tips of B tolerant and sensitive barley plants, as well as linking these observations to the characteristics of B uptake and its distribution, and the potential physiological mechanisms associated with B tolerance in barley.

Table 1. Thesis structure

Component	Chapter	Content
	Chapter 2	Review of literature on the general chemical behaviors of boron (B) chemical, the role in plant metabolism and physiology, and on the mechanism of B toxicity and tolerance.
General root and shoot growth	Chapter 3	Measurement of root and shoot growth with toxic boron (B) in the subsoil comparing within and between species.
B uptake rate	Chapter 4	Investigation of the characteristics of B uptake in two varieties of barley and barley grass.
Specific root morphology	Chapter 5	Determination of the effects of B toxicity on morphological changes in the root meristematic zone between sensitive and tolerance varieties.
Relationship between B toxicity and carbohydrate metabolism	Chapter 6	Investigation of carbohydrate status and metabolism in barley under B toxicity, linked to reduced or enhanced root cell division and elongation.
	Chapter 7	General discussion

CHAPTER 2

Literature Review

2.1 Boron

2.1.1 General chemistry

In nature B is released from rocks predominantly as ionized BO_2^-, $B_4O_7^{2-}$, BO_3^{3-}, H_2BO^{3-}, and $B(OH)_4^-$ (Kabata-pendias and Pendias, 1992). Boron dissolves readily in water to form $B(OH)_3$ which is a small, neutral molecule (MW 61.83) (Wildes, and Neales, 1971). Boric acid behaves as a very weak Lewis acid (Ka = 6×10^{-10}, pKa 9.1), and forms the tetrahedral borate ion, $B(OH)_4^-$, by accepting a hydroxyl ion (OH^-) at alkaline pH and leaving a proton , H^+, in solution (Fig. 2.1) according to the equilibrium in Equation (1) (Power and Wood, 1997).

$$B(OH)_3 + H_2O = B(OH)_4^- + H^+ \qquad (1)$$

These OH^- groups are abundant in cell wall polymers and in various compounds known to be released by plant roots into the rhizosphere (Rovira and McDougall, 1967).

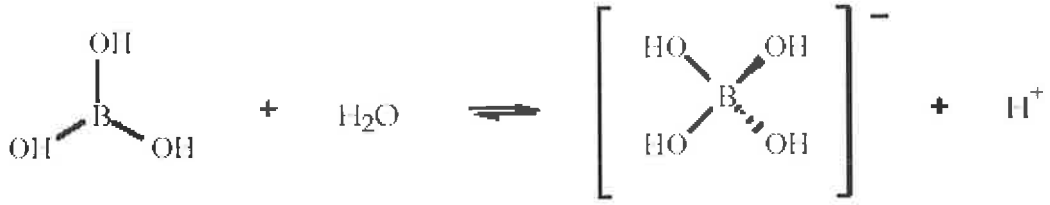


Fig 2.1 Boric acid-borate equilibrium (Loomis and Durst, 1992).

As the pK_a value (=9.1~9.25) is the same as the pH value when the acid is half dissociated, the main boron species in soil solution under the neutral or slightly acid range is undissociated boric acid that is largely absorbed by roots (Raven, 1980).

2.1.2 Distribution and chemistry of boron in soils and water

Boron is widely distributed in both the lithosphere and hydrosphere. In rocks its concentration averages about 10-20 mg B kg^{-1}, and the total B originated from soil minerals varies depends on the B content of parental rock materials. Sedimentary rocks originated from marine area have a higher amount of total B than igneous rocks (Norrish, 1975). The lowest values are found in either soils derived from acid igneous rock or from fresh water sedimentary deposits of coarse-texture and in soils low in organic acid. Higher values are found in soils derived from marine shales, loess, and alluvium with all being essentially fine-textured deposits (Fleming, 1980).

Soils may be classified into two types: those with low B content (<10 mg B kg^{-1}) or high B content (10-100 mg B kg^{-1}). Most soils have a low B content; naturally high B content soils are mainly associated with recent volcanism, or marine sediments. In seawater it can range from ca. 1-10 mg B L^{-1}, while its concentration in river water is about 1/350 that of sea water (Power and Wood, 1997).

2.1.3 Boron complexation

It is well known that boric acid readily reacts with the alcohol groups of diol or polyol compounds (e.g. glycerol or mannitol) (Fig. 2.2). The addition of polyols increases the acidity of boric acid or borate solutions by formation of complexes. This was the basis for quantitative analysis of boric acid, which could be titrated to a phenolphthalein endpoint (Power and Wood, 1997). Polyhydroxyl compounds with an adjacent *cis*-diol configuration are required for the formation of such complexes; the compounds include a number of sugars and their derivatives (e.g., sugar alcohols

and uronic acids), in particular manitol, mannan, and polymannuronic acid. These compounds are present, for example, as constituents of the hemicellulose fraction of cell walls. In contrast, glucose, fructose, and galactose and their derivatives (e.g. sucrose) do not have this *cis*-diol configuration and thus do not from stable borate complexes.

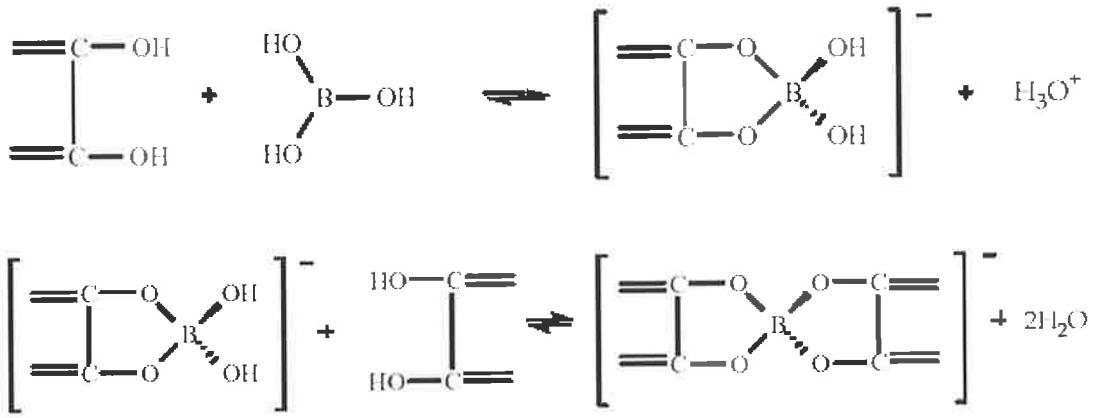


Fig. 2.2 Boric acid readily complexes with diols and polyols, particularly with cis-diols (Marschner, 1995).

The pKa of the boric acid moiety is reduced by complexation with diols, which is able to form the borate ion, producing a proton and resulting in acidification. When the B moiety is in the tetrahedral borate forms, the esterification reaction can occur again to produce a diester (Fig. 2.2).

A wide variety of boric acid esters may be synthesized in this way. The stability of the borate complex formed is strongly dependent on the type of diols on a furanoid ring. These structures are rare in nature and are limited to apiose and ribose (Fig. 2.3). There is an optimal distance between the two oxygen atoms of the diol in the complex (2.49 to about 2.63 Å) and the most strongly complexing diols favour or impose this distance (Loomis and Durst, 1992).

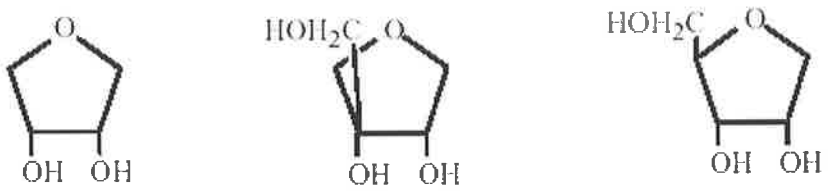


Fig. 2.3 Erythritan, ribose and apiose (cis-dihydroxyltetrahydrofurans) (Power and Wood, 1997)

2.2 Boron uptake

The major theory is that B uptake occurs by two mechanisms, passive uptake $B(OH)_3$, of at high B supply, and facilitated or active uptake at low B supply. B is taken up within the transpiration stream.

2.2.1 Passive uptake

Due to the high permeability of B through biological membranes (3×10^{-7} cm s^{-1} (Dordas *et al.*, 2000); 4.4×10^{-7} cm s^{-1} (Stangoulis *et al.*, 2001)), B can move passively through cellular membranes, its uptake being driven by changes in a plants water potential gradient that results in its accumulation at the end of the transpiration stream (Oertli and Ahmed, 1971). In reality, B uptake in plant roots is a more complicated process as new insights into this process have recently revealed (Dannel *et al.*, 1999; Dannel *et al.*, 2000; Dordas and Brown, 2001; Dordas *et al.*, 2000; Pfeffer *et al.*, 1999; Stangoulis *et al.*, 2001).

Pfeffer *at al.* (1997) conducted an experiment with sunflower plants grown at two different external B concentration (either adequate (1 μM) or sufficient (100 μM) B $(OH)_3$). However, there is no significant difference of the B concentration between the xylem exudates and the root cell sap, although the authors concluded that xylem loading of B is a passive process. In contrast, when sunflower plants were grown in high B (400 to 1600 μM), the B concentration of xylem exudates was significantly lower (by 50-60 %) than that of the root cell sap (Dannel *et al.*, 1998). This result suggests that there is a physical or biochemical barrier between the root and xylem to transfer B into the shoot at high B supply, or is an active exclusion transporter between them.

2.2.2 Active / Facilitated uptake

Active B uptake was proposed to be regulated by a high affinity uptake system (Wilders and Neales, 1971; Bowen, 1972; Bowen and Nissen, 1976). The authors suggest that the effects of the environment on B accumulation are not directly dependent on the water utilization, and B uptake and water consumption involve two separate mechanisms. However, Raven (1980) argued against the evidence of Bowen's experiment (1972) in that the concentration of B within the tissue was higher than that outside during net influx. It is difficult to assess the B concentration in the tissue (i.e. within the cell wall) and that within the external medium at the same time since it is possible that boric acid ester formation occurs and is not considered quantitatively. Moreover, borate-diol complexes have a much lower pKa value than that of the free boric acid. Compared to passive uptake, this active uptake is energetically expensive at high B concentrations. The turnover rates of channels in active transport pumps are approximately 10^2 molecules per second, whereas those of channel mediated transport pump are 10^6 to 10^8 molecules per second (Buchanan *et al.,* 2000). Thus active uptake is not likely to operate in plants supplied with high B concentration. However, one cannot rule out active uptake. For example, the estimated protons'membrane permeability is 10^{-3} to 10^{-4} cm s^{-1}, which is 100 to 1000 times more permeable than boric acid, however, active H^+ transport occurs in all living systems (Reviewed by Hu and Brown, 1997).

The recent studies suggest that two mechanisms of B uptake operate in sunflower plants depending on different levels of B concentrations in the external solution. B transport may be an active process at low B concentrations, but a passive process operates at high B concentration (Pfeffer *et al.,* 1997; Pfeffer *et al.,* 1999). Pfeffer *et al* (1999) conducted an experiment with young sunflower plants pre-cultured at low B supply (1 μM), and were then transferred to either 1 or 100 μM boric acid for a short term uptake period. Plants were then subjected to a further four different treatments; 1/1 (supplied with 1 μM, and then transferred to 1 μM), 1/100 (supplied with 1 μM, and then transferred to 100 μM), 100/1 (supplied with 100 μM, and then

transferred to 1 μM), and 100/100 (supplied with 100 μM, and then transferred to 100 μM). In plants treated with 1/100 for a short-term uptake, the B concentration of cell sap and xylem exudates was found be more than 2-folder higher than plants grown with a continuous high B supply (100/100). Interestingly, plants supplied with 100 μM B and then transferred to 1 μM B reduced the B concentration in the xylem and root cell sap. In addition plants that were continuously supplied with sufficient B (100/100) contained a slightly lower B concentration in the xylem exudates and root cell sap than in the nutrient solution. These results indicate that a B-concentrating mechanism operates at low B supply, and it was deactivated 24 hours after plants were transferred to 100 μM B, with internal B levels returning to those of plants continually supplied with 100 μM B.

Early studies (Brown and Jones, 1971) suggest that at the xylem loading, B uptake can be regulated (by an unknown mechanism). The authors found that at the low B supply the B concentration in the xylem exudates and shoots was different, depending on the B-efficiency of the tomato plant. At low B supply, B-efficient plant grew well with much higher B concentrations in the xylem exudates than in the B-inefficient plant, even though the B concentration in the exudates extracted from root was the same for both B-efficient and-inefficient genotypes. This result implies a mechanism at the xylem loading.

2.2.3 Combined uptake model

Combined B uptake models were suggested by Wides and Neals (1970) and Dannel et al (2002). Classically Wildes and Neales (1971) arranged a model for the uptake of borate, incorporating both the passive uptake diffusion of B $(OH)_3$ proposed by Bingham et al (1970) and the active uptake of the B $(OH)_4^-$ ion proposed by Bowen (1968) and Thellier and Le Guiel (1967). Wildes and Neales (1971) suggested that when tissue is first immersed in a solution of boric acid (where the internal boron concentration is lower than that in the external solution), an uptake of B will occur first by diffusive influx of $B(OH)_3$ and then a slower active uptake of B $(OH)_4^-$. The contribution of diffusion to net uptake is attributable solely to the active transport of $B(OH)_4^-$. As active transport continues, there will be a concomitant increase in the internal concentration of $B(OH)_3$ due to $B(OH)_4^- + H^+ \Leftrightarrow B(OH)_3 + H_2O$. A resultant diffusive loss of B will ensue. The rate of this diffusive loss of B will increase as the concentration gradient between the tissue and the external solution increase due to the active uptake of $B(OH)_4^-$. Finally, a stage is reached where the uptake of $B(OH)_4^-$ and the diffusive loss of $B(OH)_3$ are equal, with the result that a dynamic equilibrium is established. This scheme, then, is able to explain the apparent contradiction of active transport of B into cells, which appear to be freely permeable to B. It also explains the absence of a steady state of net influx of B into the tissues, which would be expected in the presence of a mechanism for active transport.

The latest model presented by Dannel *et al.,* (2002) suggests that the three mechanisms of B uptake by roots occur and are explained differently depending on external B levels supplied to roots. According to the model, the transport of B at high B supply occurs passively by diffusion across lipid bilayers and facilitated permeation through channels, such as major intrinsic proteins (MIP) as supported by recent work by Dordas and Brown (2001). By contrast, at low B supply the transport of B is dependant on the input of metabolic energy.

2.3 Functions of Boron in Plants

2.3.1 Cell wall structure

There is little doubt that B plays a key role in the elongation and structural integrity of plant cell walls. Boron (B) deficiency results in changes in cell wall structure including swelling of the cell walls and the formation of small irregularly shaped cells (Brown and Hu, 1997). In an earlier study, B was considered a structured component of plant cell walls, but was not associated in cell-wall metabolism (Slack and Whittington, 1964). Loomis and Durst (1992) also suggested that B did not function in nucleic acid metabolism and protein synthesis based on the rapid response of pollen tube elongation to withdrawal of B from the culture medium with abnormal swelling or even bursting in the tip region within 2-3 min of removal; this effect indicating a structural role for B. Similar effects were also reported by Cheng and Rerkasem (1993), where the authors concluded that it seems more likely that B plays a key role in cell wall construction. Recently many studies have demonstrated that a B polysaccharide complex exists in cell walls, which was identified as two chains of the pectic polysaccharide rhamnogalacturonan II (RG II), linked together by boric acid (Matoh, *et al.,* 1993). RG II, a low molecular mass (5-10 Kd) pectic polysaccharide, contains eleven different glycosyl residues (Vidal *et al.,*2000; Fig. 2.4). Matoh and colleagues (1993) demonstrated that RG II regions provide the connecting sites for two pectic polysaccharide chains through B-diester crosslink (Kobayashi *et al.,* 1997; Fig. 2.5). If B is not present, pectins chains secreted into the cell walls may not be held. In cultured tobacco cells, 80% of the cell wall bound B was present in the form of the B-RG II dimer in the pectic fraction of the cell wall (Kobayashi *et al.,* 1997). These borate-ester cross-links are critical to the plant's ability to elongate cell walls without destroying them. Since the cross-links are weak and have the acid sensitivity of a B complex, they can be broken by auxin pH changes, and then recomplexation to give the 'creep' effect that is required to allow elongation during cell wall elongation.

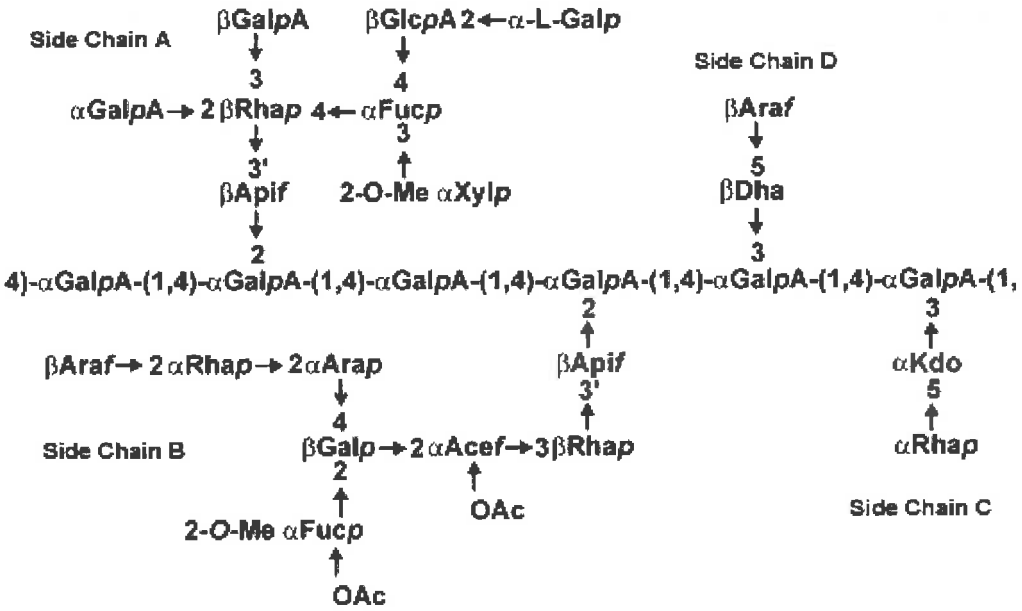


Fig. 2.4 The glycosyl sequence of RG-II. The (1→4)-linked α–D-Gal*p*A backbone of RG II is substituted with four structurally different oligoglycosyl side chains (A-D). The location of the side chains is arbitrary, since their distribution on the backbone is not known (Vidal *et al.,* 2000).

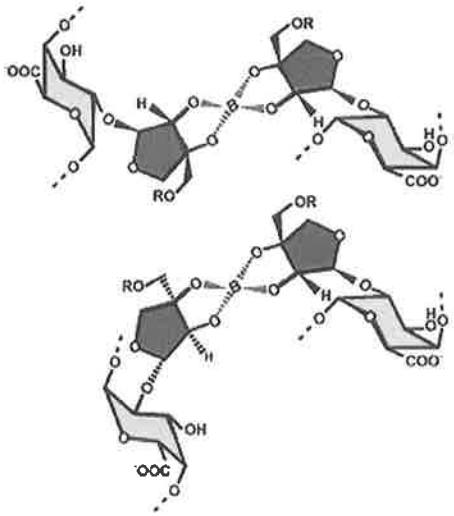


Fig. 2.5 RG-II exists as a borate cross-linked dimer that is cross-linked by a 1:2 borate-diol diester (Kobayashi, *et al.,* 1996).

Recent studies have elucidated the chemical characteristics of the B-RG II, particularly with respect to its formation. *In vitro* results demonstrate that Ca^{2+} enhanced the formation of B-RG II (Ishii *et al.,* 1999). This result is accordance with results presented by Kobayashi *et al.* (1999) that a B-RG II dimer from radish roots contained equivalent concentrations of B and Ca^{2+} and that withdrawal of the Ca^{2+} by a synthetic chelator induced breakage of the dimer. These studies help to explain that B has a structural role in maintaining cell wall by providing strength, shape, and negative charges for ionic interactions in the cell.

2.3.2 Membrane integrity

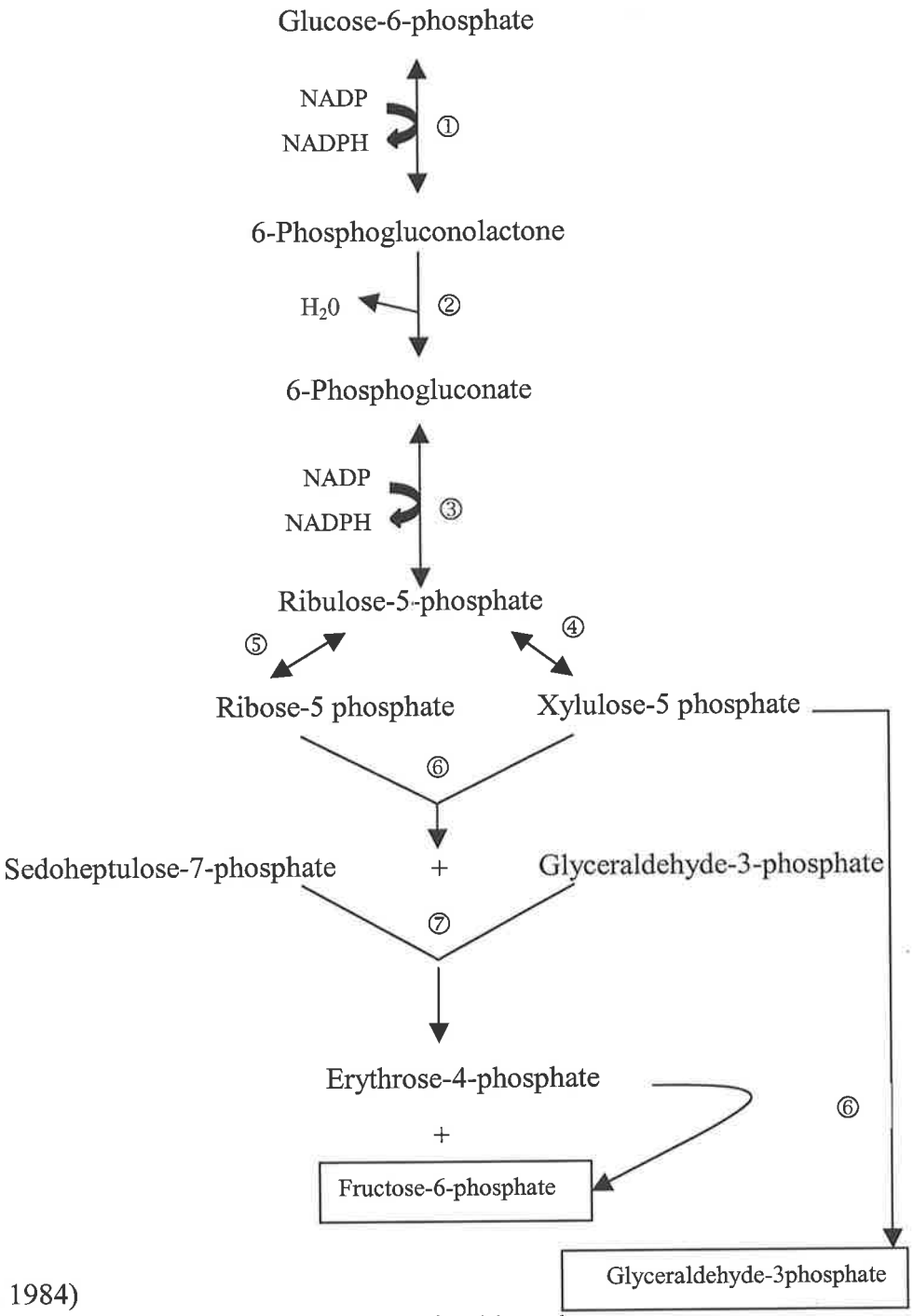
In addition to the role in cell wall structure there is evidence that B is also required for membrane integrity. B deficiency resulted in a marked reduction in Rb^{+}, K^{+}, and Cl^{-} uptake by cells (Pollard *et al.,* 1977), and the authors suggest that B may interact directly with the cell membrane by binding to polyhydroxyl compounds such as glycoproteins or glycolipids-this was consistent with a high concentration of B in the membrane. This suggestion indicated that B is associated with phenol metabolism. B complexes with 6-phosphogluconic acid and might therefore regulate the pentose shunt catabolic pathway by inhibiting 6-phosphogluconate dehydrogenase and so prevent the accumulation of phenolics (Lee and Aronoff, 1976; Dugger, 1983; Diagram 1). Shkolnik (1984) proposed that B deficiency causes membrane breakdown in growing plants, resulting in a release of RNAase from the bound, inactive form, which is followed by an alteration in nucleic acid and protein synthesis. Dugger (1973) suggests that these changes are related to the observed reduction in cellular phospholipids, membrane degeneration, increase in RNAase activity, and a possible shift in the catabolism of carbohydrates, with a larger fraction being oxidized via the pentose phosphate pathway. Under B deficient conditions, phenolics accumulate and consequently lead to necrosis of tissue (Pilbeam and Kirkby, 1983). Cara, *et al.* (2002) suggested the involvement of phenol oxidation in the loss of plasma-membrane integrity and functionality of B deficient squash roots.

However, Pfeffer *et al.* (1998) observed that B did not maintain plasma-membrane integrity by complexing phenols and inhibiting polyphenol oxidase (PPO) activity to prevent damage by oxygen-free radicals or by regulating ascorbate metabolism. The authors concluded that B has a direct function in the membrane, which may be either as a stimulant of membrane-related enzymes responsible for membrane potential, ion transport, and apoplasmic redox state, or a structural role similar to that demonstrated in the cell wall.

2.3.3 Sugar metabolism and transport

The role of B in sugar metabolism and transport has been reviewed by a number of researchers. Gauch and Dugger (1953) propose that boron might be a structural component of the plasma membrane. Thus a molecule of sucrose or another sugar would react with the borate molecule at the surface of the membrane to form a borate-sugar complex, which would then transport the sugar molecule across the membrane. One of the most significant ways in which B may act to regulate sugar metabolism is via complexation with 6-phosphogluconic acid (the first intermediate in the pentose shunt pathway) and inhibition of 6-phosphogluconate dehydrogenase (Lee and Aronoff, 1967; Dugger, 1983). This mechanism is compatible with observed changes in membrane permeability in response to B (Pollard *et al.* 1977). Under B deficiency, minimal regulation occurs leading to phenolic acid metabolism via the pentose phosphate pathway, and hence adverse effects due to phenol oxidation to reactive quinones and the enhancement of toxic oxygen species which impact on membrane integrity (Cakmak and Romheld, 1997). However, this hypothesis for a mobile borate-sugar complex to facilitate sugar transport across the plasma membrane was argued before it became known that borate complexes weakly with sucrose, and the concentration of B is particularly low in the phloem, the main conducting pathway for sugar transport (Pilbeam and Kirkby, 1983).

There is other evidence not to support the hypothesis that borate reacts with sugar to form a sugar borate and facilitate sugar transport. Although B can increase the basipetal translocation of [^{14}C] sucrose applied to leaves of bean hypocotyls cuttings, sucrose does not alleviate the symptoms of B deficiency (Whittington, 1959; Albert and Wilson, 1961). No sucrose-borate complexes have been isolated or localized in plants, and B is not redistributed by lateral or basipetal transport in plants. Other hypotheses were therefore proposed that increased translocation of sugar after adding B into B deficient plants may be an indirect effect owing to the restoration of sink activity (Albert and Wilson, 1961), except for species in which B is freely mobile as a result of sugar alcohols in the phloem.



(Duffus, 1984)

Diagram 1. The pentose phosphate pathway in chloroplast.

(1) glucose-6-phosphate dehydrogenase; (2) gluconolactone hydrolase; (3) 6-phosphogluconate dehydrogenase; (4) ribose phosphate isomerase; (5) ribulose-5-phosphate epimerase; (6) transketolase; (7) transaldolase.

2.3.4 Carbohydrate biosynthesis

Basically, B is able to form complex with substrates, enzymes, and membranes that have *cis-diols* present in their structure as a result may affect enzyme activities and metabolic pathways (Wildes and Neales, 1971). There is evidence that B deficiency inhibits the enzyme that catalyses UDPG (Uridine Diphosphate Glucose), which is involved in the biosynthesis of starch, interconversions between monosaccharides, and in the biosynthesis of sucrose (Birnaum, 1977; Heys *et al.,* 1991). This supports an early theory that B deficiency results in poor sucrose translocation due to an inhibition of sucrose biosynthesis (Dugger, 1983). There is also evidence that other components of structural polysaccharides may also be affected by B deficiency. Lee and Aronoff (1967) propose that as B is known to form a complex with 6-phosphogluconate, it might alter the activity of the enzyme 6-phophogluconate dehydrogenase by complexing its substracte (6-phosphogluconate). The authors also propose that B is able to form 6-phosphate-borate complexe and thus restrict both the flux of substrate into the a pentose phosphate pathway and synthesis of phenols, followed by glycolysis and the synthesis, for example, of hemicellulose and related cell wall materials. These authors suggest that when B is deficient, the pentose phosphate pathway is not regulated by B and associated with this is the accumulation of phenolic compounds characteristic of boron deficiency. Other evidence exists for an increase in the substrate flux into the pentose-phosphate pathway under B deficiency (Birnbaum *et al.,* 1977), supporting the observed accumulation of phenolic substances in B-deficient plants (Perkins and Aronoff, 1956). This increase of phenolic concentration within tissues can lead to the formation of quinones with photoactivation and production of reactive oxygen species that can lead to cellular damage (Cakmak and Romheld, 1997).

2.3.5 Hormones and phenolic compounds

Boron is involved in meristematic cell growth possibly through interaction with auxin, which is synthesized in the shoot and translocated to the root (Jesko, 1992). Results of Robertson and Loughman (1974) show that a reduced rate of cell division under B deficiency is not directly attributable to the availability of B but rather to its involvement in metabolism, transport or action of auxins. There are numerous accounts of the involvement of B in regulating the concentration of phenolics in plants and its effect on auxin metabolism. Baker *et al.* (1956) found leaves of B deficient plants to have a higher concentration of pentosans and pectic substances than leaves of normal plants. Coke and Whittington (1967) observed that roots of B deficient fababean accumulated excess amounts of auxin in plant tissue, followed by development of necrosis in the growing points. They conclude that under B deficient conditions, B could not form the complexes with phenolic inhibitors that restrain IAA oxidase, resulting in a build up of IAA. A fairly distinct negative correlation was found between IAA oxidase and phenolic compounds during the growth of tobacco crown gall suspension culture, suggesting the latter participates in enzyme activity (Chirek, 1990). However, there is other data that does not agree with the suggestion of a direct relationship between B and IAA/ phenolic metabolism. Hirsch and Torrey (1980) supplied IAA to roots of plants grown in deficient and adequate B, and found that the root elongation ceased in both case, a typical symptom of extreme boron deficiency, but in neither case was there a thickening of cell walls. It is possible that the accumulation of phenolic compounds may have some influence on plants in addition to that of hormone action, or independent of it. Thus, it cannot be ruled out that the involvement of B in a number of metabolic pathways could be indirect as Lovatt and Dugger (1984) proposed. Indeed, B regulates a number of metabolic pathways because of a capacity of B to complex with the large number of OH-rich compounds in plants. In addition, there are differences between B deficiency and IAA toxicity (Dugger, 1983). Generally vascular plants can obtain IAA from the hydrolysis of IAA conjugates, and IAA can be conjugated to amino acids, sugars and peptides (Bartel *et al.*, 2001).

There is also some evidence regarding the order in which B deficiency and IAA toxicity develop. Only after B deficiency symptoms and ethylene release occurred, do we see an accumulation of IAA in cucumber seedlings (Xiong *et al.,* 2001). After five days of B deficiency, cells accumulated higher IAA activity than those receiving B due to a higher IAA oxidase activity (Goldbach and Amberger, 1986). Although a relationship between B deficiency and excess IAA concentration has been shown many times, a clear mechanism linking them is still unknown. Moreover, no single IAA biosynthetic pathway is yet defined to the level that all of the relevant genes, enzymes and intermediates are identified (Bartel *et al.,* 2001).

2.4 Boron Toxicity

2.4.1 Effects on plants

Boron toxicity occurs in many plant species at levels slightly above adequate concentration for normal growth. Boron toxicity in plants is caused by excessive use of B fertilizer, high concentration of B in soil (Cartwright, 1986) derived from marine sediments (Nable *et al.,* 1997), B in irrigation water (Chauhan and Power, 1978), or B in agricultural drainage water (Smith and Anders, 1989). There are some typical and common visible toxic effects, even though exact symptoms are different depending on species and genotype. Leaf tip and margin burn, followed by necrosis are distinctive typical symptom for wheat and barley varieties (Nable, 1988; Nable *et al.* 1990; Yau, 2002; see Fig 2.1), wheat grass (Schuman, 1969), beans (Gupta, 1979), and lentil (Yau and Erskine, 2000). These symptoms lead to poor shoot growth, dry mass reduction, and less seed yields (Nable *et al.* 1997). There are some species, such as almond, apricot, pear, apple, cherry, apricot, celery, and olive, where B is retranslocated through the phloem to the developing sink, and in these species, they do not exhibit typical symptoms B toxicity. In these species, the increase of B in the older leaves is little, but B accumulates in sink areas, and thus affects stems and petioles leading to brown corky lesions and bud tissues leading to abscission and death (Brown and Hu, 1996). Excess B supply on plant resulted in shorter root axes and less lateral roots with a reduction in root dry weight (Huang and Graham, 1990; Fig.2.6).

Fig. 2.6. B toxicity symptoms in B-sensitive variety Clipper (CL). The photograph was taken 21 days after transplanting. Seedling were grown at ¼ Hoagland solution containing 0.015 mM B (control) in 10 litre trays. High B treatment (5 mM) was applied for 7 days after 14 days in control solutions.

2.4.2 Toxic boron in soils

Generally, B-toxicity in crop plants occurs when the level of hot water soluble B in the soil exceeds 5.0 mg kg^{-1} (Mengel and Kirkby, 1987). These high B soils mainly occur naturally from marine and volcanically active areas (Stangoulis, and Reid 2002; Nable *et al.,* 1997). The sea has around 4.5 mg B L^{-1} (Chesworth, 1991; Weast *et al.,* 1985), and soils of marine origin have high boron level (Erd, 1980). These soils are observed in the dry and semi arid land of Southern Australia, the Middle East, the west coast of Malaysia, the southern coast of Peru, northern Chile, in the solonchaks and solonetz soils of USSR, ferralsol of India, rendzinas in Israel, and Lake California (reviewed by Nable *et al.*, 1997). In addition, West Asia, North Africa (Yau *et al.,* 1994), and the Central Anatolian Plateau of Turkey (Kalayci *et al.,* 1997) have been recognized as a B toxicity region.

The other potential source of high B soil is irritation water Keren and Bingham (1985) classified the maximum permissible concentration of B in irrigation water from 0.028 to 0.093 mmol B L^{-1} for sensitive crops, from 0.093 to 0.19 mmol B L^{-1} for semitolerant crops, and from 0.19 to 0.37 mmol B L^{-1} for tolerant plant species. B toxicity may also be caused by industrial pollution as has been reported by Judel (1977). Where practical, soils polluted with B can be corrected by irrigation with B free water. Boron availability and thus the uptake of excess B can also be depressed by liming (Judel,1977). Boron toxicity occurs only when plants uptake high concentrations of B from the soil solution, and is not related to the amount of B adsorbed by the soil (Ryan *et al.,* 1977).

Boron adsorption in soil increases with increasing pH in the range 8.5 to 10, and this B fixation reduces the uptake of B by plant roots from the soil solution (Scott *et al.,* 1975). However, B-rich soils found in semi arid and dry land, such as southern Australia (Cartwright *et al.,* 1984, 1986), have high soil pH values with high $CaCO_3$

contents, yet the B concentration in shoot and grain of crops grown in these soils has been reported at toxic levels (Cartwright *et al.,* 1984, 1986). These observations are widespread across many soil types with high B concentration including red-brown earths (calcic natrixeralfs), calcareous earths (calciorthids) and calcareous sand (xerochrepts) at extremely high pH and with no carbonates in subsoil (Cartwright *et al.,* 1984). Cartwright *et al.* (1984) mentions that both adsorbed B and solution phases of B in the soil influence the severity of B toxicity. The influence of high B concentrations, causing toxicity to plants in these soils, varies not only with soil pH, but also with the natural variation in distribution, with high B generally occurring at depths in the soil profile. B toxicity is more of an issue when water availability is restricted in the topsoil and causes plant roots to penetrate B rich subsoil.

2.4.3 Toxicity mechanism

Although the mechanism of B toxicity is not completely understood yet, the phenotypic effects of B-toxicity such as impaired root growth, reduced shoot growth, chlorosis and necrosis in leaf tissue are well known (Stangoulis and Reid, 2002). Studies have demonstrated effects of B toxicity on shoot photosynthesis, where accumulation of B at the end of the transpiration stream decreases chlorophyll concentrations, followed by a reduction in CO_2 fixation (Lovatt and Bates, 1984). Subsequently, visible toxicity symptoms develop, such as chlorotic and necrotic patches, at the margins and tips of older leaves. However, the mechanism of B toxicity remains elusive. In this part of literature review focus on some physiological aspects as potential candidatures, such as disruption of 1) root elongation 2) carbohydrate metabolism, and 3) enzyme activity.

2.4.3.1 Disruption of root elongation

Most studies about the relationship between B and cell division and differentiation were conducted with B-deficient plants. For example, root growth of squash plant was shown to be restricted by B deficiency with a failure of cell division in meristematic cells (Cohen and Lepper, 1977). B deficiency has a very rapid effect on root growth and cessation of root elongation has been observed within 12 hours of transferring plants to a B deficient medium (Hirsch and Torrey, 1980; Hirsch *et al.*, 1982). This effect may be attributed to the inhibition of mitosis and disintegration of the meristematic region (Cohen and Lepper, 1977; Hirsch and Torrey, 1980; Hirsch *et al.*, 1982). However, the effect of B toxicity on cell division is not well known as much as the effect of B deficiency. The root elongation and lateral root development of *Cucurbita pepo* was inhibited 72 hours after high B was supplied to five-day-old plant (Lovatt and Bates, 1984). Recent research has shown that B toxicity reduces the root growth of cereals and peas (Paull, 1985; Nable, 1988; Holloway and Alston, 1992). Chantachume *et al.* (1995) reported a toxic effect of B was on plant root elongation very early after B application. Huang and Graham (1990) demonstrated variation for B tolerance by culturing excised root tips at high B supply. They found elongation of primary and lateral roots of sensitive genotypes was severely inhibited in response to B toxicity, while tolerant genotypes either produced callus or developed longer root axes. High B supply (10 mM B) has been shown to inhibit mitosis in the root meristematic zone in broad bean (V. Faba L.), changing chromosome fragments, chromosome stickiness and micronuclei (Liu, *et al.* 2000). In an earlier study, mitotic activity of the pea (Pisum sativum L. cv. 'Alaska') root meristematic zone ceased within 24 hr after exposure to 10 mM borate (Klein and Brown, 1981). Comparatively little is known about the effect of toxic B on root morphology and in particular, whether a response would be due to a direct effect on B on the root itself, or a consequent effect. No experimental result explained the associations of inhibition of cell division and cell elongation to reduced root elongation under B toxicity.

2.4.3.2 Disruption of carbohydrate metabolism

There are only a few studies that support the effect of high B on carbohydrate metabolism. Conditions of B-deficiency (0.5 – 0.05 mg B L^{-1}) and B-toxicity (30 mg L^{-1}) result in a substantial decrease of the sugar levels in the sap (glucose level) and in the root (sucrose level) of sugar beet (Bonila *et al.*, 1980). Interestingly, this effect of boron levels on the sugar levels in the root is very similar to that observed in the sap. This result indicates that boron toxicity may inhibit the sugar metabolism in both the aerial part of the plant and root. One study showed that high B concentration inhibited the production of α-amylase in barley (*Hordeum vulgare* L. var. Atif. De Grignon) seed, indicating that high B effects the production of reducing sugars (Jimenez and Barea, 1979). B toxicity also influenced the balance between starch and reducing sugars. Excess B results in increased levels of reducing sugars and sucrose (Dugger and Humphreys, 1960; Scott, 1960)) and decreased levels of starch content (Scott, 1960; Lovatt and Dugger, 1986). Scott (1960) reported that the increased sugar levels in leaves of sunflower was not a result of impeded transport due to B imbalance as the concentrations of sugars were the same in the roots as in the shoots.

2.4.3.3 Disruption of enzyme activity

Recent studies have improved our understanding of the processes leading to toxicity development with respect to biochemistry and molecular biology. Loomis and Durst (1992) propose that B toxicity may influence the activity of enzymes directly. The high B concentration in the cytosol may inhibit dehydrogenase enzymes by complexing pyridine nucleotide coenzymes, in particular NAD^+, or ribosomal RNA. The conversion of NAD to NADH is slowed as the NAD-borate complex is more stable than NAD^+ (Johnson and Smith, 1976; Pfeffer *et al.*, 1999; Ralston and Hunt, 2000).

In a very recent study, the effect of B toxicity on antioxidant enzymes was measured (Karabal *et al.* 2003). The authors reported that the membrane damage in leaves of the sensitive cultivars increased and this included increased electrolyte leakage, whereas no significant change was observed in the roots. This data suggest that a possible site of B-toxicity is within the plasma membrane. This experiment resulted also a significant increase in total SOD (superoxide dismutase) and a decrease in GR (glutathion reductase) activities in roots of sensitive cultivars, but not in leaf tissues of both tolerant and sensitive cultivars. Moreover, tolerant and sensitive cultivars exhibited similar behavior in terms of enhanced root CAT (catalase) and leaf APX (ascorbate peroxidase) activities. The authors conclude that antioxidant enzyme activity is not a critical indicator of the tolerance of the genotypes against stress condition.

Interestingly, there is evidence that high B influences tissue phenolic compound contents and the activity of some enzymes (Fawzia *et al.,* 1994). When high B solution (50 mg B L^{-1}) was applied to date palm and sorghum seedlings, there was a negative correlation between B concentration in the media and the tissue content of phenolic compounds and IAA-oxidase, polyphenol oxidase, and phenylalanine ammonia-lyase. Excess B supply to tomatoes resulted in significantly reduced concentrations of the polyphenolic flavonoid glycosides (Carpena *et al.*, 1984). An increase in phenolic concentration within tissues can lead to the formation of quinones with photoactivation, production of reactive oxygen species that can lead to cellular damage (Cakmak and Romheld, 1997).

A study conducted to test combined stresses of salinity and boron toxicity found that the pectin content was reduced in salt-affected leaves in combination with increased soluble B concentrations (Wimmer, et al., 2003). The authors propose that a reduced pectin content may affect intercellular proteins in two ways: (1) less protein could be bound to the cell wall due to a lower amount of binding sites; and (2) larger pore sizes due to excessive B supply could facilitate access of enzymes to the apoplastic space.

2.5 Boron Tolerance

Tolerance to B toxicity has been investigated not only at the whole plant, but also at the cellular level. Many Australian breeders have identified B-tolerant germplasm and bred B tolerant crop varieties (Paull *et al.*, 1988 (a, b), 1990; Jefferies *et al.*, 1999, 2000; Rathjen, *et al.*, 1995; Moody, *et al.*, 1993; Hollamby, *et al.*, 1994 a, 1994 b) with the major genes. B tolerance in bread wheat is controlled by at least there major genes, namely, *Bo1*, *Bo2*, *Bo3* (Paull *et al.*, 1988 a). These genes were additive, non-maternal and partially dominant, based on dry matter production and tissue B concentration in F1, F2, and F3 populations derived from five genotypes that differed for level of tolerance. Transgressive segregation was observed in the progeny between the moderately tolerant Halberd and tolerant G61450, suggesting that another gene, designated Bo4, was involved in the control of tolerance to B (Paull, 1990).

Compared to B–sensitive plants, B-tolerant plants generally accumulate less B in the shoot tissue (Nable, 1988). The mechanism may consist of a specific gene expression in the tolerant plants that can control a reduced B accumulation. Mahboobi, *et al.*, (2000) obtained protein profiles of seedlings from both B-tolerant and B-sensitive cultivars grown under high and low B conditions using 2 dimensional gels and silver staining. It was found that B-toxicity either increased or decreased the expression level of a range of proteins in root and leaf tissue. In the B-sensitive cultivar, Hamidiye, protein levels declined due to the effects of B toxicity, while conversely in the B-tolerant cultivar, protein levels were enhanced. Multiple effects on gene expression were observed in shoot tissue, while the effects in the root were less evident. Alternatively, the variation in cell wall structure due to changes in protein among different species or genotypes may act to reduce B uptake (Wimmer *et al.*, 2003). However, a few species (e.g. sunflower) are able to tolerate higher B concentration in the tissue (Reuter *et al.*, 1997; Blamey *et al.*, 1997; Dannel *et al.*, 1999), and this could be attributed to an internal mechanism, such as differential

distribution or compartmentation of B at the cellular or organ levels. Possibly, altered distribution of B between cytosol and vacuole might contribute to differential tissue tolerance to B toxicity between barley cultivars (Torun, *et al.,* 2003).

2.5.1 Inter-and intra-specific variation in response to boron toxicity

All investigations have shown a variable range in B concentration across within and between plant species. Tanaka (1967) reported boron concentration in roots from different crop plants, ranged from 3 to 30 mg B kg^{-1} with barley and cucumber having the lowest and highest B concentrations, respectively. The average B concentration in plants is 20 mg kg^{-1} dry weight (Epstein, 1973), and the excessive or toxic range is 50-200 mg kg^{-1} in mature leaf tissue (Kabata-Pendias and Pendias, 1984). Toxic levels of B were highest in celery leaf (720> mg B kg^{-1} dry weight), pasture grasses (>800) and sugar beet (>800) (Reviewed in Jones, 1991). Ferreyra *et al.* (1997) observed variations in B-tolerance between species grown in the coastal region of Northern Chile at Calama. The more B-tolerant species were asparagus (*Asparagus officinalis*), onion (*Allium cepa*), celery (*Apium graveolens*), red beets (*Beta vulgaris*) and sugar beets (*Beta vulgaris*), and the more sensitive, strawberry (*Fragaria* spp.), sweet potato (*Ipomoea batata*) and suddex (*Graminia*, hybrid sudan × sorghum).

More recent investigations have demonstrated a wide range of intra specific variation. In cereals, many genotypes more tolerant to high concentrations of B than Halberd have been identified at the Waite Institute (Moody *et al.*, 1988). Of 1576 lines from the Australian Wheat Collection, screened in pots containing 80 mg B/kg of soil, 12% were highly sensitive (growing slowly to the second leaf stage and dying), 35% sensitive, 33% moderately sensitive, 14 % moderately tolerant and 6% tolerant (virtually no symptoms). The field experiments showed that tolerant varieties accumulated less B in vegetative tissues than sensitive varieties, but B accumulation in the grain was not strongly correlated with seedling sensitivity. Yau *et al.* (1997)

have identified B-tolerant durum wheat (*Triticum turgidum* L. var. *durum* Desf., syn. *T. durum*) adapted to the high B soils of the Mediterranean region. Peas (based on the most tolerant of the Australian varieties) are more tolerant than chickpeas, which are more tolerant than lentil (Bagheri, 1994). Bagheri *et al.* (1994) identified that 4 % of 617 pea accessions originating from Afghanistan, India, China and South America are more tolerant than the most tolerant Australian variety, Alma. B tolerant lines of barley varieties (e.g. Sahara) maintain lower levels of B in shoots and roots than the more sensitive varieties, such as Sloop. The variation of B concentration in shoots between tolerant and sensitive varieties was from 91 to 1226 mg B kg^{-1} dry weight, respectively (Nable, 1988). The more tolerant Australian varieties of pea were Early Dun, Partridge, Dun, Derrimut, and Parraggio, Ghor and Cyprus were the most tolerant of the Australian medics (Paull, *et al.,* 1992). The major mechanism of B tolerance in these legumes consists of exclusion of B from the root and shoo1ts of the tolerant line, and is consistent with previous results for wheat and barley (Paull, *et al.,* 1992).

2.5.2 Synthesis of proteins and activity of enzymes

Protein synthesis is known to vary according to different environmental stresses, including B toxicity, although there are only limited reports of such an occurrence. Mahboobi *et al.* (2000) found that the expression of over 20 proteins was affected by transferring plants to high B conditions, and the affect was different in a tolerant and sensitive variety. They subjected the total protein extracted from roots and leaves of 5 day-old barley seedlings to two-dimensional gel electrophoresis and silver staining. A completely new polypeptide (35.0 kDa) was detected only in roots of the tolerant variety, but not the sensitive variety. There were more changes in polypeptide composition in leaves than roots, with the expression of at least seven proteins increased in the tolerant variety, but no changed in the sensitive variety. In addition, a group of high molecular weight proteins were decreased in the sensitive variety. This result may imply that there are tolerance genes controlling the B-tolerance mechanism in tolerant plants.

Although one experiment was conducted under combined stresses between salinity and boron toxicity, there is also evidence for quantitative and qualitative changes in intercellular protein composition. A 25 kDa protein was induced, and an increased amount of a 33 kDa protein was also observed (Wimmer *et al.*, 2003). The authors suggest that these changes in protein might be due to structural modifications of the cell wall.

2.5.3 Composition of membrane and cell wall

The composition of the lipid membrane and the cell wall could affect on the permeability of B resulting in differences in B accumulation (Nable, 1988; Nable *et al.*, 1990b; Nable and Paull, 1991; Dordas and Brown, 2001). Dordas and Brown (2001) found that the permeability coefficient for boric acid across the membrane made only of phosphatidylcholine was 4.9 $\times 10^{-6}$ cm s^{-1}, but with the addition of sterols, the permeability was reduced to between 7 $\times 10^{-6}$ cm s^{-1} to 9.5 $\times 10^{-6}$ cm s^{-1}. The reduction in the B permeability with the addition of more sterol is attributed to changes in the length of the fatty acyl chain or the type of phospholipid head group. This result indicates that a B-tolerant plant could control B uptake by changing their membrane composition to restrict permeability of boric acid.

Not only membrane, but also the composition of the cell wall could function to block passive transport. While the majority of the water soluble B is localized in the apoplastic region as boric acid, the water insoluble B is associated with RG-II (Thomas *et al.*, 1989). In the RG II, B crosslinks two chains of pectic polysaccharide through borate-diester bonding, forming a network of the pectic polysaccarides in the cell wall. Variation in this B complex have been identified depending on plant species. In duckweed, a different type of B-polysaccharide complex has been recognized. Duckweed cell walls contain a lot of B, as much as 780 mg B kg^{-1}, while only 0.87 % of the B is associated with the high molecular weight fragments, and 70 % of the B remains in the cell wall (reviewed by Matoh, 1997).

The responsibility for fixing B to the polysaccharide chains by the apiosyl residue, one of the components of RG II indicates that the increased capacity of the duckweed cell walls to fix B may be related to its higher apiose content. An excessive cross-linking in the cell walls may help to reduce B diffusion into the symplast of the cell, while in theory this is possible, evidence has shown that the cell wall saturated with B at quite a low B supply, and for the cereals where we see genotypic variation in B uptake, it is hard to see how this mechanism would work given the very low requirements in cereal cell walls and the very high levels of external B supply.

Despite the major role of B in the cell wall structure as part of the RG-II complex, there is no strong evidence that high B has adverse effects on apoplastic process. For example, to investigate the ability of sunflower to tolerate high internal levels of B, 1mM B was applied in a short-term treatment (6h) (Dannel, *et al.*, 1999). The short-term treatment resulted in a high internal B concentration within the root (about 70 % of the external B), thereby achieving diffusion equilibrium. The majority of the additional B was found in the cell sap, but a small amount of it was bound in the water insoluble residue of the cell sap. This indicates that the root cell wall was not able to bind any additional B refuting the hypothesis that the cell wall acts as a detoxifying mechanism.

Long-term experiments with excess B also resulted in a 10-fold higher B concentration in the leaves than in the roots with the root unable to retain B to prevent the shoot from excess accumulation of B (Dannel *et al.* 1998). These two results of both short and long-term experiments imply that B is first accumulated in the root cell sap, mainly representing soluble B in the symplasm, and then with time, B is translocated into the shoots. The leaf cell wall also had a very limited capacity to bind additional B in response to the short-term treatment with 1 mM B suggesting the binding of B in the leaf cell wall does not contribute substantially to the detoxification of excess B in sunflower leaves.

The hypothesis that the cell wall can detoxify excess B is not supported by more recent studies. For example, when the cell wall uronic acid (a significant structural components of cell wall pectins) content of root and leaves was measured after addition of toxic levels of B, no significant changes were observed in both sensitive and tolerant varieties (Mahboobi *et al.,* 2001).

2.5.4 Phloem immobility

Internal detoxification of internal high B has been demonstrated in some species, such as almond, apricot, and celery (Brown and Shelp, 1997; Hu et al, 1997 a). Using boron isotopes, studies have been carried out to investigate boron mobility that could act to remove excess B from plant tissues in the phloem. For example, changes in B isotopic concentrations following [^{10}B] labeling in celery (*Apium graveolens* L.) have been observed (Hu *et al.*, 1997 a). 1 day after labeling, B was absorbed by the leaf tissue with an 8 times increase of [^{10}B] in the labeled leaf. However, with time, the high concentration of [^{10}B] in the labeled leaf decreased rapidly. The distribution B within celery leaves was 104, 49, 32 μg g^{-1} B in the youngest growing tissue, fully expanded leaflets, and the oldest leaves, respectively, showing a characteristic pattern of a phloem-mobile element. In addition, boron polyol complexes were isolated and characterized in the phloem sap of celery, giving an indication of the mechanism responsible for B retranslocation (Hu, *et al.*, 1997 b). Immobility of B in the phloem B may be considered as an internal B tolerance mechanism, thereby keeping more B away from key metabolic sites, retaining it instead in leaf margins, where regardless of leaf burn, plants can still retain considerable photosynthetic area (Nable *et al.* 1997). For example, with increasing B supply the B concentration linearly increased in all parts of the citrus tree in the following order: basal leaves> top leaves> bark> root> stems> wood (Papadakis *et al.,* 2003), and thus the top leaves could grow normally.

2.6 Summary and Implications of the thesis research

B is an essential plant micronutrient that is released predominantly from variable rock type and origin, and the highest concentrations are in marine sediments. The uptake of B by plant root is not absolutely passive, but there is a degree of controlled active uptake. The major roles of B for plant metabolism that have been proposed are 1) maintenance of membrane or cell wall integrity and functioning and 2) enzymatic regulations for carbohydrate metabolism and cell growth interacted with hormone and phenolic compound. However, if B is present in soils in high concentration, it can be toxic to plants. B toxicity has been recognized as a problem in the dry regions of the world and associated with irrigation water. B toxicity in crops including barley, wheat, and grain legumes grown in southern Australia under dry land conditions has been identified, and genotypic variation for tolerance of B toxicity exists within and between species and varieties. The breeding programs in South Australia have produced tolerant varieties of wheat, barley, peas, and medics using selection criteria of leaf symptom, root and shoot dry weight, and root length. The results from the programs have also proved that B tolerance is associated with at least more than one mechanism. The tolerant cultivars in cereals have the ability to exclude B from plant tissue, and have a tolerance to higher tissue B. In addition to these mechanisms, there may be potential mechanisms associated with root development and morphology that may allow tolerant plants to exploit water and nutrients from subsoil with toxic B. However, it is still necessary to introduce a broad range of B tolerant plant roots that may have different growth and morphology. Although plant researchers, who have been trying to understand B chemistry with plant physiology and biochemistry, have improved the understanding of B-toxicity and B-tolerance mechanism, there is still little information on the comparative responses of genotypes in terms of the tolerance to B. Thus it will be useful to assess and identify whether the variable tolerance to B is associated with diverse morphology and physiology within and between species and variety under conditions of B toxicity.

CHAPTER 3

Exploring the Response of Crop and Weed Species to Increased Concentrations of Boron in Subsoil

3.1. Introduction

Across a large area of the low rainfall agricultural regions in southern Australia, crop growth and yield are restricted by an accumulation of excess B in the subsoil (Cartwright *et al.,* 1986; Holloway and Alston, 1991; Coventry *et al.,* 1998). Typically, an extractable B concentration in soil between 0.5 and 5.0 mg B kg^{-1} soil is considered not toxic for normal plant growth (Bell, 1999), however, considerably higher concentrations (from 5 to 50 mg/kg) of extractable B have been reported for soils in some regions across southern Australia (Isbell, 1996), particularly at depths below 30 cm (subsoil). Recognition that these high levels of B ultimately reduce crop yields (Cartwright *et al.,* 1984, 1986) has resulted in substantial investment into breeding tolerant cultivars over the past 20 years, with good success (Rathjen and Moody *et al.,* 1993).

Selection criteria commonly used in screening for B tolerance in barley include shoot dry weight, tissue B concentration, severity of symptom expression in leaves, and less often, relative root length (Nable, 1988; Chantachume *et al.*, 1995). More recently, quantitative trait loci have been developed using these traits as tolerance indicators (Jefferies *et al.*, 1999). The definitive processes involved in B tolerance are not clearly understood (Stangoulis and Reid, 2002) although it is agreed that more than one mechanism may operate, involving either exclusion or restricted uptake of B, or both together. It has been suggested that some perennial or summer weeds in southern Australia may possess a B tolerance mechanism since they survive and grow under conditions where they are likely to be accessing water from depths in the soil profile that contain high B concentrations. However, there is no clear evidence to

support or refute this suggestion. Clearly, more information on the shoot and root response of tolerant and sensitive crop and weed species may assist in defining B tolerance mechanisms, and provide additional genetic resources from which to source traits for breeding tolerant crop species.

The overall aim of the work reported in this chapter was to investigate root responses to varied subsoil B concentrations for a range of crop and weed species common to agricultural areas of South Australia. The specific objectives being to determine, for each species (a) the morphological response of the whole root system in both the topsoil and subsoil layer to different subsoil B concentrations, and (b) the available B concentrations in sub-soil critical for expression of selection traits and (c) to elucidate plant responses that may provide further insight into the mechanism of B tolerance as it operates under field conditions.

3. 2 Materials and Methods

Two experiments were carried out to test the shoot and root response of a number of crop and weed species to a range of $CaCl_2$ extractable B concentrations in subsoil. The experiments were carried out, at the Roseworthy campus of the University of Adelaide, South Australia (34°50` S and 138°69` E) during the period April to November 2001, in a glasshouse with refrigerated cooling to maintain a maximum temperature of 22°C.

3.2.1 Experiment 3(a) Shoot and root response of barley (Clipper and VB9953), barley grass, lincoln weed and evening primrose to different extractable boron concentrations in subsoil

Annual crop species grown in this first experiment included two barley (*Hordeum vulgare*) cultivars, the variety Clipper (B sensitive) and an advanced breeding line of barley (VB9953) selected for tolerance to B. Weed species were selected from those commonly growing on soils in crop production areas of SA with known high B concentrations in the subsoil. These included the annual weed barley grass (*Hordeum*

glaucum) and the perennial weeds evening primrose (*Oenothera Stricta*) and lincoln weed (*Diplotaxis tenuifolia*). Seeds of the crop species and lincoln weed were germinated under glasshouse conditions in seedling trays filled with finely sieved sand and seedlings were transplanted to cylinders of soil approximately two weeks after germination. Seeds of barley grass and evening primrose were germinated in petri-dishes on filter paper moistened with de-ionized water, incubated at 5°C for two days, 20°C for a further two days (7 days for evening primrose), and then transferred to the glasshouse for one week prior to seedlings being transplanted into cylinders of soil.

3.2.1.1 Boron treatment and watering regime

PVC cylinders (154 mm diameter and 50 cm depth), sealed at the base and filled with a sandy soil to a bulk density of 1.6 g cm^{-3} were used for growing the plants. During the preparation of the cylinders, boric acid (H_3BO_3), at rates of 0, 15, 25, 50 or 100 mg kg^{-1} soil, was added to 8.9 kg of air-dried soil in the 20-50 cm depth of each cylinder and thoroughly mixed. The pH ($_{H2O}$) of the soil was then measured and ranged from 6.8 to 7.2. Soil without any added B was then packed into the top 20 cm section in each cylinder and the entire soil column was adjusted to approximately 12-13 % moisture content (70 % of field capacity) by addition of a low strength McKnights nutrient solution (according to Bergersen, 1980). Following these additions the resultant extractable (hot $CaCl_2$) B concentrations in the sub-soil were 0.5 (control), 2.4, 4.3, 6.8 or 12.2 mg B kg^{-1} dry soil, and 0.5 mg B kg^{-1} in the topsoil. Other available nutrients (mg kg^{-1}) were; NO_3-N: 22, NH_4-N 45, Colwell P 22, Colwell K 92, S 89, EDTA Fe 32, EDTA Cu 20, EDTA Zn 1, EDTA Mn 2.2, and Ca (Exc.) 1.2 mmol L^{-1}, Mg (Exc.) 26 mmol L^{-1}, Na (Exc.) 19 mmol L^{-1}, and K (Exc.) 25 mmol L^{-1}.

The soil water content at different depths in each column was monitored frequently (every 2- 3 days) using a calibrated portable TDR probe and meter (Microscan Electronics). The wave-guides were 7.5 cm long and holes in the pot were sealed using tape after measurement. During the first two weeks after transplanting there were no additions of water to the columns. Subsequently, regardless of B treatment,

the moisture content (w/v) of the soil columns was maintained for the remainder of the experiment at approximately 5-6 % in the 0-20cm depth, 12-13 % in the 20-30cm depth and 18-19 % in the 30-50cm depth (i.e at 30 %, 70 % and 100 % field capacity, respectively) by frequent (twice weekly) injection of deionised water via ports in the side of the cylinder. Destructive sampling of the soil columns was carried out when it could be inferred from the TDR probe readings that roots were extracting water from the 30-50 cm soil depth, and therefore had clearly colonized the subsoil.

3.2.1.2 Design

Each column contained one plant and each sub-soil B treatment was replicated three times. The columns were arranged in a completely randomized design on a bench in the glasshouse. Data for shoot and root parameters were subject to 'General ANOVA' analysis of variance using the statistical package GENSTAT 5, Release 4.2 (Lane *et al.*, 1988). Significant mean separation was evaluated using the least significant differences (LSD) at the 5% level where the F value was significant.

3.2.1.3 Root sampling and image analysis

After the shoot was removed each cylinder was placed horizontally on to a plastic sheet and the base cap was removed. The intact soil-root column was then carefully pushed out via the top of the cylinder and sectioned into the top 0-20cm and the remaining 20-50cm depths. Each section was initially separated by hand with the larger roots picked out and washed. The remaining entire soil depth sample was then gently washed through a sieve to recover roots greater than 0.5mm diameter. Root samples were stored in water at 4-5°C prior to analysis.

Each root sample was placed in a shallow transparent tray (20 × 30cm) filled with 2 to 3 mm of water to facilitate separation of roots and thus minimizing overlap. Root segments longer than the tray were cut into shorter pieces for ease of analysis. Root imaging of the sample in each tray was carried out using a flat-bed scanning technique (Richner *et al.*, 2000). Images were analysed for total length (cm), total surface area (cm^2), diameter (mm), total number of root tips and number of root forks

using a commercially available image analysis software package (WinRhizo®). Care was taken to adjust threshold levels to enable accurate analysis of both fine fibrous root and thick taproot images. Root length density (cm cm^{-3}) was calculated as follows: root length (cm) divided by the known soil volume (cm^3) from which the roots were extracted.

3.2.1.4 Dry weight and boron concentration

Each shoot was oven-dried at 65°C for two days, the dry weight was recorded and the sample ground in a stainless steel mill to pass a 1mm screen. Root samples were also oven-dried, weighed and ground following image analysis. The ground materials were analyzed for B using ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometry).

3.2.2 Experiment 3(b) Shoot and root response of barley (Clipper), fababean and prickly lettuce to different extractable boron concentrations in subsoil

This second experiment investigated Clipper barley, fababean ((*Vicia faba* var. Fiord) was moderate to high tolerance to B (J. Paull, personal comm.)) and one weed species, prickly lettuce (*Lactuca serriola*). Prickly lettuce seeds were germinated in petri-dishes, as already described for evening primrose. The experiment differed slightly from the previous one in that there were two plants per cylinder and only two extractable B treatments (i.e. the control with no added B (Ext. B; 0.5mgB/kg) and high B (Ext. B; 12.2 mg B/kg)). The treatments were applied in duplicates. Glasshouse conditions, nutrient solutions and watering regime were similar to the first experiment except that the soil contained some peat (42.8 % by volume) and therefore had a slightly different bulk density (1.21 g cm^{-3}).

Sampling and analytical procedures were also similar to those in the previous experiment, except that destructive sampling of the soil columns was carried out at two growth stages. Root image analysis was not conducted because of difficulties with separating root material from the peat. The first sampling for Clipper and fababean at 35 days after sowing coincided with the development of B toxicity

symptoms in the shoots (Table 3.1), and a second sampling was carried out at 55 days when it could be inferred from the TDR probe readings that roots had clearly colonized the 30-50 cm soil depth. There was a single sampling at 45 days after sowing for prickly lettuce.

Table 3.1. Number of days after sowing to the initial appearance of B toxicity symptoms in mature leaves, the minimum B concentration for symptom appearance and total number of growing days (TGD), for the crop and weed species used in the two glasshouse experiments

Species	Days to symptom development	Minimum B conc. for symptom appearance	TGD
Experiment (a)		mg B kg^{-1}	
Barley VB9953	37	≥ 2.4	57
Barley Clipper	33	≥ 2.4	54
Barley grass	35	≥ 12.2	57
Evening primrose	86	≥ 4.3	113
Lincoln weed	70	≥ 2.4	97
Experiment (b)			
Barley Clipper	31		58
Fababean	32		61
Prickly lettuce	48		48

3.3 Results

3.3.1 Experiment 3(a) Shoot and root response of barley (Clipper and VB9953), barley grass, lincoln weed and evening primrose to different extractable boron concentrations in subsoil

3.3.1.1 Boron toxicity symptoms

Red-brown necrotic lesions symptomatic of B toxicity were initially observed one month after sowing with symptoms localized to leaf margins and tips on the barley plants at the B concentrations greater than 2.4 $mg.kg^{-1}$ in the subsoil (Table 3.1 and Plate 3.1). While symptoms first occurred on the margins of the leaf sheaths and also the stems, this gradually extended to affect a large proportion of the leaf, and eventually other younger leaves, with the timeframe for this inversely proportional to extractable B concentration in the subsoil. The cultivar Clipper demonstrated a higher proportion of leaf damage than the advanced breeding line VB9953. The monocotyledonous weed, barley grass, showed minor B toxicity symptoms 35 days after sowing when grown at the highest extractable B concentration (12.2 mg kg^{-1}) in the subsoil. The minor toxicity symptoms included necrosis on the leaf tips of the older leaves. Boron toxicity symptoms on the leaves of the dicotyledonous weeds appeared more slowly and were not evident in lincoln weed or evening primrose until at least ten weeks after sowing. The two weeds differed slightly in B-tolerance with clear visual symptoms appearing on the leaf margins and tips in lincoln weed at an extractable B concentration in subsoil of 2.4 mg kg^{-1} and greater, whereas the threshold for evening primrose was 4.3 mg kg^{-1} .

Overall, increasing the concentration of B in the subsoil decreased root dry weight in this region, but the response of the root in the topsoil and subsequent responses in the shoot differed between species (Table 3.2, Fig 3.1). Increasing extractable B concentration in subsoil up to 12.2 mg kg^{-1} dry soil did not reduce shoot dry weight of VB9953, barley grass, and evening primrose, whereas it was significantly

($P<0.001$) decreased by 24% in Clipper and by 21% in lincoln weed at subsoil B concentrations of 2.4 mg kg^{-1}.

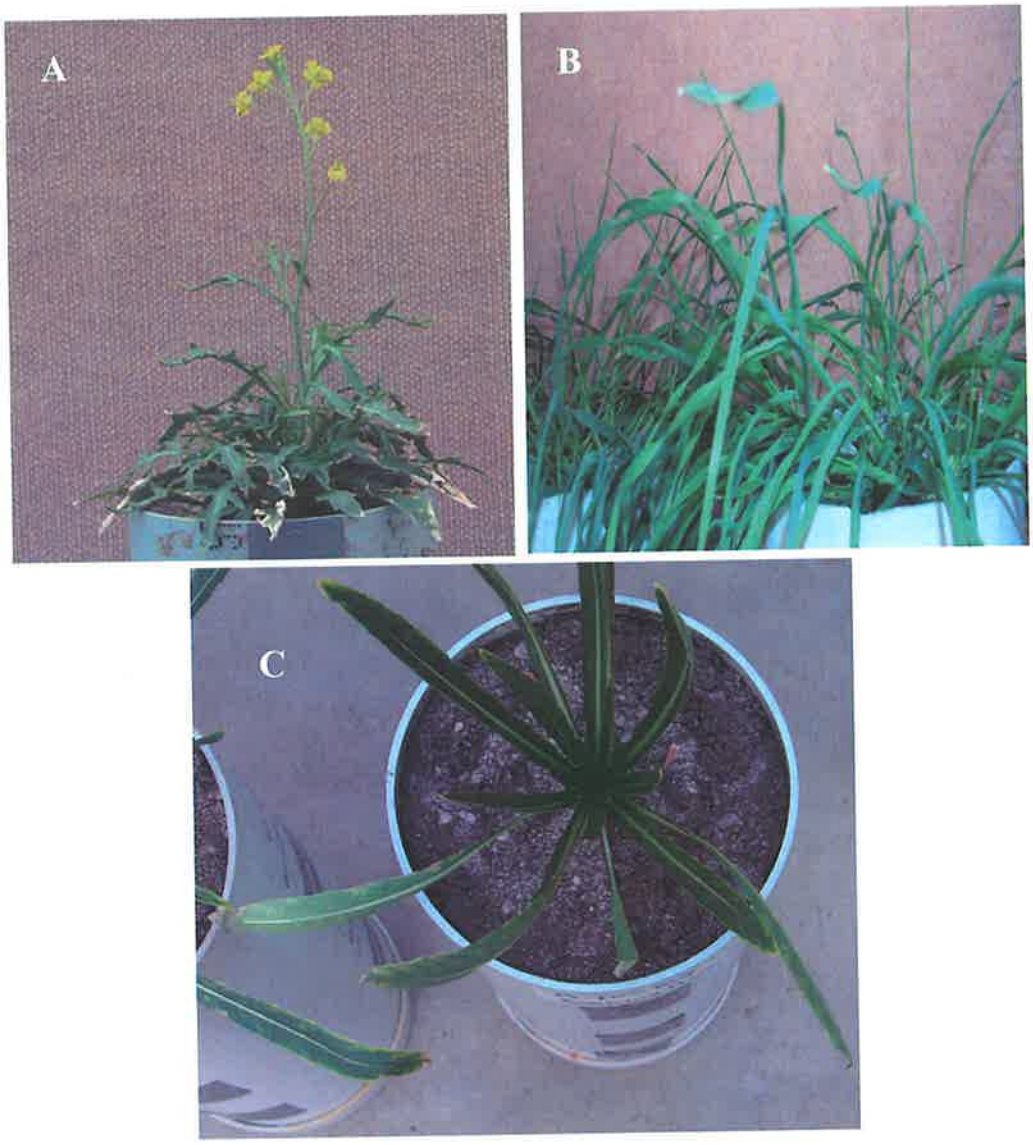


Plate 3.1. Boron toxicity symptoms in three weed species (A) lincoln weed, (B) barley grass and (C) evening primrose grown at an extractable B concentration in subsoil of 12.2 mg kg^{-1}.

3.3.1.2 Plant Growth

Root growth appeared more sensitive than shoot growth to high B supply. Although overall growth of Clipper was greater than VB9953, Clipper exhibited a greater reduction in total root dry weight than VB 9953, but at 12.2 mg kg^{-1} extractable B in the subsoil there were no differences in root dry weight between the two barleys (Table 3.2, Fig 3.1). Root growth of Clipper, lincoln weed, and evening primrose was reduced not only in the subsoil layer, but also in the topsoil. Root dry weight in the topsoil for Clipper, lincoln weed and evening primrose grown at 12.2 mg kg^{-1} extractable B in subsoil was 42% ($P < 0.001$), 68% ($P < 0.001$), and 47% ($P < 0.05$), respectively, of the control. The reduction was associated mainly with a decrease in taproot and not the fine root fraction in lincoln weed and evening primrose. Taproot dry weight for lincoln weed at 12.2 mg kg^{-1} extractable B in subsoil was 0.84 g $plant^{-1}$ (± 0.035), 80% less than the control value of 4.28 g $plant^{-1}$ (± 0.08), and for evening primrose was 0.18 g $plant^{-1}$ (± 0.034), 58 % less than the control at 0.43 g $plant^{-1}$ (± 0.015). Total root dry weight for VB9953 at 12.2 mg kg^{-1} extractable B was 2.40 g $plant^{-1}$ (±0.015), 19 % less than the control, 2.95 g $plant^{-1}$ (±0.13) and this reduction resulted mostly from a decrease in root dry weight in the subsoil. In contrast, the reduction of total plant dry weight compared to the control in Clipper (45%) and lincoln weed (57%) was due to a combined decrease in shoot, topsoil root, and subsoil root dry weight.

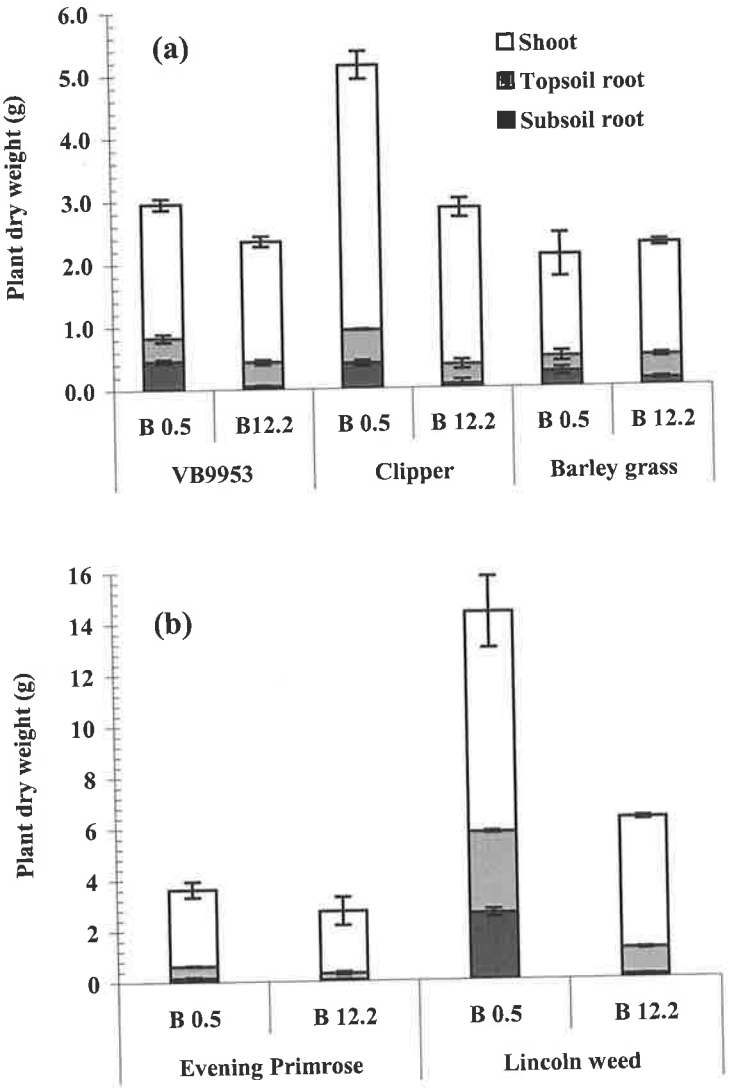


Fig. 3.1. Dry mass of shoot, topsoil root and subsoil root for (a) VB9953 (VB) and Clipper (CL) barley and barley grass (BG), and (b) evening primrose (EP) and lincoln weed (LW), grown under control (B 0.5) and elevated extractable boron (B 12.2) concentrations in subsoil. Bars represent mean of three plants±standard error.

Table 3.2. Shoot, topsoil root, subsoil root, and total root dry weight (g plant^{-1}) of VB9953 and Clipper barley, lincoln weed, barley grass, and evening primrose for different extractable B concentrations in subsoil. Values are mean ± standard error of 3 replications.

Subsoil B (mg kg^{-1})	Dry weight (g plant^{-1})				
	Species				
	VB9953	Clipper	Lincoln weed	Barley grass	Evening primrose
			Shoot		
0.5	2.13±0.09	4.21±0.22	8.59±1.39	1.61±0.35	3.00±0.31
2.4	1.98±0.05	3.20±0.06	6.79±0.59	1.36±0.09	2.71±0.24
4.3	2.59±0.17	3.30±0.20	7.07±0.10	1.98±0.13	3.94±0.18
6.8	2.05±0.01	3.00±0.09	5.81±0.39	1.56±0.04	3.64±0.30
12.2	1.92±0.08	2.49±0.15	5.10±0.07	1.79±0.05	2.43±0.55
			Topsoil root (0-20 cm)		
0.5	0.37±0.06	0.53±0.01	3.17±0.04	0.24±0.06	0.47±0.02
2.4	0.36±0.03	0.41±0.04	2.01±0.20	0.23±0.02	0.42±0.01
4.3	0.40±0.05	0.37±0.05	1.83±0.24	0.35±0.01	0.35±0.01
6.8	0.39±0.04	0.36±0.02	1.24±0.07	0.34±0.02	0.34±0.07
12.2	0.38±0.04	0.31±0.07	1.03±0.03	0.36±0.03	0.25±0.06
			Subsoil root (20-50 cm)		
0.5	0.45±0.03	0.40±0.05	2.61±0.15	0.24±0.08	0.16±0.04
2.4	0.29±0.01	0.18±0.04	0.68±0.19	0.16±0.01	0.14±0.01
4.3	0.24±0.02	0.20±0.04	0.57±0.01	0.24±0.03	0.16±0.02
6.8	0.21±0.01	0.13±0.01	0.32±0.01	0.10±0.01	0.08±0.04
12.2	0.05±0.01	0.05±0.07	0.14±0.01	0.11±0.02	0.06±0.03
			Total root		
0.5	0.82±0.06	0.93±0.05	5.78±0.12	0.47±0.14	0.62±0.02
2.4	0.67±0.03	0.59±0.06	2.69±0.30	0.39±0.02	0.56±0.03
4.3	0.64±0.06	0.57±0.09	2.40±0.23	0.59±0.03	0.51±0.01
6.8	0.60±0.04	0.49±0.03	1.56±0.06	0.44±0.02	0.43±0.07
12.2	0.43±0.04	0.37±0.06	1.17±0.04	0.48±0.04	0.31±0.08

3.3.1.3 Ratio of root:shoot

Lincoln weed (a dicotyledonous plant) had the highest root:shoot dry weight ratio (Table 3.3) across all genotypes in the control treatment; a reflection of investment in tap roots (Fig 3.4). It was considerably higher than the root:shoot ratio for evening primrose which was closer to that of the monocotyledonous plants. The relative sensitivity of the various genotypes in the study, both monocots and dicots, to high B supply in subsoil can be related to the magnitude of the reduction in the root:shoot ratio (Table 3.3). For example, the reduction in root:shoot ratio for Clipper was greater than VB9953, and for lincoln weed was far greater than evening primrose. Overall, the two weeds with taproot systems (lincoln weed and evening primrose) exhibited the greatest reductions in root:shoot ratio indicating a more negative impact on root growth due to B toxicity than for the others species. The root:shoot ratio for barley grass was not altered in any subsoil B treatment suggesting a lack of sensitivity to concentrations of extractable B in subsoil up to 12.2 mg kg^{-1}.

Table 3.3 Root: shoot dry weight ratio of VB9953 and Clipper barley, barley grass, lincoln weed and evening primrose grown under different extractable subsoil B. Values are mean ± standard error of three replications.

Subsoil B ($mg\ kg^{-1}$)	Ratio of root: shoot				
	Species				
	VB 9953	Clipper	Barley grass	Lincoln weed	Evening primrose
0.5	0.387±0.03	0.222±0.008	0.282±0.029	0.684±0.061	0.221±0.024
2.4	0.341±0.02	0.186±0.016	0.292±0.022	0.398±0.048	0.159±0.015
4.3	0.250±0.03	0.169±0.017	0.303±0.023	0.339±0.031	0.130±0.010
6.8	0.293±0.02	0.164±0.006	0.280±0.006	0.269±0.012	0.104±0.012
12.2	0.218±0.01	0.149±0.029	0.266±0.024	0.229±0.005	0.126±0.020

3.3.1.4 Boron concentration in shoot and root

Whole shoot B concentration (mg kg^{-1}) for the control plants was 83 (±17) for VB9953, 64 (±14) for Clipper, 11(±0.8) for Barley grass, 53 (±3) for evening primrose, and 93 (±12) for lincoln weed. Whole shoot B concentration increased as extractable B concentrations in the subsoil increased (Fig.3.2) with the highest concentration measured for lincoln weed (3500 mg B kg^{-1} shoot dry weight), almost double that in VB9953 and much greater than evening primrose and barley grass. A whole shoot B concentration greater than 570 (±125) mg kg^{-1} (in the 2.4 mg B in subsoil treatment) caused a 24 % reduction in shoot dry weight of Clipper (data not shown), whereas a shoot concentration of 430 (±66) mg B kg^{-1} did not significantly reduce shoot dry weight of VB9953.

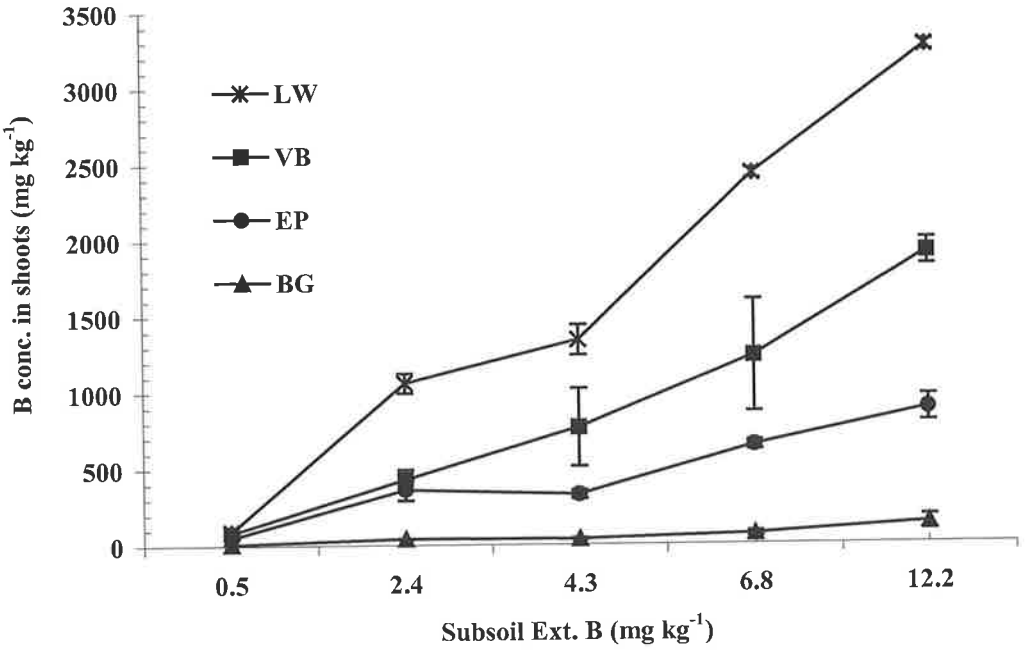


Fig. 3.2 Whole shoot B concentration (mg kg^{-1}dry weight) for VB9953 (VB) barley, barley grass (BG), evening primrose (EP) and lincoln weed (LW) grown at different concentrations of extractable B in subsoil. Values are mean ± standard error of three replications.

The rate of B accumulation in barley grass was extremely low with a maximum concentration of 150 mg B kg^{-1} shoot dry weight causing minimal visible toxicity symptoms, and no significant effect on the shoot dry weight. B accumulation in Clipper shoots at 6.8 and 12.2 resulted in extensive leaf damage followed by secondarily infection with disease and thus shoots were not analyzed by ICP.

The extensive root washing procedure would remove all symplastic B most likely leaving only tightly bound cell wall B. The B concentration of washed roots for the two barleys was less than the limit of detection of determination (< 2-3 mg kg^{-1}) of the method in the ICP analysis. However, B was likely to be mainly associated with structural components, such as cell walls, detected in washed roots of the three weed species (Table 3.4). Overall, B concentration in the washed roots of barley grass was much lower than for the other two weed species. The B concentration was significantly higher in subsoil roots than in the topsoil roots for barley grass, but there were no significant differences between the control plants and those grown at elevated extractable B concentrations in subsoil. Whereas, in evening primrose and lincoln weed, increasing the extractable B concentration in the subsoil significantly ($P<0.05$) increased B concentration in the washed tap and fine roots in both topsoil and subsoil. In the presence of elevated extractable B in the subsoil, there was a trend for the washed taproots (where present) to have a higher concentration than the washed fine roots (Table 3.4). However, the taproot of evening primrose did not actually extend into the subsoil in any treatment or in the control, and was absent in the subsoil at the highest extractable B treatment in lincoln weed. Interestingly, the range of B concentrations for washed roots of evening primrose (18-159 mg B kg^{-1} dry weight) was similar to that for roots of lincoln weed (24-146 mg B kg^{-1} dry weight) despite the much higher shoot B concentrations measured for lincoln weed (Fig 3.2).

Table. 3.4 B concentration (mg kg^{-1} dry weight) of washed tap and fine roots in the topsoil (0-20cm) and subsoil (20-50 cm) for weed species grown at different concentrations of extractable B in subsoil.

Subsoil B (mg kg^{-1})	Barley grass		Evening Primrose			Lincoln weed			
			Tap	Fine		Tap		Fine	
	Top	Sub	Top	Top	Sub	Top	Sub	Top	Sub
0.5	4.22	4.45	22.3	29.0	18.4	24.3	25.4	34.6	32.8
2.4	4.89	7.31	53.2	31.6	32.5	47.9	37.9	38.9	48.4
4.3	4.60	7.31	48.7	32.8	38.6	53.8	59.7	40.2	44.1
6.8	5.15	5.63	77.0	51.6	44.8	145.7	52.3	61.8	49.1
12.2	5.05	6.29	159.3	53.0	52.9	67.9	-	57.9	48.9
Depth	0.481***			*NS*		33.38*		*NS*	
Boron	*NS*		24.7***	8.31***		46.97*		6.96***	
Depth·Boron	1.407*			*NS*		*NS*		*NS*	

***, *P<0.001*
*, *P<0.05*
NS; not significant

3.3.1.5 Root length density

Whilst root growth, and thus root length density, in the subsoil was significantly reduced with increased B supply in all genotypes except barley grass; the subsoil B treatments had no effect on the root length density of VB9953 and barley grass in the topsoil (Fig. 3.3). In fact, in barley grass, lincoln weed and evening primrose there appeared to be a redistribution of fine root biomass with root length density increasing in the topsoil (Fig. 3.4).

In VB9953, subsoil root length density linearly decreased (R^2 =0.91, *P<0.001*) with increasing concentration of extractable B in subsoil. Subsoil root growth for barley grass was not significantly reduced, although at the higher extractable B concentrations (6.8 and 12.2 mg kg^{-1} dry soil) there was a slight reduction in root length density (not significant). Root length density in topsoil for barley grass linearly increased (R^2 =0.84, *P=0.026*) with increasing subsoil B. Only in Clipper was there a clear reduction in root length density with increasing subsoil B in both topsoil (R^2 =0.60, *P<0.05*) and subsoil (R^2 =0.83, *P<0.001*). Even at a relatively low concentration of extractable B in the subsoil (2.4 mg kg^{-1}) root length density of Clipper in the topsoil and subsoil was 21 % and 51 % less than the control, and at the highest concentration of extractable B in subsoil (12.2 mg kg^{-1}) root length density in the subsoil had decreased by 85 %.

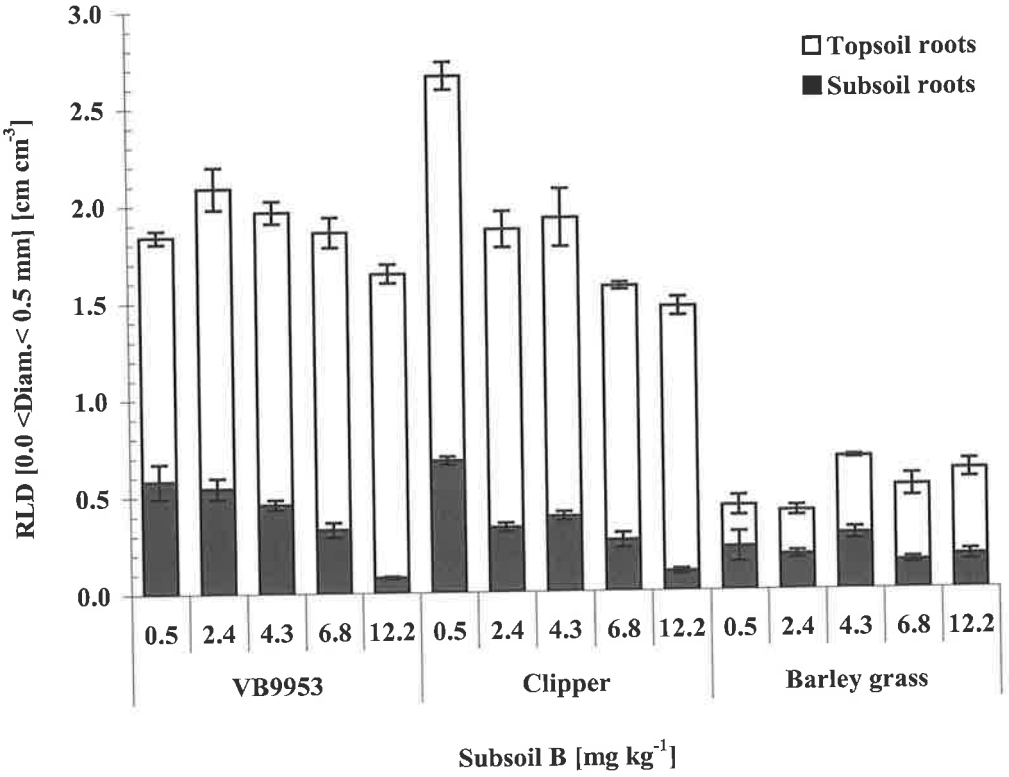


Fig. 3.3 Root length density [cm cm^{-3}] for fine roots (less than 0.5 mm diameter) of VB9953 and Clipper barley and barley grass grown under different concentrations of extractable B in subsoil. Values are mean ± standard error of three replications.

The increase in root length density for lincoln weed in the topsoil with increasing subsoil B treatment (Fig. 3.4) resulted from an increase in fine roots, whereas root length density of lincoln weed taproot (> 1 mm diam.) in the topsoil linearly decreased (R^2 =0.83, *P<0.001*) with increasing subsoil B concentration to 58 % of the control. Lincoln weed taproot length density in the subsoil also linearly declined (R^2 =0.78, *P<0.005*) with increasing subsoil B and the taproot was completely absent from the subsoil at the highest concentration of extractable B in subsoil (12.2 mg kg^{-1}) (Fig 3.4).

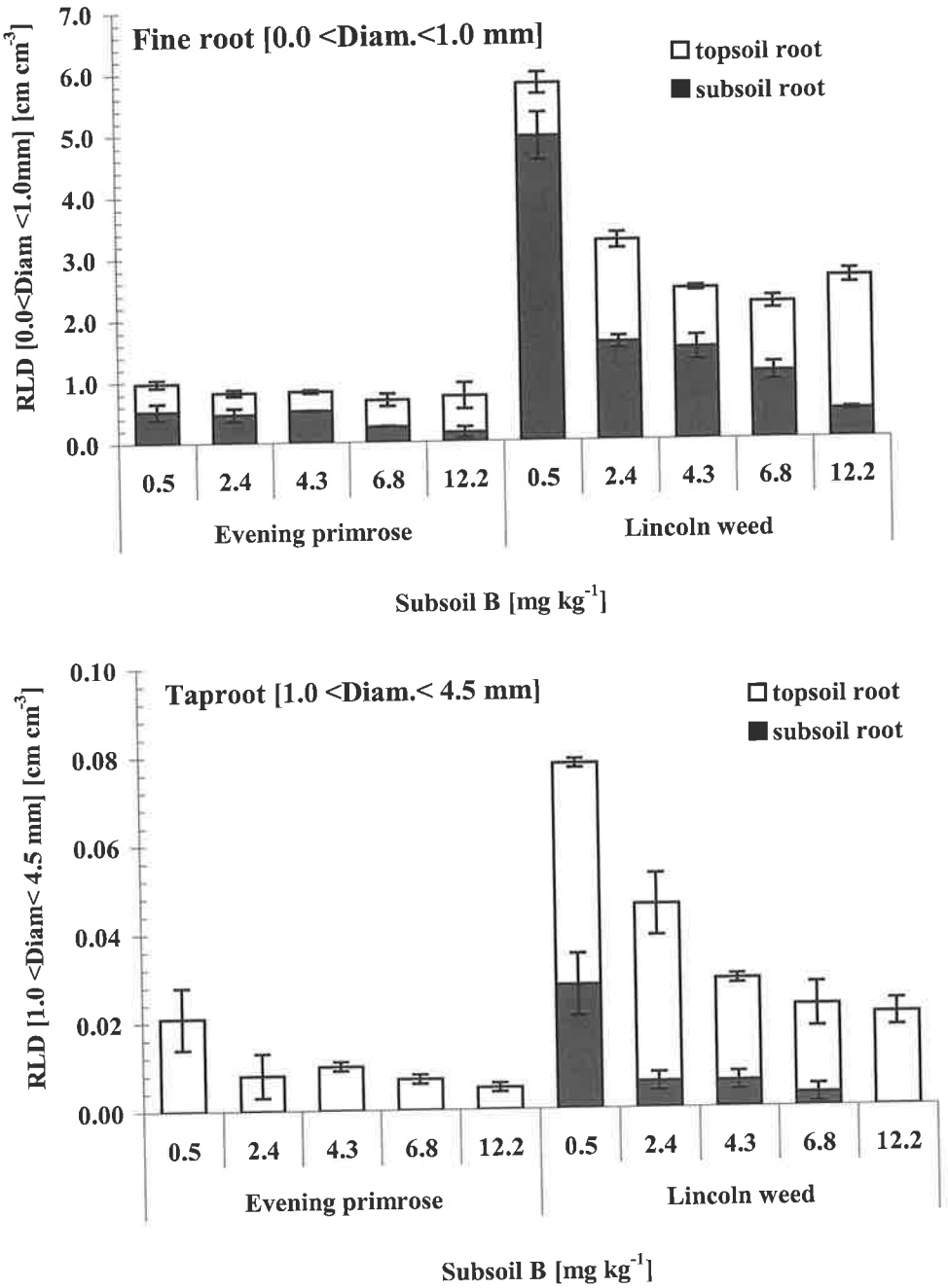


Fig. 3.4 Fine root (diam. <1.0 mm) and taproot (diam. < 4.5 mm but >1.0 mm) length density [cm cm^{-3}] for evening primrose and lincoln weed grown under different concentrations of extractable B in subsoil. Values are mean ± standard error of three replications.

Root length density for evening primrose was much less than that of lincoln weed (Table 3.5) both for taproots and fine roots (Fig 3.4). The fine roots maintained a similar root length density in the topsoil regardless of subsoil B treatment, but were significantly reduced ($P<0.05$) at the higher concentrations of extractable B in subsoil, 6.8 and 12.2 mg kg^{-1}. The taproot of evening primrose was significantly reduced in topsoil by increasing subsoil B ($P<0.001$) (Fig 3.4).

3.3.1.6 Root branching

Overall, the response of root branching (number of forks and links) to subsoil B treatment was in accordance with the responses observed for root length density. Increasing subsoil B did not significantly change the branching of VB9953 roots in the topsoil, whilst in the subsoil, at concentrations of extractable B greater than 4.3 mg kg^{-1}, root branching was reduced, and at the highest subsoil extractable B concentration (12.2 mg kg^{-1}) number of forks and links in subsoil was only 9 % of the control (Table 3.5). Root branching for Clipper was reduced significantly, not only in the subsoil but also in the topsoil (Table 3.5) with a decrease in the number of forks and links even in the lowest subsoil B treatment and a severe reduction in branching at the highest concentration of subsoil B. Root branching in the subsoil in barley grass was unaffected by subsoil B concentration, but the number of forks and links appeared to increase in the topsoil in response to increasing subsoil B concentrations (Table 3.5). The number of forks and links in topsoil did increase significantly with increasing subsoil B concentrations in both evening primrose and lincoln weed (Table 3.6), which correlates well with the observed increase in fine root density in the topsoil (Fig. 3.4).

Table 3.5 The mean number of forks and links for root systems of VB9953 and Clipper barley, and barley grass, grown under different concentrations of extractable B in subsoil.

Branching	Subsoil B (mg kg^{-1})	VB9953		Clipper		Barley grass	
		Topsoil	Subsoil	Topsoil	Subsoil	Topsoil	Subsoil
No. of Forks[z]	0.5	13384	8514	25412	11918	2425	1694
	2.4	15636	7761	21110	6353	3200	1570
	4.3	14265	6475	20662	7312	6875	3694
	6.8	15842	5198	20461	5754	5890	1447
	12.2	15537	971	22273	1709	7622	1874
Depth		1522.8***		2046.8***		*1586.5***	
Boron		*NS*		4099*		*NS*	
Depth ·Boron		*3810.9**		*NS*		*NS*	
No. of Links	0.5	30754	18714	57856	27040	6553	5350
	2.4	37005	17194	47015	13794	7823	4512
	4.3	34879	15078	46341	16038	15992	9842
	6.8	37405	11549	44547	12396	14228	3954
	12.2	37817	2330	50061	3817	17554	4712
Depth		2963.5***		4139.6***		3116.3***	
Boron		*NS*		7975.0*		*NS*	
Depth·Boron		7364.8*		*NS*		*NS*	

[z]: Number of length subtraction
***, $P<0.001$
*, $P<0.05$
NS; not significant

Table 3.6 The mean number of forks and links for root systems of evening primrose and lincoln weed grown under different extractable boron concentrations in subsoil.

Branching	Subsoil B (mg kg^{-1})	Evening Primrose		Lincoln weed	
		Topsoil	Subsoil	Topsoil	Subsoil
No. of Forks	0.5	3249	7430	11383	78966
	2.4	2742	6851	20713	33474
	4.3	2635	7178	12896	29760
	6.8	4057	1444	14624	22435
	12.2	7575	3100	28262	9030
Depth		*NS*		3911.8***	
Boron		*NS*		8291.1***	
Depth ·Boron		6674.5*		9734.6***	
No. of Links	0.5	11383	78966	25966	177514
	2.4	20713	33474	46932	73479
	4.3	12896	29760	29249	68393
	6.8	14624	22435	33065	49907
	12.2	28262	9030	64576	19607
Depth		*NS*		8140.1***	
Boron		*NS*		18002.5***	
Depth·Boron		16694.6*		20857.9***	

***, *P<0.001*
*, *P<0.05*
NS; not significant

3.3.2 Experiment 3(b) Shoot and root response of barley (Clipper), fababean and prickly lettuce to different extractable boron concentrations in subsoil

3.3.2.1 Boron toxicity symptoms

In fababean, B toxicity symptoms on the oldest leaf developed in the subsoil B treatment (12.2 mg kg^{-1}) around 30 days after sowing, followed by necrosis of a large proportion of leaf tissue, but not on the stem. Clipper exhibited the same severe symptoms observed in the previous experiment. Symptoms in prickly lettuce developed more slowly, appearing at the edges of leaves 40 days after transplanting, and progressing to a browning and drying of margins and tips for most of the fully expanded leaves.

3.3.2.2 Plant growth

Five days after B toxicity symptoms were expressed, the subsoil B treatment (12.2 mg kg^{-1}) reduced shoot dry weight of Clipper by 9 % ($P<0.05$), but did not significantly reduce the shoot dry weight, or topsoil root dry weight of fababean (Table 3.7 and Fig 3.5). However, the dry weight of Clipper roots in the subsoil (79 % $P=0.028$), and fababean (52 % $P<0.05$), were significantly lower than the controls. By day 55, the shoot dry weight of Clipper decreased significantly (44 %, $P=0.006$) in the subsoil B treatment, whilst fababean also decreased (9.5 %), but not-significantly. In addition, there was a significant reduction in topsoil root growth for both Clipper ($P<0.05$) and for fababean, but only in Clipper did we observe a significant reduction in subsoil root growth. Shoot dry weight for prickly lettuce was significantly lower in the subsoil B treatment, as was total root dry weight due to a reduction in subsoil root only (Table 3.7).

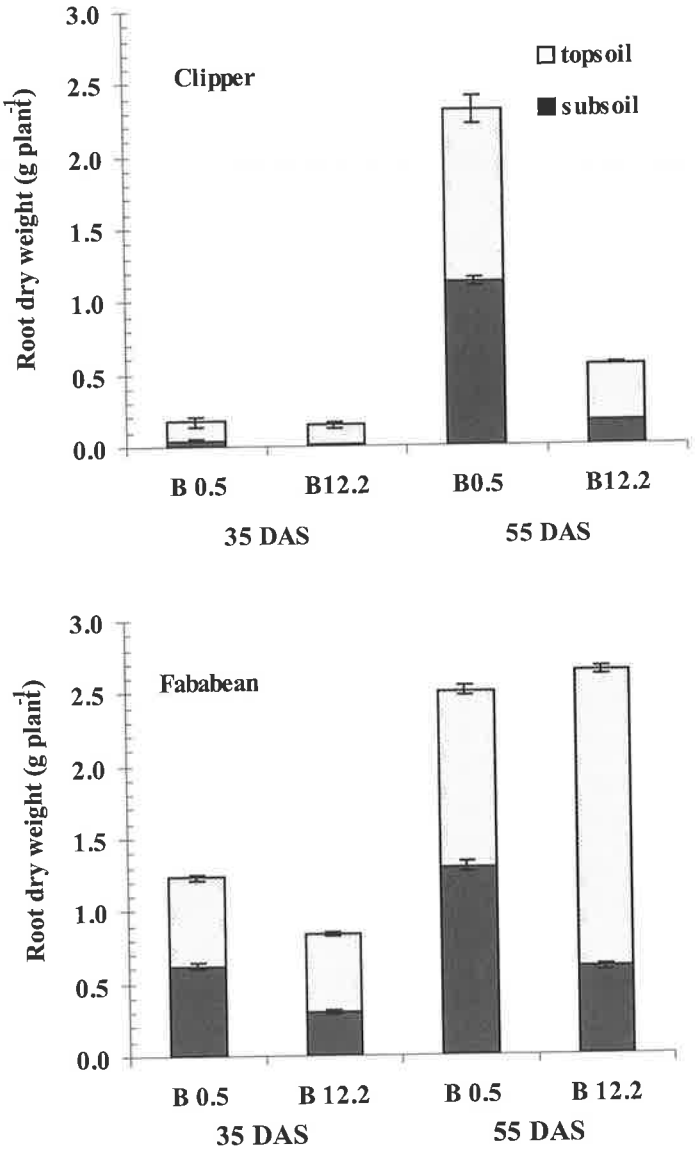


Fig. 3.5. Root dry weight in the topsoil (0-20cm) and subsoil (20-50cm) for Clipper barley and fababean 35 and 55 days after sowing (DAS) in the control (0.5 mg B subsoil kg^{-1}) and B treatment (12.2 mg B subsoil kg^{-1}). Values are mean ± standard error of 2 replications.

Table 3.7 Shoot and root dry weight (g plant^{-1}) of Clipper barley, fababean, and prickly lettuce 35, 45, and 55 days after sowing (DAS) in the control (0.5 mg B kg^{-1} subsoil; B 0.5) and subsoil treatment (12.2 mg B kg^{-1} subsoil; B 12.2). Values are mean ±standard error for two replications.

Subsoil B (mg/kg)	Dry weight (g plant^{-1})				
	Species				
	Clipper		Fababean		Prickly lettuce
	35DAS	55DAS	35DAS	55DAS	45DAS
	Shoot				
B 0.5	0.74±0.01	7.19±0.19	2.50±0.01	5.03±0.04	4.91±0.10
B 12.2	0.68±0.01	4.04±0.16	2.27±0.28	4.55±0.18	3.41±0.15
	Topsoil root (0-20 cm)				
B 0.5	0.13±0.03	1.19±0.30	0.61±0.02	1.21±0.03	0.39±0.01
B 12.2	0.14±0.02	0.39±0.01	0.54±0.01	2.04±0.03	0.40±0.01
	Subsoil root (20-50 cm)				
B 0.5	0.05±0.003	1.13±0.03	0.63±0.03	1.30±0.04	0.31±0.003
B 12.2	0.01±0.01	0.17±0.01	0.30±0.01	0.60±0.02	0.21±0.01
	Total root (0-50cm)				
B 0.5	0.17±0.03	2.31±0.27	1.24±0.01	2.51±0.07	0.70±0.01
B 12.2	0.15±0.02	0.56±0.01	0.84±0.02	2.64±0.05	0.61±0.01

3.3.2.3 Ratio of root:shoot

The subsoil B treatment resulted in a decrease in the root: shoot dry weight ratio in Clipper barley (as was measured in the previous experiment), and in fababean also (Table 3.8). In contrast prickly lettuce had a higher ratio in the B treatment, representing a greater sensitivity of shoot growth than root growth to B toxicity.

Table 3.8 Root: Shoot dry weight ratio of Clipper barley, fababean, and prickly lettuce 35 or 45 and 55 days after sowing (DAS) in the control (0.5 mg B kg^{-1} subsoil; B 0.5) and subsoil B treatment (12.2 mg B kg^{-1} subsoil; B 12.2). Values are mean ± standard error for two replications.

Subsoil B (mg/kg)	Root: shoot dry weight ratio				
	Clipper		Fababean		Prickly lettuce
	35DAS	55DAS	35DAS	55DAS	45DAS
B 0.5	0.23±0.04	0.32±0.011	0.49±0.002	0.50±0.010	0.14±0.001
B 12.2	0.22±0.03	0.14±0.007	0.37±0.036	0.58±0.011	0.18±0.007

3.3.2.4 B concentration in shoot

Compared to the species in the previous experiment (Fig 3.2) fababean and prickly lettuce accumulated relatively low concentrations of B in shoots 55 days after sowing (Table 3. 9).

Table 3.9 The shoot B concentration of fababean (55 DAS) and prickly lettuce (40 DAS) in the control (0.5 mg B kg^{-1} subsoil; B 0.5) and subsoil B treatment (12.2 mg B kg^{-1} subsoil; B 12.2).

Subsoil B ($mg \cdot kg^{-1}$)	Boron ($mg \cdot kg^{-1}$)	
	Fababean	Prickly lettuce
0.5	45	36
12.2	730	515
LSD (5%)	127.2***	76.3***

***, *P<0.001*

3.4 Discussion

These results show that an extractable B concentration of 2.4 mg B kg^{-1} in the subsoil can cause a reduction in both root and shoot growth of crop and weed species with subsequent visible toxicity symptoms (i.e. necrosis in leaf tissue). Thus, the common 'anecdotal' rule often quoted by agronomists in southern Australia, that B has little influence on crop growth and yield at extractable concentrations in soil less than 15 mg kg^{-1} (B. Holloway – pers.comm.) should, perhaps, be applied cautiously. An important implication from the data is that the influence of B on crop growth in agricultural areas of southern Australia may be more widespread than currently believed (Cartwright et al 1984, Sadras *et al.,* 2002) and relatively low concentrations of extractable B in upper regions of the soil profiles could be restricting root growth for some crops. This may apply more so where soils are becoming acidified as a result of farming practices since it has been reported that in an acidic sandy soils extractable B concentrations as low as 1 mg B kg^{-1} soil resulted in a decrease in shoot and root dry weights of the barley variety, Stirling (Riley, 1987). Indeed, from a study of 16 barley crops at sites spread across South Australia, Cartwright (1986) concluded "In some cases, even the control samples (i.e plants showing no foliar symptoms) may be considered slightly affected by the toxicity....". However, it should be acknowledged that the effects of B under field conditions are moderated by seasonal conditions that affect the concentration of B in soil solution, absorption rates of water (and boron), and patterns of root growth (Cartwright *et al.*, 1986, Holloway and Alston, 1992). Clearly, further studies are warranted to investigate crop plant root development in relation to the temporal and spatial dynamic of extractable B in the soil profile, particularly as influenced by seasonal factors such as rainfall (Yau, 2002, Sadras *et al.,* 2003). In addition, there is a need to test the response of roots to the complex of edaphic constraints that often occur in conjunction with B (Nuttall *et al.*, 2003a), and thus attempt to identify, at the farm level, where and when B may be a limitation to wheat yield (Nuttall *et al.,* 2003b).

The work reported in this chapter also provides evidence that some perennial dicotyledenous weed species, commonly found growing in southern Australia on

soils with high subsoil B concentrations, are affected by excess B. Only in lincoln weed did shoot dry weight production decrease, but growth of taproots in lincoln weed and evening primrose were severely reduced or completely impeded in soil with high B, fine roots were reduced, and necrosis of shoots was induced, albeit more slowly than in the crop species. However, it could be inferred that prickly lettuce exhibited some 'tolerance' in that the topsoil roots were unaffected by increasing concentration of subsoil B. Furthermore, in barley grass, as well as in the crop species fababean, there was actually a positive response by roots in the topsoil to increasing subsoil B, indicating a compensation mechanism potentially mediated via root-shoot signaling. Barley grass was also extremely slow-growing, accumulating very low concentrations of B in shoots and only developing minor symptoms of necrosis in shoots at the highest B concentration in the subsoil, where shoot B concentration was around 100 mg kg^{-1}. It may be that this slow growth trait, coupled with a B avoidance response of the roots, enables barley grass to survive on soils with elevated B concentrations. The hypothesis that barley grass is tolerant to B will be revisited in the next chapter (Chapter 4). Overall, the data suggest it is unlikely that the weed species tested represent a major genetic resource for engineering B tolerant crop species.

The reported work also provides comparisons between different traits used to assess tolerance to B, and in particular highlights the necessity for assessing crop/plant sensitivity to B under soil conditions similar to those encountered in the field (e.g. non-uniform B distribution in the profile (Ryan *et al.*, 1998)). If absence of toxicity symptoms were the sole indicator for B tolerance in this study then one would conclude that, of the species/genotypes tested, only barley grass was tolerant (Table 3.10). Whereas, an analysis based on shoot dry weight response indicates that evening primrose and VB9953 are also tolerant (Table 3.10). Indeed, it has been suggested (Nable, *et al.*, 1997) that symptom expression may not be the most appropriate trait for selection of B tolerance in crop species and that dry weight response may be a better indicator. Relatively recent studies have suggested using root length responses when screening for B tolerance (Jefferies *et al.*, 1999;

Chantachume *et al.*, 1995), although in this study root growth in the subsoil was reduced in response to increasing B in the subsoil in all species/genotypes regardless of 'tolerance' as indicated from other traits such as shoot dry weight. However, some of the species were clearly able to compensate for root sensitivity to subsoil B by maintaining or increasing root growth in the upper soils depths, and thus a ranking for B tolerance based on total root productivity per plant suggests that barley grass and fababean are the most tolerant (Table 3.10), with VB9953 barley next in the ranking. The marked reduction in root growth throughout the soil profile for the sensitive barley variety, Clipper, is the same response as that reported for another sensitive barley line where there was 21mg.kg^{-1} extractable B in the subsoil (Yau, 2001, 2002). Adaptive aspects of root system growth in response to spatial variability in soil B concentrations are not easily incorporated into glasshouse screening work for B tolerance (Paull *et al.*, 1990) but clearly require consideration when field testing breeding lines, although it should be remembered that the advantage of such an avoidance strategy may be less under water-limited conditions experienced in the field.

Shoot B concentration has widely been correlated with symptoms of B toxicity (for example: Grieve and Poss, 2000) and therefore could be considered as an indicator of tolerance or sensitivity to B toxicity. Many authors, including Nable (1988), Paull *et al.*, (1992), and Bellaloui and Brown (1998), observed reduced B uptake in tolerant genotypes (such as the barley variety Sahara) and suggested they all possess a similar B tolerance mechanism involving exclusion of B. However, the results of this study indicate that reduced B uptake may not be the only B tolerance mechanism in barley since high B concentrations in the shoot of VB9953 (2000 mg kg^{-1} dry weight) did not impact negatively on shoot growth, and only resulted in slight toxicity symptoms. Thus, the ranking based on shoot B concentration (Table 3.10) does not give a true indication of B tolerance for all the genotypes tested. A boron exclusion mechanism does not seem to be evident in VB9953, which appears to have the capacity to tolerate a high B concentration in the shoot, a feature commonly termed 'internal tolerance' (Stangoulis and Reid, 2002). Indeed, there are some species that are more

tolerant to high tissue B than others; for example, sunflower can tolerate B in the youngest mature leaf up to 1150 mg kg^{-1} whilst barley showed toxicity in the youngest mature leaf containing 30-100 mg kg^{-1} (Reuter *et al.,* 1997). Overall, identification of a critical concentration of B in the subsoil for expression of B toxicity was not easy due to the wide range of response observed for different traits and across the species and genotypes. The research reported in this chapter also highlighted the difficulty for assessing B concentration of roots grown in soil since the process of obtaining clean roots involves washing with relatively large volumes of water that cause soluble B to be leached from the root cells (R. Reid- pers.comm.). However, it was interesting that the B concentration for taproots of evening primrose and lincoln weed was still relatively high after washing, suggesting B had accumulated in the cell walls of these root system components. This is understandable given that two species are dicots and therefore have a higher B requirement.

It is not known with certainty why B is toxic to plants (Stangoulis and Reid, 2002) although the phenotypic responses are diverse, as observed in this study, and range from impaired root growth to chlorosis and/or necrosis in leaf tissue and reduced shoot growth. On the basis of measurements showing an increase of sucrose contents in the leaf tissue and decreased glucose in the roots in response to excess B (Picchioni *et al.,* 1991), it was suggested that a direct consequence of high leaf B concentrations in pistachio seedlings is alteration in carbohydrate status of the roots, and ultimately a reduction in root growth. They quoted an earlier study (McNairn and Currier, 1965) suggesting excessive callose accumulation in the leaf tissue with excess B causing impaired export of leaf sugars to root. However, in the study (Picchioni *et al*, 1991), the content of sucrose, the main carbon energy exported to root, was not significantly decreased in the root, although starch levels increased. In this chapter, a significant reduction of subsoil root caused by which subsoil B was observed before any serious effect on the shoot (Experiment 3 (b)) although topsoil root mostly maintained growth. Thus it should not only consider indirect influence of B on root growth, but also a direct effect of B on roots, especially root tips, should

not be ruled out. High B ceases miotic activity in fababean (*Vicia* faba L.) and in pea (*Pisum sativum* L. cv. Alaska) root tips over a 24 hr period (Liu, *et al.*, 2000; Klein and Brown, 1981). Further work is necessary to observe the effects of B toxicity on ultra-structural responses of the root meristematic zone (see Chapter 5), and the carbohydrate status in root growing, at the site of phloem unloading of sucrose (see Chapter 6).

In summary, the work reported by this chapter has important implications for several groups of people working in the area of B tolerance/toxicity and should influence:

- Agronomists assessing the impact of B toxicity under field conditions,
- Breeders screening for B tolerance and,
- Plant physiologists investigating the mechanisms of B tolerance.

Table 3.10 Relative order of B tolerance for crop and weed species/genotypes exposed to 12.2 mg ext. B kg^{-1} ranked using either shoot toxicity symptoms, shoot dry weight (DM), shoot B concentration, total root DM, subsoil root DM or root:shoot ratio.

Scale	Toxicity Symptom	Shoot DM	Shoot B conc.[Y]	Root total DM	Subsoil root DM	Ratio root: shoot
Tolerant	barley grass	barley grass	barley grass	barley grass		barley grass
		VB9953		fababean		fababean
				VB9953		prickly lettuce
Partly tolerant	evening primrose	evening primrose	prickly lettuce	prickly lettuce	prickly lettuce	VB9953
	prickly lettuce	fababean	evening primrose	evening primrose	barley grass	
	fababean	prickly lettuce	fababean		fababean	
	VB9953					
Sensitive	Clipper	lincoln weed	VB9953	lincoln weed	evening primrose	Clipper
	lincoln weed	Clipper	lincoln weed	Clipper	VB9953	evening primrose
			Clipper[Z]		Clipper	lincoln weed
					lincoln weed	

[Z]: Based on data from chapter 6

[Y]: tolerance refer to low shoot B concentration

CHAPTER 4

Genotypic Variation in Boron Uptake Under High Boron Supply

4.1 Introduction

Given the high permeability of B through biological membranes (3 x 10^{-7} cm s^{-1} (Dordas *et al.*, 2000); 4.4 x 10^{-7} cm s^{-1} (Stangoulis *et al.*, 2001)), B can move passively through cellular membranes, its uptake being driven by changes in a plants water potential gradient that results in its accumulation at the end of the transpiration stream (Oertli and Ahmed, 1970). In reality, B uptake in plant roots is a more complicated process as new insights into this process have recently revealed (Dannel *et al.*, 1999; Dannel *et al.*, 2000; Dordas and Brown, 2001; Dordas *et al.*, 2000; Pfeffer *et al.*, 1999; Stangoulis *et al.*, 2001). B uptake consists of two components, the first operating at low B supply where B uptake is either facilitated or actively concentrated in the symplast against the concentration gradient, while the other involves the passive permeation of B across plant membranes when B supply is high.

Under B-toxic conditions, excluding B through active efflux or through the generation of ligands able to complex and "detoxify" the excess B, would be energetically expensive to the plant, however, there are numerous reports demonstrating a reduced B concentration in B-tolerant plants (Nable, 1988; Nable *et al.*, 1997; Paull *et al.* 1992). While there have been recent efforts to understand the reasons for these differences, the mechanism, or mechanisms, are still not clear (Stangoulis and Reid (2002) and references therein). Evidence suggests that the major B-tolerance mechanism is associated with B-exclusion from the root and hence the shoot, although there are genotypes' that are able to withstand higher levels of B in leaf tissue (e.g. sunflower (Blamey *et al.*, 1997; Dannel *et al.*, 1999)). The aim of this study was to investigate the characteristics of B uptake in two varieties of barley

and barley grass that are known to differ in their B-tolerance. In particular, the distribution of B in both B-tolerant and B-intolerant varieties, and whether a barrier to B-uptake may exist at xylem loading.

4.2 Materials and Methods

4.2.1 Experiment 4(a) Evaluating the sensitivity of barley and barley grass to boron toxicity

4.2.1.1 Experimental design

This experiment was modeled on a factorial design, with three genotypes' (two barley (*Hordeum vulgare*) varieties (Sahara and Clipper) and barley grass (*Hordeum glaucum*)), and six B treatments (15, 200, 500, 1000, 2000, and 5000 μM B). The experiment was replicated three times.

4.2.1.2 Seed preparation and planting

Seeds were germinated on moist filter paper in glass petri-dishes with 4 ml of distilled water. The seeds of the barley grass were stored in a dark growth cabinet at 5°C for 3 days and then a further 2 days at 20°C. The barley varieties, Clipper and Sahara, were germinated at 20 °C for 2 days in a dark growth cabinet. Germinated seed were selected for uniformity and transplanted into seedling trays with aerated nutrient solution for 3 days, and then transferred to PVC containers filled with 2 L of aerated nutrient solution.

4.2.1.3 Nutrient solution and growth conditions

The plants were grown in aerated quarter-strength Hoagland's solution that consisted of the following nutrients: Ca $(NO_3)_2$, 1.25 mM; KNO_3, 1.25 mM; $MgSO_4$, 0.5 mM; KH_2PO_4, 0.25 mM; $MnSO_4$ 5.9 μM; ZnSO4, 8.0 μM; CuSO4, 0.01 μM; NaMoO4, 0.01 μM; KCl, 50mM; FeEDTA, 20 μM; $B(OH)_3$, 15 μM. The solution was adjusted to pH 6.0 with KOH and HCl every 2 days once the plants were in the growth solution. B treatments were applied at rates of 15, 200, 500, 1000, 2000, and 5000

μM B as boric acid. The rates of B were chosen from previously published work by Nable *et al.* (1988). Plants were grown in a growth chamber at 20/10 °C day/night temperature and 14/12 h day/night photoperiod. The nutrient solution was changed with the fresh solution every week. At d 7 and d 14, the two barley varieties were harvested, while the barley grass was harvested at d 14 and d 28. Harvested plants were quickly rinsed in high purity water (18 MΩ cm^{-1} resistance) for 10 s, quickly blotted dry with tissue paper, and plant parts separated into root and shoot. The length of primary roots were measured and then oven dried at 80°C for 24 h. Samples were weighed and digested at 140°C in 70 % (w/w) HNO_3, then analysed for essential elements (except N) by inductively coupled plasma optical emission spectrometry, using an ARL 3580 B ICP analyzer.

4.2.1.4 Calculation of boron uptake rate and plant relative growth rate

The B uptake rates per unit of root dry weight (μg B g^{-1} root DW day^{-1}) were calculated according to Williams (1948).

$$\text{B uptake rate} = [(m_1-m_0)/(DWr_1-DWr_0)] \times [(\ln DWr_1-\ln DW\ r_0)/(t_1-t_0)]$$

Where m_1 and m_0 are the total B content and DW r_1 and DW r_0 are the root dry weights at day t_1 and t_0, respectively.

Plant relative growth rate (g g^{-1} day^{-1}) was calculated according to the formula:
Plant relative growth rate (RGR) = (ln(DWn)-ln(DWn-1))/T

Where DWn = the average dry weight of whole plants at harvest (n), DWn-1 = the average dry weight of plants at the preceding harvest (n-1), and T = time (days) between the two harvests.

4.2.1.5 Statistical analyses

The data were subjected to analysis of variance using the Genstat 5 statistical package. Significant mean separation is indicated by the use of the least significant difference (LSD) at the 5% level where the F value is significant.

4.2.2 Experiment 4(b) Barriers to boron uptake: Boron uptake into root, xylem, and shoot

4.2.2.1 Experimental design

This experiment was modeled on a factorial design, with two barley varieties (Sahara and Clipper) and two B treatments (15 and 5000 μM B). The experiment was replicated three times.

4.2.2.2 Seed preparation and planting

Details regarding seed preparation and planting are as reported in section 4.2.1.2.

4.2.2.3 Nutrient solution and growth conditions

Nutrient solution and growth conditions were as reported in section 4.2.1.3 with the following exceptions. B treatments were applied at rates of 15 μM B and 5000 μM B. The rates of B were chosen from results taken from experiment 4 (a), with the highest B concentration, 5000 μM B, visually affecting root growth of the sensitive variety. Both nutrient solutions were changed weekly, and after 3 weeks, changed two times per week.

4.2.2.4 Xylem sap collection and harvesting of plant material

Sahara and Clipper were grown for seven weeks in 15 μM B solution. After seven weeks, xylem sap was collected in light-on conditions from 4 plants over a 1.5 h period before the high B (5000 μM B) treatment was applied (classified H1). Xylem sap was collected by cutting the shoots about 1cm from the solution surface with root pressure sap immediately collected on exudation to minimize drying out of the sap and subsequent concentrating of the nutrients. Exudates were kept on ice in Eppendorf® tubes.

Root and shoot were also harvested for analysis of tissue concentration by ICP-OES with analytical procedures as reported in section 4.2.1.3. Plants (3) that were not harvested in H1 were transferred to nutrient solutions containing 5000 μM (toxic) B

for 4 days, and then a second sampling for xylem sap and tissue analysis was conducted (classified H2) with the same procedure as outlined for H1. After H2, 3 remaining unharvested plants were transferred to fresh nutrient solutions containing 5000 μM B for a further 4 d, and subsequently the third sampling of sap, root, and shoot was conducted. This last harvest was designated, H3. Root material harvested in all of the three harvests were rinsed for 10 s in high purity water, and patted dry on clean paper towel. After measuring shoot and root fresh weights, samples were dried at 80 °C prior to B analysis using ICP-OES.

4.3 Results

4.3.1 Experiment 4(a) Evaluating the sensitivity of barley and barley grass to boron toxicity

4.3.1.1 Plant Growth

Both barley and barley grass responded differently to high B supply with no significant effect of high B treatment on the variety Sahara, while Clipper appeared more affected (Fig. 4.1). Barley grass showed more a decline in root dry weight than in shoot growth, which also indicated, along with the results observed in Clipper, that root growth is more sensitive than shoot growth under high B supply.

At the highest concentration B supply (5000 μM) shoot dry weight was reduced by 28 % and 44 % of adequate B treatment for barley grass and Clipper respectively, while in Sahara, no reduction was observed. In contrast, root growth was reduced by 63 % in barley grass and 53 % in Clipper.

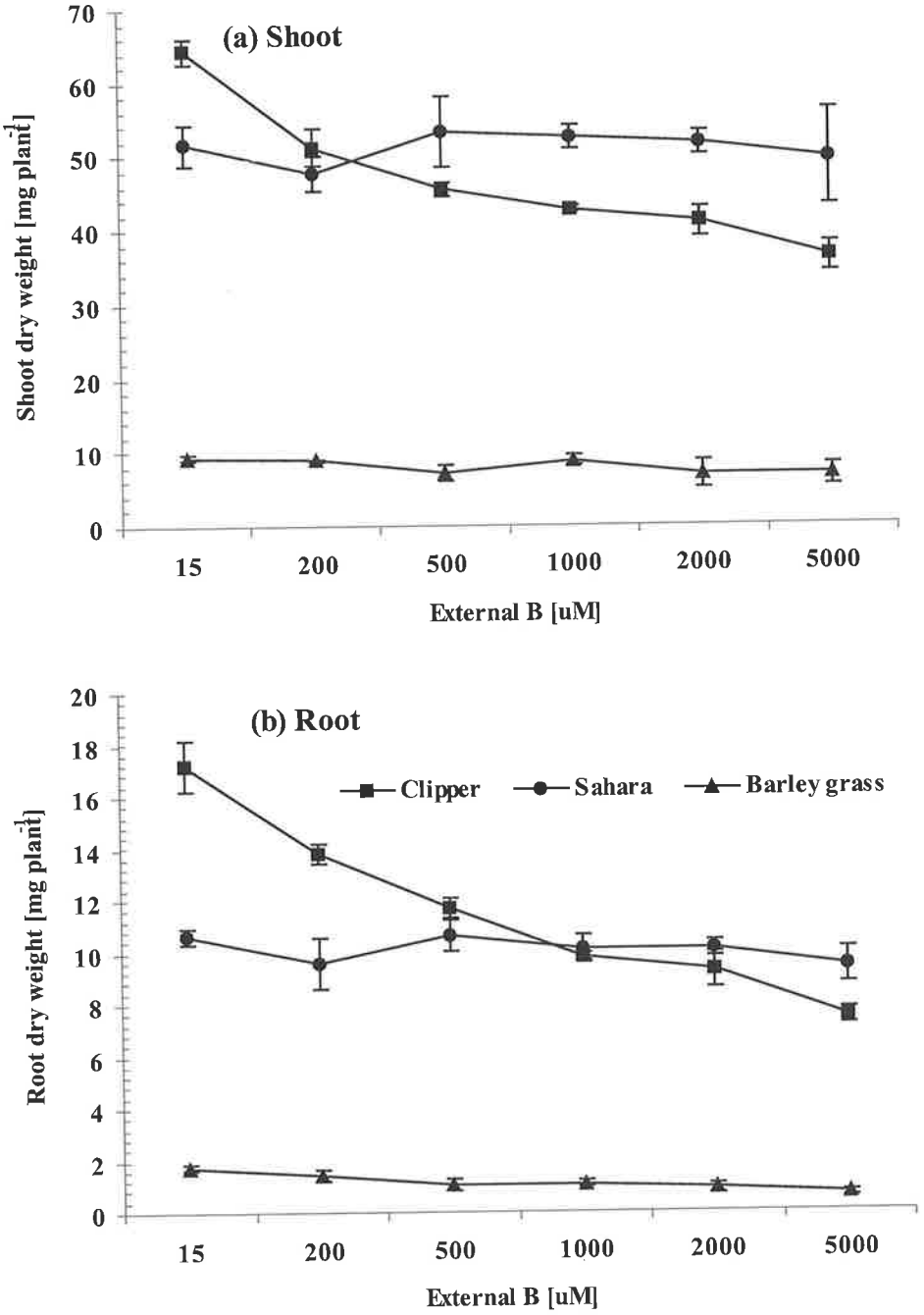


Fig. 4.1. The effect of B (15, 200, 500, 1000, 2000, and 5000 μM B) on (a) shoot and (b) root dry weight of two barley varieties, Clipper and Sahara and barley grass grown for 14 days in nutrient solution. Bars represent the mean of 3 plants ± SE. LSD (*P<0.001*): Variety (shoot: 2.7 and root: 0.5), Boron (shoot: 3.8 and root: 0.8), and Variety x Boron (shoot: 6.6 and root: 1.3).

4.3.1.2 Boron concentrations in shoot and root tissue

Boron concentrations in both the shoot (Fig 4.2 a) and root (Fig 4.2 b) increased with external B supply and varied between genotypes. Shoot B concentration was in the order Clipper > barley grass > Sahara with a 3.4 fold higher B concentration in Clipper than in Sahara at 5000 μM B, while barley grass was 2.5 fold higher than Sahara. Root B concentration was in the order barley grass > Clipper > Sahara with a 2 fold higher B concentration in barley grass when compared to Sahara and a 1.2 fold higher B concentration in Clipper over Sahara in the 5000 μM B treatment. Results highlight the sensitivity of both barley grass and Clipper to B toxicity, as well as the tolerance of the variety Sahara. The very high B concentration in the root of barley grass at 5000 μM B supply may indicate a barrier to B uptake from root to shoot. However when one considers the B content (% of total plant B) of the root and shoot, there appears to be little evidence for restrictive translocation as shoot B content increases while root B content decreases in barley grass (Table 4.1).

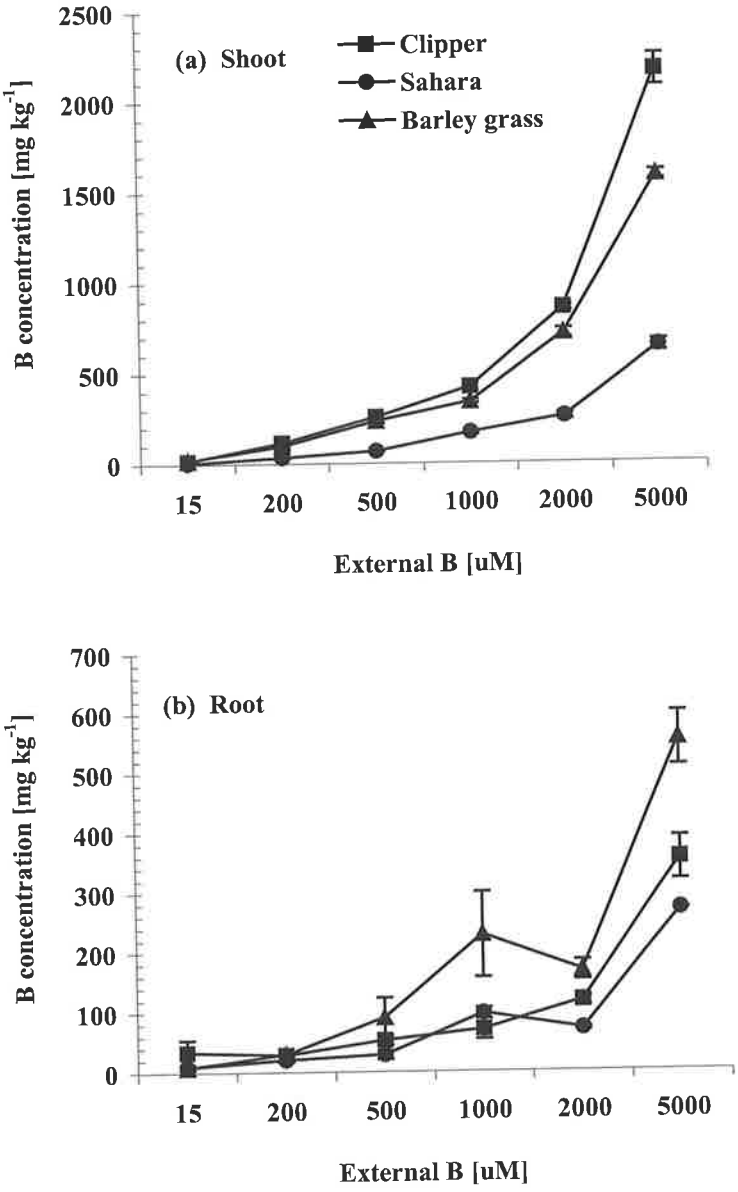


Fig. 4.2 The effect of B (15, 200, 500, 1000, 2000, and 5000 μM B) on (a) shoot and (b) root B concentration in two barley varieties, Clipper and Sahara and barley grass grown for 14 days in nutrient solution. Bars represent the mean of 3 plants ± SE. LSD (*P<0.001*): Variety (shoot: 33 and root: 26), Boron (shoot: 47 and root: 36), and Variety x Boron (shoot: 81 and root: 63).

Table 4.1. B content (% of total plant B) of root and shoot tissue of two barley varieties Clipper and Sahara and barley grass grown for 14 days at adequate (15 μM B) and high (5000 μM B) B supply.

B Conc. (μM)	Clipper		Sahara		Barley grass	
	Root	Shoot	Root	Shoot	Root	Shoot
15	29.7 ± 8.7	70.3 ± 8.7	20.5 ± 6.9	79.7 ± 6.9	7.1 ± 0.8	92.9 ± 0.8
5000	3.2 ± 0.3	96.8 ± 0.3	8.5 ± 1.0	91.5 ± 1.0	3.6 ± 0.1	96.4 ± 0.1

4.3.1.3 Boron uptake rate and relative growth rate (RGR)

The rate of B uptake was linear over the B treatment range for the two barley varieties (Sahara, r^2= 0.98; Clipper, r^2=0.98) and barley grass (r^2= 0.82) (Fig 4.3) indicating passive uptake of B within the B concentrations used in the growth medium. The B-intolerant variety, Clipper, had a greater B uptake rate when compared to Sahara and barley grass. The sensitivity of both Clipper barley and the barley grass was observed in their relative growth rates as they were reduced by the toxicity in the growth medium, although in barley grass not until the highest external B concentration when growth was suddenly severely reduced (Fig. 4.4). The very high B concentration in the root of barley grass, at 5000 μM compared to Clipper (Fig 4.2 (b)) may be due to the severely impeded relative growth rate coupled with a slower uptake rate at the highest B concentration. It is doubtful that the tolerance in barley grass, as shown in the soil based pot experiment (Chapter 3), is associated with a restricted translocation mechanism. In contrast, while B-tolerant variety Sahara had the lowest B uptake rate, it showed no apparent reduction in growth rate.

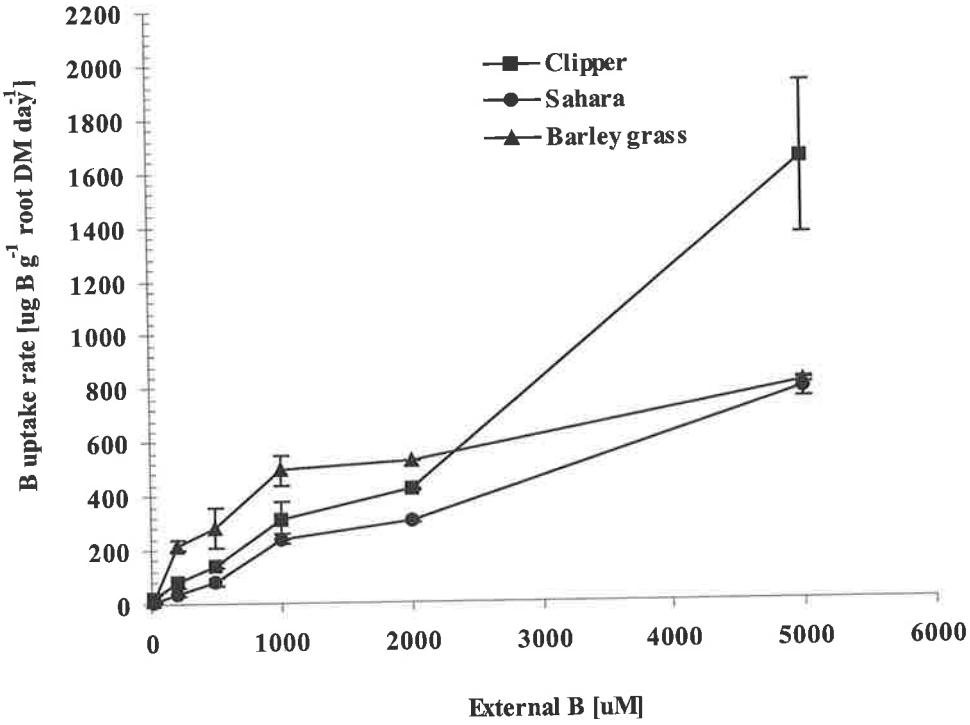


Fig. 4.3. The effect of B (15, 200, 500, 1000, 2000, and 5000 μM B) on B uptake rate in two barley varieties Clipper and Sahara, and barley grass. For Sahara and Clipper, the uptake period was measured between d 7 and d 14, while for barley grass it was between d 14 and d 28 due to very slow growth. Bars represent the mean B uptake rate for 3 plants ± SE. LSD (*P<.001*); Variety (106), Boron (150), and Variety x Boron (261).

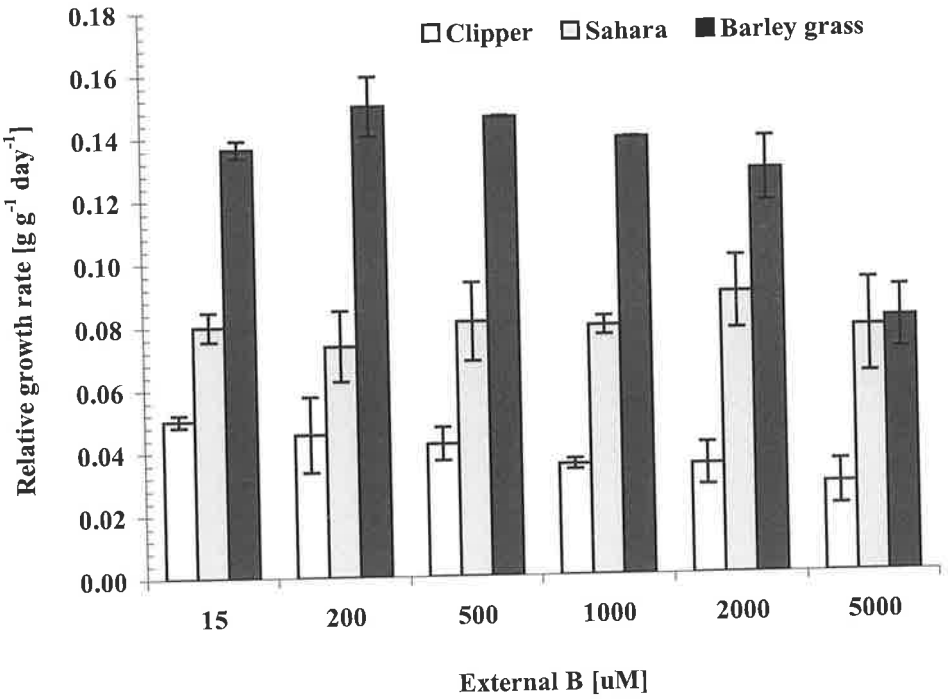


Fig. 4.4. The effect of B (15, 200, 500, 1000, 2000, and 5000 μM B) on relative growth rate between d 7 to d 14 for two barley varieties Sahara and Clipper, and between d 14 to d 28 for barley grass. Bars represent the mean RGR for three plants ± SE. LSD (*P<0.05*), Variety (0.01), Boron (0.01), and Variety x Boron (0.02).

4.3.2 Experiment 4(b) Barriers to boron uptake: Boron uptake into root, xylem, and shoot

4.3.2.1 Boron concentration in leaf, root, and xylem sap

The B concentration in both barley varieties increased within the first 4 d after application of high B supply with the B-tolerant Sahara having a significantly lower B concentration in the shoot and a higher concentration in the root when compared to the B-intolerant Clipper (Fig 4.5 a and b). By d 8 (H3), varietal differences in shoot B concentration increased with a much lower B concentration in the shoot of Sahara. By contrast with H1 results at H3, the B concentration in the root of Sahara was lower when compared to Clipper.

Four days after plants were transferred to the high B treatment (5 mM), the increase in B concentration within the xylem sap was much greater in Clipper (4.7 ± 0.35 mM) compared to Sahara (2.7 ± 0.25 mM), whereas differences in fresh root B concentration were not significant (4.5 ± 0.43 mM and 5.1 ± 0.6 mM for Clipper and Sahara, respectively). Importantly, the B concentration of the xylem sap in Sahara (Fig 4.6 a) was significantly lower than the B concentration in the whole fresh root (by 47 %), suggesting a barrier to B uptake at the xylem loading.

Eight days after imposing the high B treatment, the B concentration in the xylem sap greatly decreased for Sahara and slightly less so for Clipper (Fig. 4.6). A restriction of B uptake occurs in both Clipper and Sahara with the barrier not as evident in the former. The apparent rise in the xylem B concentration 4 days after imposing the high B treatment, and subsequent decline from days 4 to 8 indicate that B exclusion from the root is not immediate and constitutive. In Clipper, the root B concentration equilibrated with the external medium B concentration, without a subsequent decline over the 8 d treatment period that was observed in Sahara. However, the mechanism of excluding B from the xylem is present in Clipper, as evidenced in a 44 % reduction in xylem B concentration between the second (H2) and third (H3) harvests.

The changes in B uptake into the root and xylem were also observed in the B uptake rate within the two varieties as the rate of B uptake declined in both varieties between the two harvest times, although the variety Clipper had a higher B uptake compared to Sahara (Fig. 4.7).

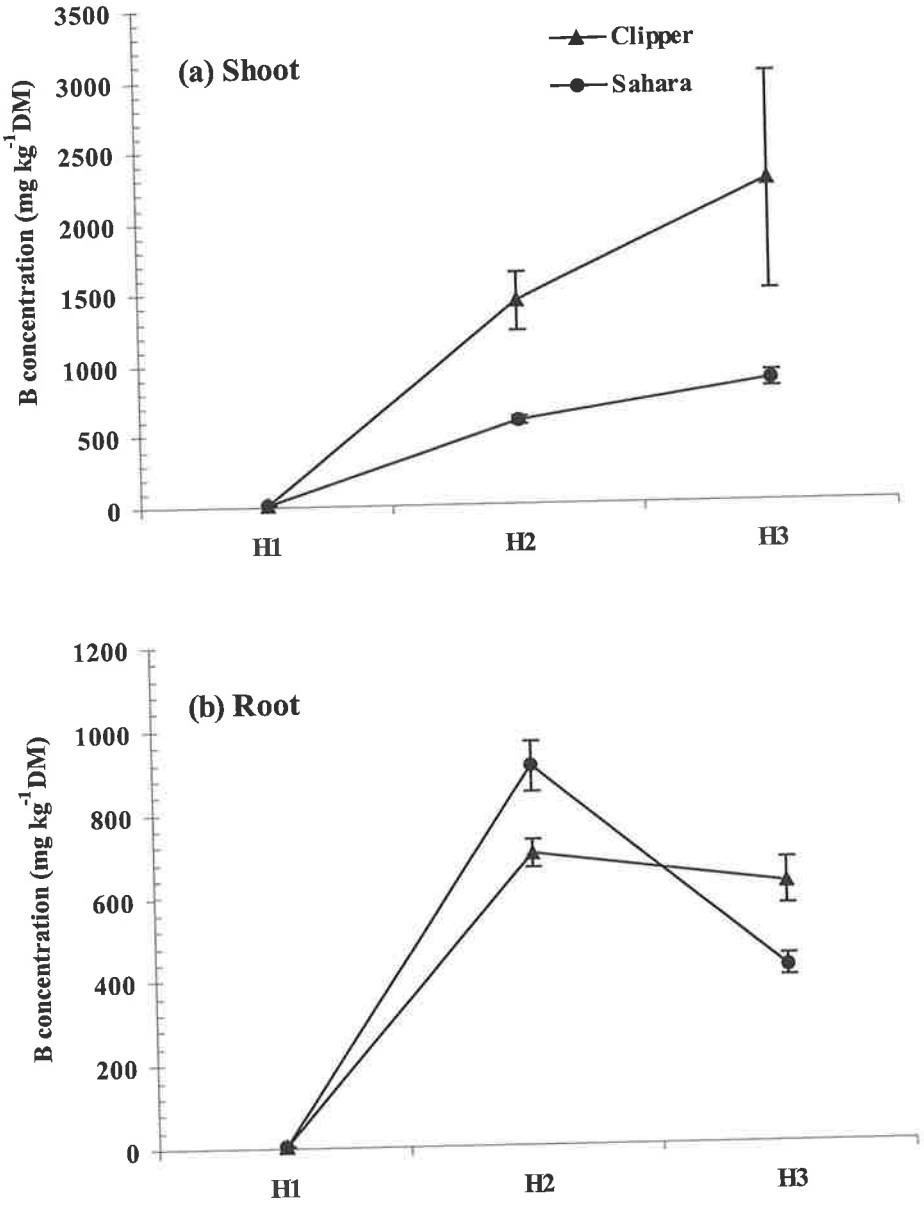


Fig. 4.5. The effect of high B (5000 μM B) supply on the (a) shoot and (b) root B concentration in Clipper and Sahara barley. Plants were grown for 7 weeks in ¼ strength Hoagland's solution prior to the application of the high B treatment. Three plants were harvested prior to high B application (H1) with subsequent harvests at d 4 (H2) and d 8 (H3). Bars represent the mean B concentration of 3 plants ± SE. LSD (*P<0.001*), Boron (shoot: 249 and root: 51.6), Variety × Boron (shoot: 352 and root: 73).

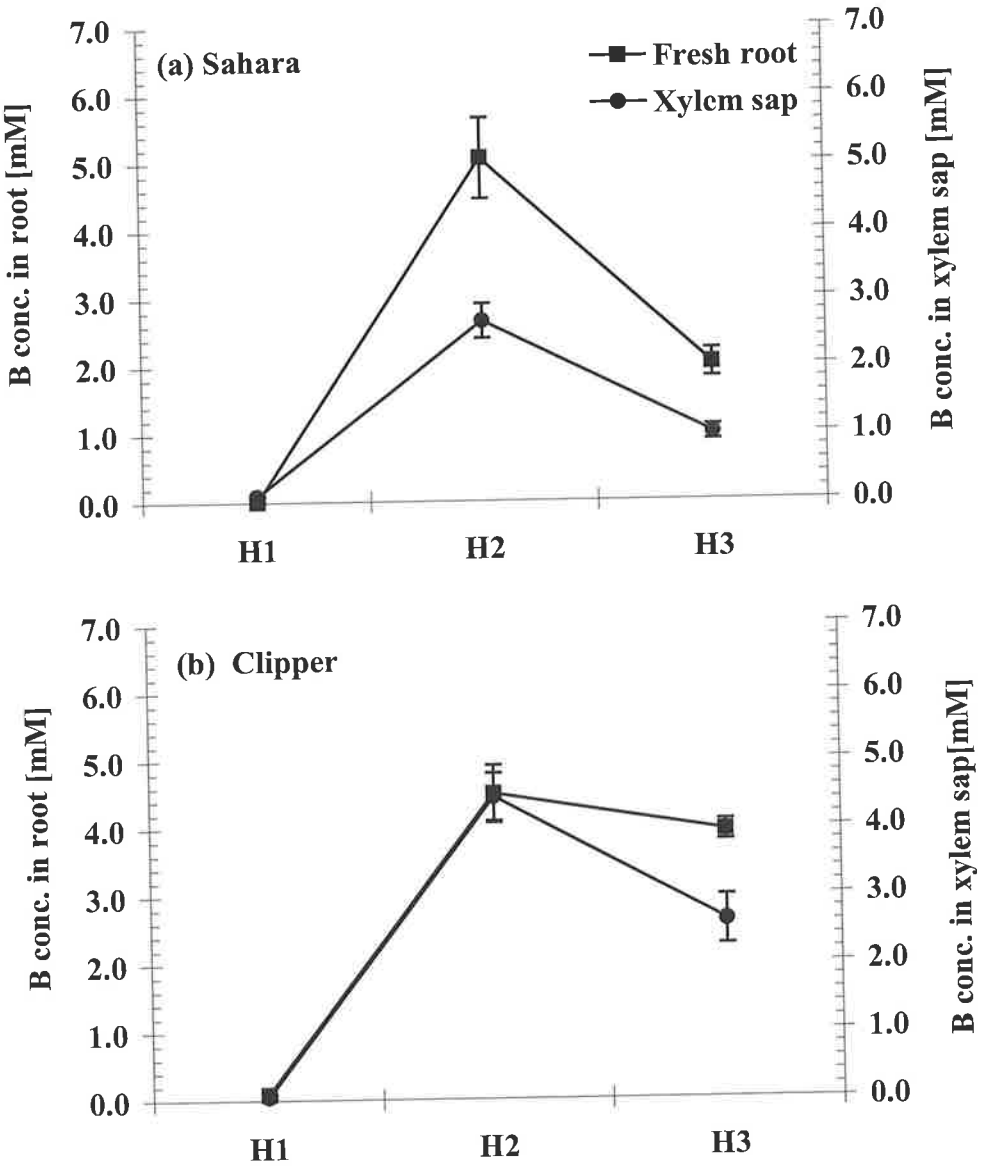


Fig. 4.6. The effect of B (5 mM) on xylem sap and fresh root B concentrations in two barley varieties, (a) Sahara and (b) Clipper. B concentrations (mM) in fresh roots were calculated from [μg B g^{-1} root fw] divided by [10.8 = Boron atomic weight]. Plants were grown for 7 weeks in ¼ strength Hoagland's solution prior to the application of the high B treatment. Plants harvested prior to application of high B (H1) with subsequent harvests at d 4 (H2) and d 8 (H3). Bars represent the mean B concentration of three plants ± SE. LSD (*P<0.001*) Boron (xylem sap: 0.3 and fresh root: 0.5) and Variety x Boron (xylem sap: 0.4 and fresh root: 0.7).

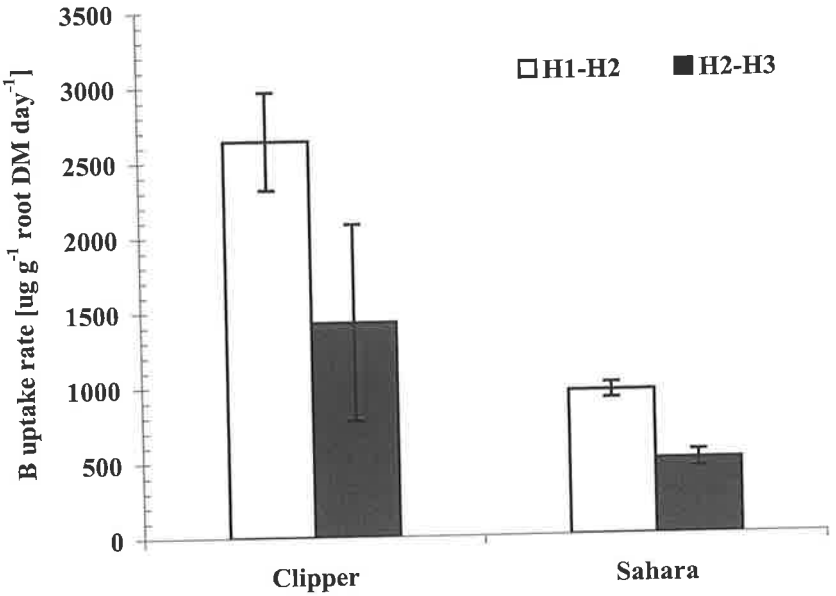


Fig. 4.7. The effect of high B (5000 μM B) supply on the rate of B uptake in two barley genotypes, Clipper and Sahara. Plants were grown for 7 weeks in ¼ strength Hoagland's solution prior to the application of the high B treatment. Plants were harvested prior to high B application (H1) with subsequent harvests at day 4 (H2) and day 8 (H3). Bars represent the mean B uptake rate of three plants ± SE.

4.4 Discussion

Results from this study concur with those from the literature (Nable, 1988; Nable *et al.,* 1990b; Nable and Paull, 1991) that the barley variety Sahara is more B-tolerant than Clipper, and the B-tolerance mechanism is associated with a reduced B-uptake. Whilst Clipper was affected by B-toxicity and this was manifest in both reduced root and shoot dry weight, there were no obvious adverse effects of B toxicity on root and shoot growth in Sahara over the range of B treatments imposed.

There is little reported information concerning the susceptibility of barley grass to B-toxicity, although anecdotal evidence suggests a level of B-tolerance when grown in the field on B-toxic soil (Dr Glenn McDonald, personal communications). Data presented in this chapter show a reduction in the dry weight of barley grass at both low and higher levels of B supply, with susceptibility to B-toxicity very evident since there was a 50 % reduction in root dry weight when plants were exposed to high B supply. This result contrasts with observation for barley grass grown in a soil profile where only the sub-soil was B-toxic (cf. Chapter 3), and it was identified as B-tolerant. The most likely reason for this observed variation in B-tolerance is that barley grass is unable to avoid toxic levels of B in a solution culture environment. Whereas, a gradient toxic B is present in a soil environment (i.e. more toxic B deeper down in the soil profile), it clearly redistributes its root biomass to the top-soil where the levels of B are not as toxic, and where it can continue to maintain growth and reproduction. The B-tolerance of field grown barley grass may therefore be more an avoidance strategy and not an exclusion mechanism as found in Sahara.

Results support passive B uptake at high B supply (Fig. 4.3) which is in agreement with more recent reports of passive B uptake across biological membranes at high B supply (Stangoulis *et al.,* 2001; Dordas and Brown, 2001; Dordas *et al.,* 2000; Pfeffer *et al.,* 1999). Yet it is clearly obvious that the B-tolerant Sahara has a mechanism by which it reduces the B concentration over time; first manifest in a reduced xylem B concentration, and then within the rest of the root. Interestingly, the B-intolerant variety, Clipper, also has a mechanism to reduce the B concentration within the

xylem when challenged by toxic B, yet its mechanism is not sufficiently developed as in Sahara. The results clearly highlight the region of the root in which the B-tolerance mechanism is initiated, and that is at xylem loading, thereby preventing B translocation from root to shoot. These results are also supported by Pfeffer *et al.* (1999), who reported a reduced uptake of B into the xylem and root cell sap when young sunflower plants (*Helianthus Annuus* cv. Frankasol) were supplied with sufficient levels of boron. With time, the B concentration in the xylem and root cell sap was lower than the B concentration in the external growth medium. The authors propose that roots receive a signal from various organs (e.g. in the shoot) that B demand is adequate and therefore a reduced B uptake is required. This hypothesis may also be used to explain the results observed in this Chapter. In addition, Dannel *et al.* (1998) reported a reduced B uptake into the xylem when plants were grown with high (400 to 1600 μM) B that resulted in a 50-60 % lower B concentration in the xylem when compared to the root cell sap.

There are a number of mechanisms that may be involved in the B-tolerance mechanism and these include: 1. active or facilitated efflux of excess B from root tissue 2. the formation of a borate ligand and its subsequent efflux from the root 3. a change in the membrane permeability to boric acid thereby reducing B uptake, and 4. a physical barrier to B transport in the root either through suberin or lignin deposition in the cell wall. For mechanism 1, results presented in this Chapter suggest that B uptake is a passive process, which is in keeping with our understanding of the permeability of B across plant membranes (Dordas and Brown, 2001; Dordas *et al.,* 2000) and the kinetics of B uptake (Dannel, *et al.,* 2001). Basically, under such conditions, one may consider active efflux to be energetically expensive to the plant as the turnover rates of channels in active transport pumps are approximately 10^2 per second, whereas those of channel mediated transport pumps are 10^6 to 10^8 molecules per second (Buchanan *et al.,* 2000). While realizing that this is energetically expensive to the plant, the theory should still be tested as the same logic was used to disclaim facilitated or active uptake at low B supply, which is now known occur (Stangoulis *et al.,* 2001; Takano, *et al.,* 2002). Secondly, the formation of a borate

ligand and its subsequent removal from the root is plausible (as seen in Al tolerance (Ma, 2000)) although from personal communications with Drs' James Stangoulis and Robert Reid who looked into this mechanism, they could not find any increase in organic acid or sugar release from the root of the B-tolcrant genotype Sahara when grown in B-toxic solution. This mechanism can probably be discounted, as an increase in the B concentration of the root tissue of the tolerant variety would be observed whereas the opposite actually occurred. Thirdly, there is recent evidence to support changes in the permeability of boric acid across plant membranes as a result of changes in membrane composition (Dordas and Brown, 2001) where *Arabidopsis thaliana* mutants that differed in membrane composition also differed in their B uptake. In the mutant, *chs-1-1* that had a lower proportion of sterols, B uptake was reduced by 30 % when compared to the wild type. The *act-1-1* mutant exhibited a 35 % lower uptake than the wild type. It is possible that the effects witnessed in this study are also due to changes in the membrane composition of the plasma-membrane thereby influencing B-uptake. This hypothesis requires further testing. Finally, the fourth hypothesis is related to the deposition of suberin and or lignin that may act to reduce the permeability of boric acid in both the appoplast and symplast of the root cortical cells and in the xylem parenchyma. Recent evidence suggests that under B toxicity, both suberin and lignin increase in the cell walls (Ghanati, *et al.,* 2002). In the normal process of adcrustation, suberin is deposited in cork cells that replace the root epidermis (Lauchli, 1976). Suberized walls may also contain waxes in alternating layers (Sitte, 1962). The lamellar structure of suberized cell walls is of significance as this reduces the permeability through the cell wall and causes an isotropic diffusion resistance. Lignin also affects apoplasmic transport as adcrustration of cell walls leads to a restriction on diffusion and solutes (Lauchli, 1976). The reduced uptake of B in Sahara may therefore be due to the induction of suberin or increased lignin in the cell wall thereby reducing the permeability of boric acid and its uptake.

In summary, the barley variety Sahara in mixed solution culture is more B-tolerant than Clipper and barley grass. The B-tolerance mechanism in Sahara is associated with a reduced B-uptake and its capacity to reduce B is distinctively developed at the site of xylem loading, indicating a barrier between the root cortex and xylem. The barrier may be due to the induction of suberin or increased lignin in the cell wall, or to an active efflux mechanism.

CHAPTER 5

Effects of Toxic Boron on Root Morphology and Cellular Structure in the Root Meristematic Zone

5.1 Introduction

It was observed in Chapter 4 that the B-tolerant Sahara has the capacity to reduce B uptake into the root as well as into the xylem. A mechanism for reduced B uptake into the xylem was also expressed in the B-sensitive Clipper, but not total root exclusion, and was therefore insufficient to limit B translocation from root to shoot via the xylem.

A reduced B concentration in the root of the B-tolerant genotype may prevent the toxicity effect on root growth that is generally accompanied with shorter root axes and reduced lateral roots (Huang and Graham, 1990). It has been postulated that inhibition of root elongation under B deficiency might be due to a cessation of cell division (Cohen and Lepper, 1977), yet the phenotypic effects of B-toxicity on root morphology remain elusive. However, there is some support for the effect of B toxicity on root morphology being due to B interfering with cell division in the root tip. For example, high B (10 mM in solution culture) inhibited mitosis in the root meristematic zone of broad bean (*Vicia* faba L.) altering chromosome fragments, chromosome stickiness and occurrence of micronuclei (Liu, *et al.* 2000). Borate at 10 mM caused cessation of mitotic activity in pea (*Pisum sativum* L. cv. Alaska) root-tips over 24 hr periods (Klein and Brown, 1981). Although these results indicate adverse effects of toxic B on DNA synthesis and mitosis during cell division, the actual mode of action is still not fully clear. Few studies of the effect of B toxicity on root morphology and cell division have been undertaken. Therefore the aim of this study from morphological studies is to determine the effects of B toxicity on

morphological changes in the root meristematic zone and whether genotypic variation in these changes may help to explain the B-toxicity of B tolerance mechanism.

5.2 Materials and Methods

5.2.1 Experiment 5(a) Effect of boron toxicity on root morphology

This was a factorial experiment with two barley (*Hordeum vulgare*) varieties, Sahara (B-tolerant) and Clipper (B-sensitive) and barley grass (*Hordeum glaucum*). Adequate (15 μM B (B 15)) and toxic (5000 μM B (B 5000)) B supply was imposed when the seedlings were 2 days old. The experiment was replicated four times. Information on seed germination is given in Chapter 4, section 4.2.1.2. Nutrient solution and growth conditions were identical to those in Chapter 4, section 4.2.1.3., except for the two B treatments (see above).

5.2.1.1 Measurement of root morphology

Plants were harvested four, eight and 12 days after the B treatments were imposed, and shoots were separated from roots. The length of the primary roots was measured manually. Additionally, root imaging was carried out using a flatbed scanning technique (Richner *et al.,* 2000), reported in chapter 3. Individual primary roots of each plant were placed in a shallow transparent tray (20 × 30 cm) filled with 2 to 3 mm of culture solution in which they had been grown to facilitate separation of roots and to enable correct imaging. Images were analyzed for root diameter and surface area of the root tip (0-10 mm from root apex) in individual primary roots, using a commercially available image analysis software package (WinRhizo ®). Data is presented as the mean value of all individual primary roots in each treatment.

5.2.1.2 Shoot and root dry weights

Shoot material and root material (following morphological measurement) was dried at 65 °C and weighed.

5.2.2 Experiment 5 (b) Effect of boron toxicity on cell structure in the root meristematic zone

5.2.2.1 Staining roots

To investigate cell structure in the root meristematic zone, four days after B treatment were imposed, a segment of root was cut 10 mm from the root apex of four plants grown at adequate (15 μM B) or high (5000 μM B) B supply. The segments were stained with aniline blue using a method modified from Kaneko *et al.* (1999). Root tips were washed in deionized water, boiled in 95 % (v/v) ethanol for 1h, soaked in water for 5 min, and stained with 0.05 % (w/v) aniline blue (water soluble, BDH Limited Poole England) solution in 0.15 M K_2HPO_4 (pH 11.0) for 2h at room temperature and overnight at 5°C. The stained samples were washed in deionized water for 5 minutes before observation with confocal microscope.

5.2.2.2 Measurement of root meristematic zone and cell size

Stained root tips were observed with confocal microscopy (MRC 1000 UV; Bio-Rad) using a ×10 lens and an argon laser with an excitation filter of 488 nm and an emission filter of 527 nm. The same settings for confocal aperture, laser power, camera gain, and camera black level were used for examination of all roots. The length and width of the root meristematic zone was measured using confocal images 1733×1155 μm in size. To calculate the total area of meristematic zone, image pixel number in the aniline-blue labelled zone was measured, and then the value multiplied by pixel area (2.26 × 2.26 μm = 5.1076 μm^2). The distal end of the meristematic zone was estimated to occur at a cell length of 2.5 times the length of the shortest cell (Lenoble, *et al.,* 1996). Electronically zoomed-in images of 866×578 μm size for Sahara and Clipper, and of 578×385 μm for barley grass were taken to measure the cells in the meristematic zone using image analysis software Volume J (version 1.28, NIH). Measurements were made for 10 adjacent cells for each meristematic zone and repeated for 10 roots per treatment. The cell area was calculated by multiplication of cell length and width. Cell number along the meristematic zone was calculated using the length of the meristematic zone divided by the average length of a single cell.

Cell number across the meristematic zone was calculated from the width of the meristematic zone divided by the average width of a single cell.

5.3 Results

5.3.1 Experiment 5(a) Effect of boron toxicity on root morphology

5.3.1.1 Root and shoot growth

The root systems of representative plants grown for 4 or 8 days at adequate or high B are shown (Fig 5.1 a and b). For plants grown at adequate B supply, the mean length of the primary root was in the order Clipper > barley grass > Sahara (Fig 5.2). However, four days after B treatment were imposed, the mean primary root length of Clipper and barley grass was reduced in the high B treatment by 8 % and 22 %, respectively, while the root length of Sahara increased two fold when compared to the adequate treatment. Mean length of the individual primary roots at day 12 in the high B treatment decreased relative to the adequate treatment by 41 % for Clipper and 35 % for barley grass. Root elongation did not completely cease for either genotype, but continued at a slower rate than in the control. The length of the main root in Sahara at day 12 was longer than in the adequate treatment; there was a 58 % increase in root length in the adequate treatment between days 4 to 12, but a 73 % increase at high B supply. The emergence of lateral roots in all plants in the high B treatment was inhibited due to B toxicity. In addition, although there is no quantitative data, it could be seen that lateral root emergence of Sahara occurred further from the root apex at high B supply (Fig 5.1 a, b).

B toxicity caused severe inhibition of shoot growth in Clipper four days after initiation of the B treatment, but this was not observed in Sahara (Fig. 5.3). There was no significant decrease of shoot dry weight in barley grass within 12 days of treatment, despite a significant decrease in root dry weight (observed in Chapter 4, Fig 4.1 (b)).

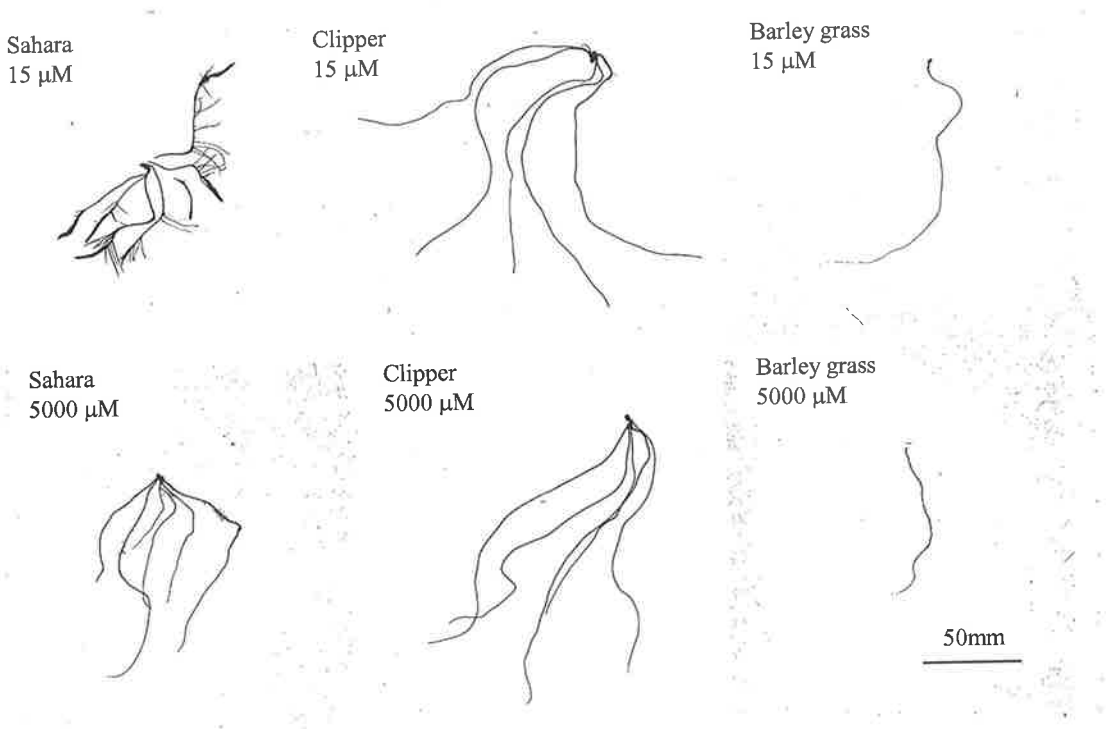


Fig 5.1 a. Root systems of representative plants grown for four days at adequate (15 μM) or high (5000 μM) B supply.

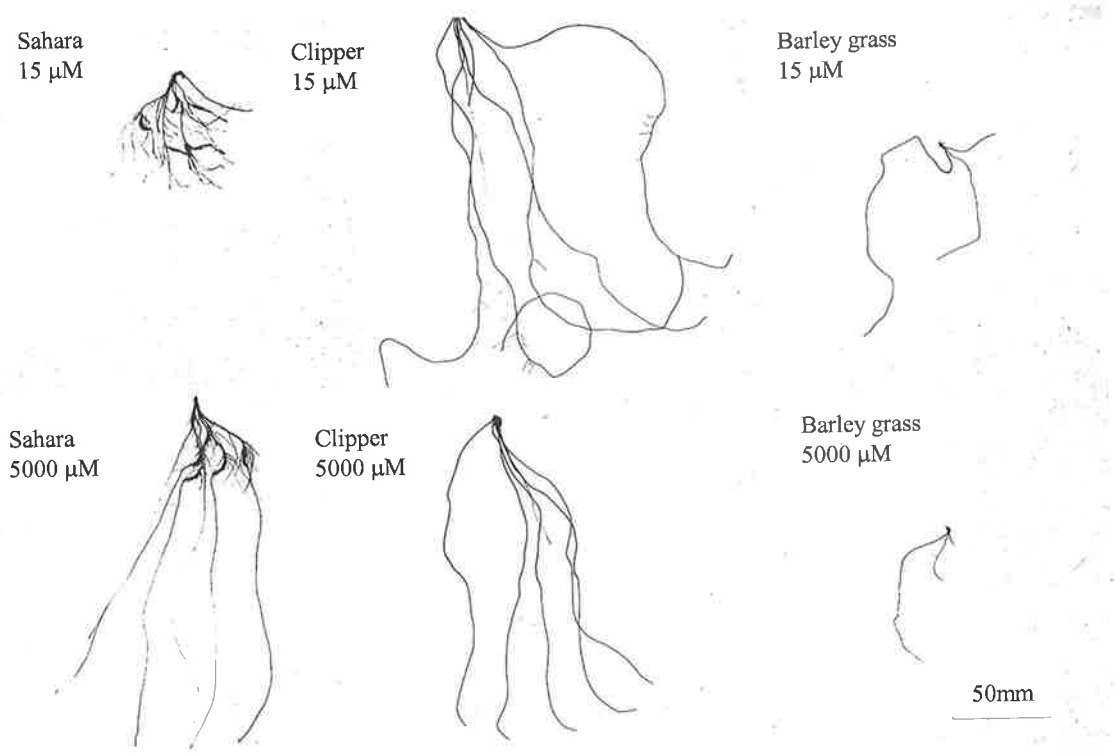


Fig 5.1 b. Root systems of representative plants grown for 12 days at adequate (15 μM) or high (5000 μM) B supply.

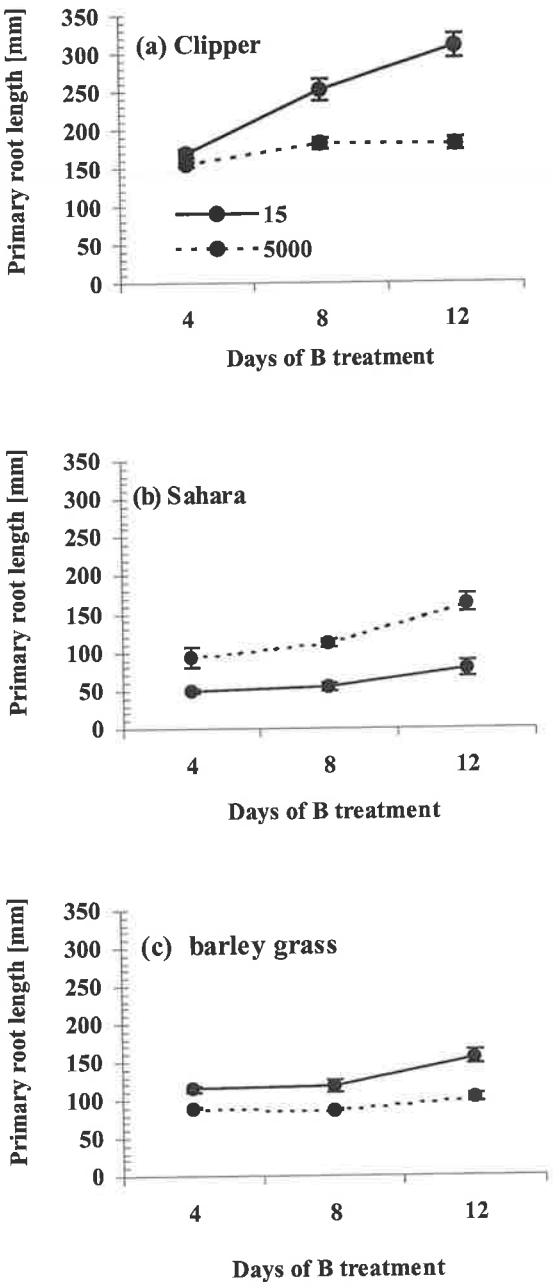


Fig. 5.2. Primary root length for two barley varieties, (a) Clipper and (b) Sahara, and (c) barley grass grown for 4, 8, and 12 days at adequate (15 μM B; straight line) or high (5000 μM B; doted line) boron supply. Values represent mean ± standard error for 10 individual primary roots.

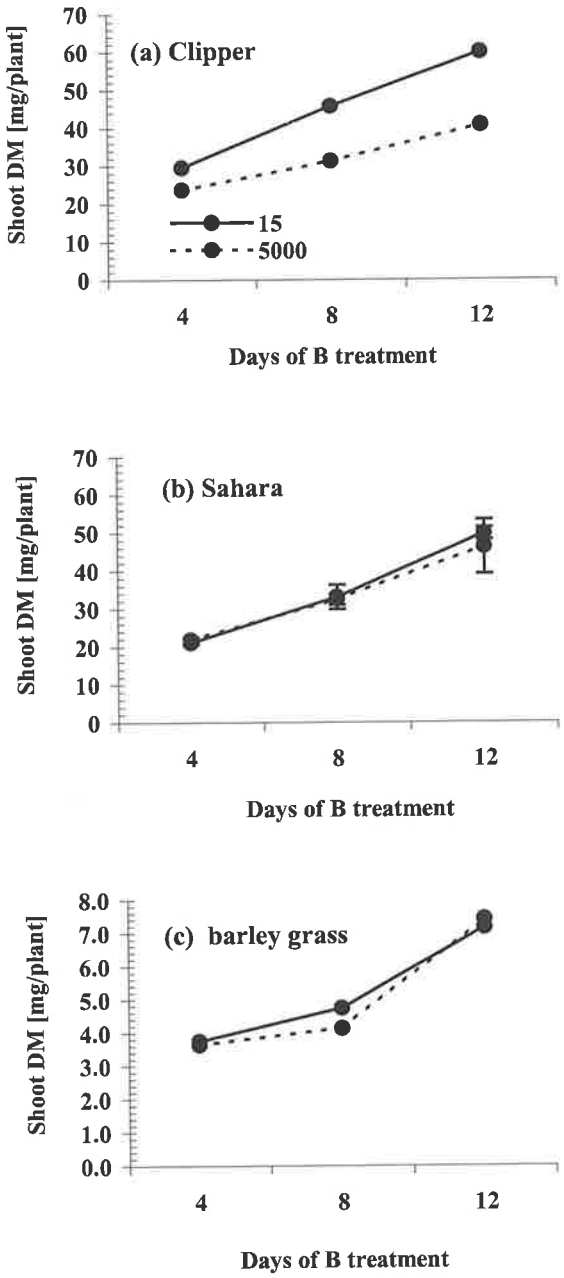


Fig. 5.3 Shoot dry weight for two barley varieties, (a) Clipper and (b) Sahara, and (c) barley grass grown for 4, 8, and 12 days at adequate (15 μM B) or high (5000 μM B) boron supply. Values represent mean ± standard error for four plants.

5.3.1.2 Diameter and surface area

Sahara grown at adequate B supply had much thicker root tips (0.53 mm ± 0.06) due to root hairs (Fig 5.1 a), than those of Clipper (0.30 mm ± 0.005) and barley grass (0.17 mm ± 0.010) 4 days after the B treatments were initiated. However, this difference between the two barley varieties had disappeared by day 8 of treatment (Fig 5.1 a; Fig 5.4 and 5.5). B toxicity significantly reduced the surface area and diameter of the root tips in Sahara and Clipper, but not in barley grass (Fig 5.4 and 5.5). High B affected both the tolerant and sensitive barley variety, although a reduction in surface area and diameter of root tips was more apparent in Sahara than in Clipper 4 days after the B treatments were imposed. There was no difference in root tip diameter and surface area between Sahara and Clipper treated with the high B treatment.

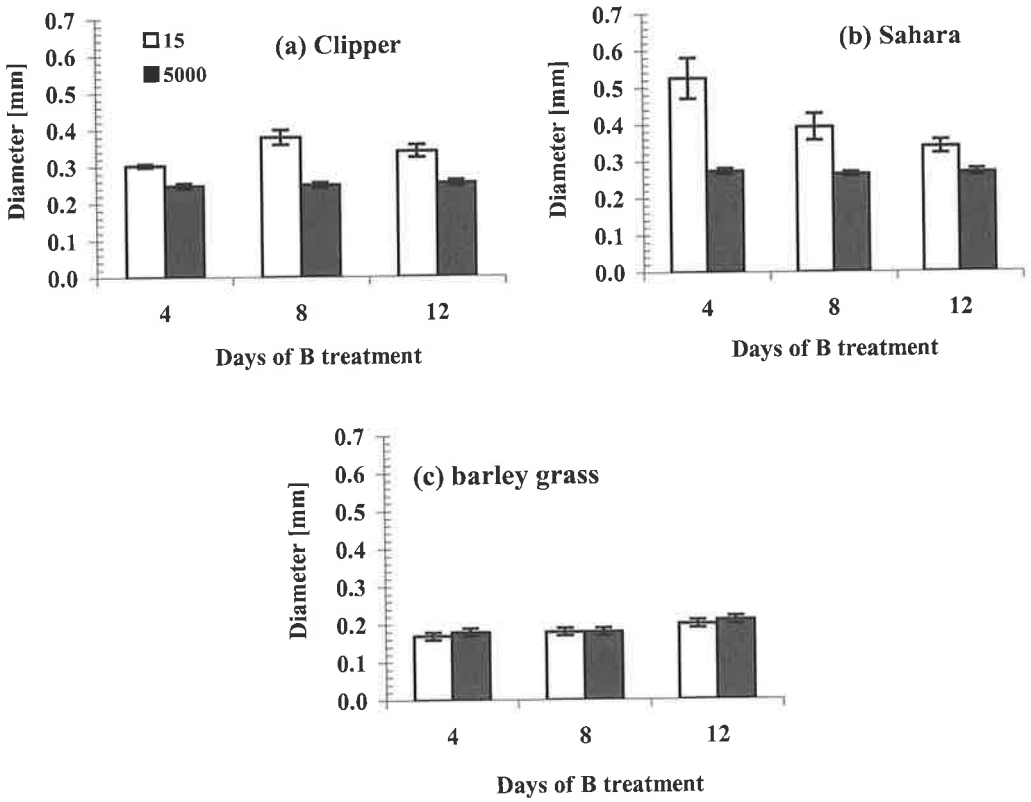


Fig 5.4 Diameter of root tips (0-10 mm) for two barley varieties, (a) Clipper and (b) Sahara, and (c) barley grass grown for 4, 8, and 12 days at adequate 15 μM B or high 5000 μM B supply. Values represent mean ± standard error for 10 individual primary roots.

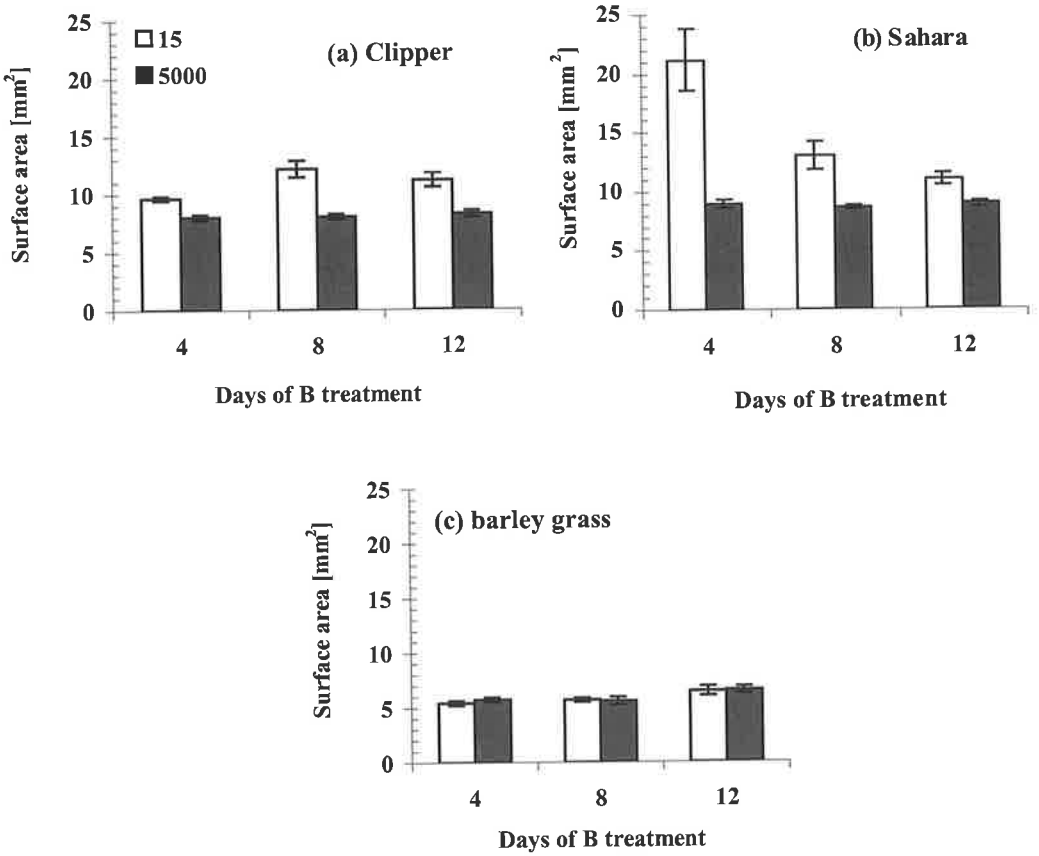


Fig 5.5 Surface area of root tips (0-10 mm) for two barley varieties, (a) Clipper and (b) Sahara, and (c) barley grass grown for 4, 8, and 12 days at adequate 15 μM B or high 5000 μM B supply. Values represent mean ± standard error for 10 individual primary roots.

5.3.2 Experiment 5(b) Effect of boron toxicity on cell structure in the root meristematic zone

5.3.2.1 Length and width of the root meristematic zone

Four days after commencing the treatment at either adequate or high B supply, the length and width of the root meristematic zone differed between genotypes (Fig 5.6). In plants grown at adequate B supply, the average length of the root meristematic zone was in the order Clipper (636 μm) > Sahara (557 μm) > barley grass (365 μm). High B concentration increased the length of the meristematic zone by 15 % in Sahara compared to the control. By contrast, Clipper and barley grass reduced their root meristematic zone length by 20 % and 49 % respectively compared to the adequate B treatment. At high B supply, the length of the root meristematic zone was in the order Sahara (638 μm) > Clipper (506 μm) > barley grass (186 μm). Clipper and barley grass had smaller mean root length at high B supply compared to the adequate B treatment, while Sahara had greater mean root length than the adequate B treatment. There was no significant difference in width of the root meristematic zone between adequate and high B treatment in Sahara and Clipper, whilst there was a 22 % reduction in the root meristematic zone width of barley grass compared to the adequate B treatment.

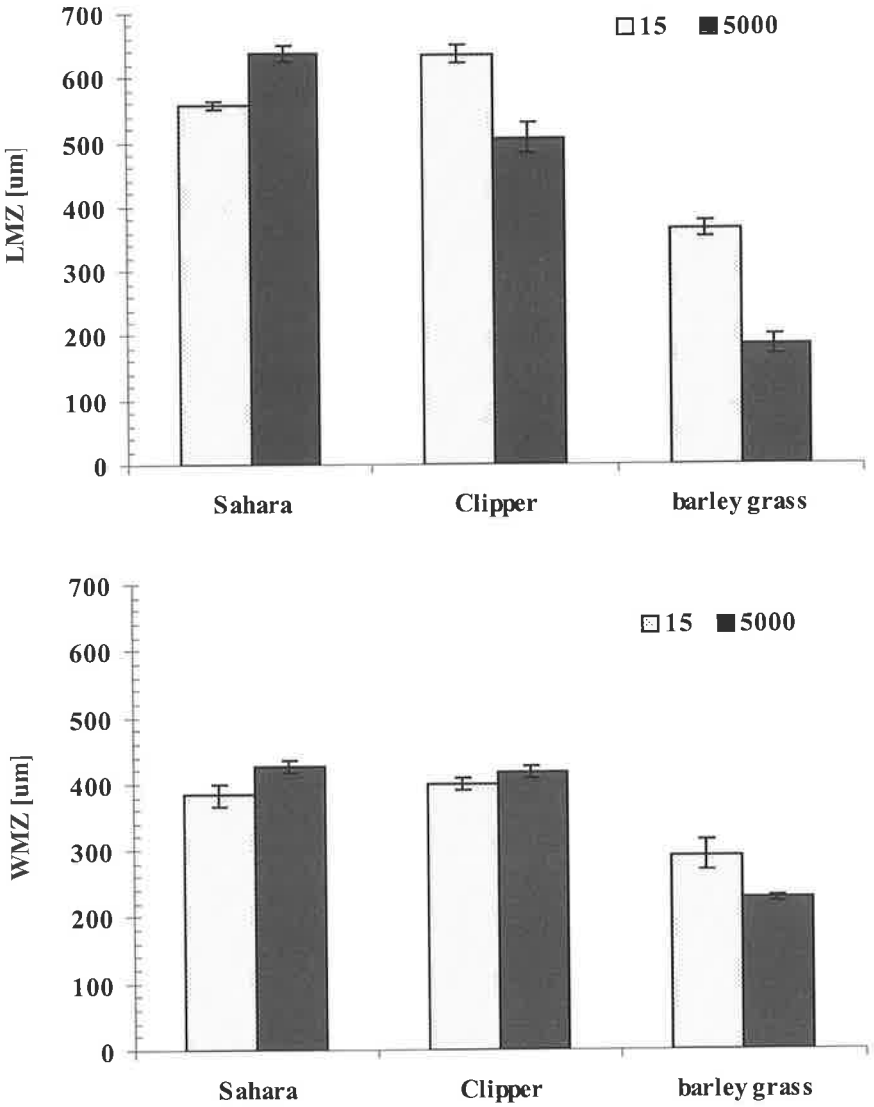


Fig. 5.6. Length of meristematic zone (LMZ, [μm]) and width of meristematic zone (WMZ, [μm]) for root of two barley varieties, Clipper and Sahara, and barley grass grown for four days at adequate (15 μM) or high (5000 μM) B supply. Values represent mean ± standard error for 10 root tips. LSD (*P<.001*) for LMZ: variety 30.57, boron 25.07, and variety × boron 43.42. LSD (*P<.001*) for WMZ: variety 16.92 and variety × boron 24.03.

5.3.2.2 Cell structure in the root meristematic zone

The length, width, area, and number of cells in the root meristematic zone were measured after four days of adequate and high B treatment (Fig. 5.8). The number of cells in the meristematic zone for the high B treatment was reduced in Clipper, drastically reduced in barley grass, but increased in Sahara (Table 5.1). Mean cell length in the adequate B treatment for Sahara and Clipper was similar, and larger than in barley grass. Clipper and barley grass had larger cell length at high B supply when compared to the adequate treatment, while in Sahara, the cell length was not changed by B treatment (Table 5.2). Toxic B concentration increased the cell length by 11 % for Clipper and 120 % for barley grass compared to the adequate treatment. Increased cell width due to B toxicity occurred in Sahara and Clipper, but not in barley grass, which was consistent with differences in the width of the meristematic zone. No significant genotypical variation in cell area within the root meristematic zone was observed for Clipper (99.2 μm^2), Sahara (105 μm^2), and barley grass (100 μm^2) grown at adequate B supply (Fig 5.7). However, B toxicity increased the cell area in Clipper (135 μm^2) and barley grass (221 μm^2), while in Sahara there was no significant change.

Table 5.1 Cell number in the meristematic zone roots in plants grown for four days at adequate (15 μM) or high (5000 μM) B supply. Values represent mean ± standard error for each replication.

B conc. [μM]	Cell numbers along meristematic zone*		
	Clipper (n=100)	Sahara # (n=40)	Barley grass (n=100)
15	66.10[a] (±2.73)	60.31[a] (± 2.65)	41.01[a] (±3.85)
5000	49.22[b] (±2.95)	70.16[b] (±2.57)	9.93[b] (±0.80)
	Cell numbers across meristematic zone*		
15	37.19[a] (±1.51)	36.11[a] (±2.62)	24.47[a] (±2.37)
5000	33.24[b] (±1.24)	36.94[a] (±1.13)	20.24[b] (±0.94)

n: number of replicates
#: non-hairy roots measured
*: measured in longitudinal optical section
a,b : different indices denote statistically significant difference, identical indices denote no statistical difference ($p<0.05$)

Table 5.2 Length and width [μm] of cells in the meristematic zone of roots in plants grown for four days at adequate (15 μM) or high (5000 μM) B supply. Values represent mean ± standard error for each replication.

B conc. [μM]	Cell length *		
	Clipper (n=100)	Sahara # (n=40)	Barley grass (n=100)
15	9.43 [a] (±0.15)	9.28 [a] (±0.20)	8.56 [a] (±0.17)
5000	10.47 [b] (±0.21)	9.19 [a] (±0.14)	18.84 [b] (±0.38)
	Cell width *		
15	10.88 [a] (±0.15)	10.81 [a] (±0.35)	11.69 [a] (±0.21)
5000	12.74 [b] (±0.21)	11.70 [b] (±0.20)	11.46 [a] (±0.24)

n: number of replicates
#: non-hairy roots measured
*: measured in longitudinal optical section
a,b : different indices denote statistically significant difference, identical indices denote no statistical difference ($p<0.05$)

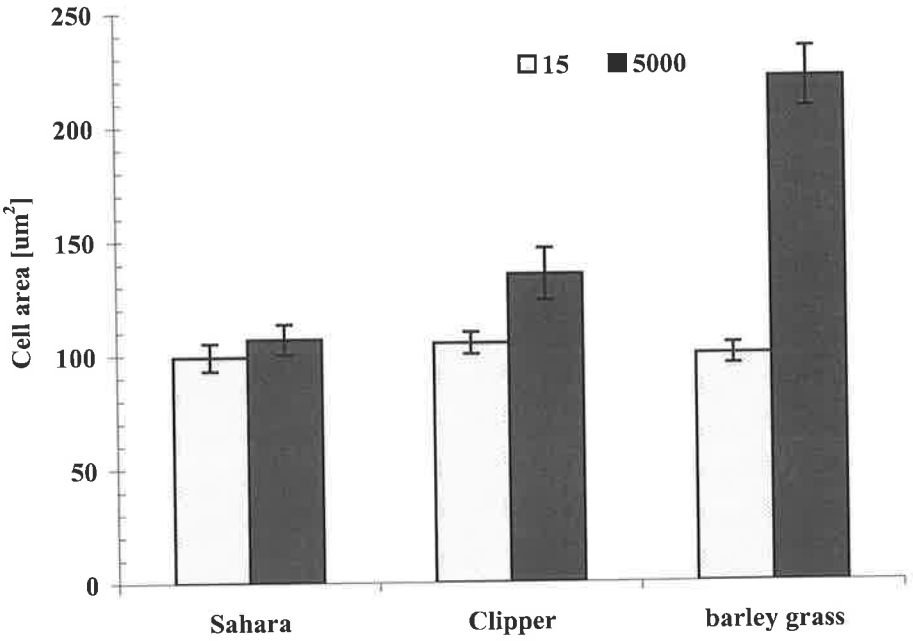


Fig. 5.7. Cell area (μm^2) in the root meristematic zone of two barley varieties, Clipper and Sahara, and barley grass grown four days at adequate (15 μM) or high (5000 μM) B supply. Values represent mean ± standard error for 40 cells for Sahara and 100 cells for Clipper and barley grass. LSD (*P<.001*): variety 16.36, boron 13.41, and variety ×boron 23.23.

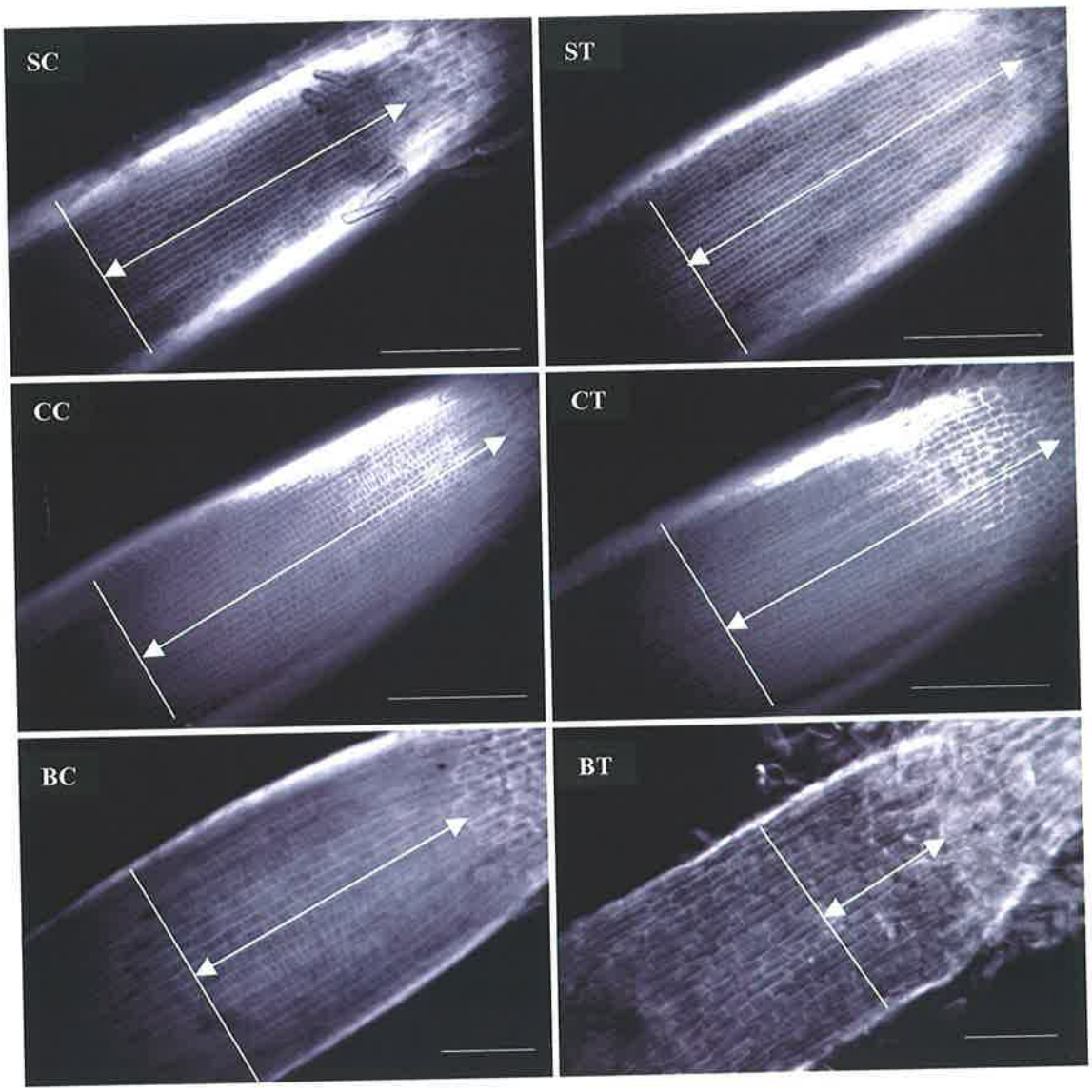


Fig. 5.8. Confocal images of root tips. SC: Sahara control, ST: Sahara treated, CC: Clipper control, CT: Clipper treated, BC: barley grass control, BT: barley grass grown for four days at adequate (15 μM) or high (5000 μM) B supply. Scale bars equal to 200 μm, cross line indicates the distal end of meristematic tissue.

5.4 Discussion

This study shows that B toxicity has a significant effect on root morphology including root length and diameter, as well as the length of the root meristematic zone and cell density in the meristematic zone. It suggests that the change in root morphology under high B supply is associated with altered activity in the root meristematic zone with subsequent structural changes to these cells that affect root elongation. The lower cell density in a shorter root meristematic zone found in the B-sensitive variety Clipper and barley grass, coupled with increased cell length, indicates inhibition of root division at high B supply. In contrast, the B-tolerant variety Sahara increased length of the root meristematic zone at high B supply when compared to a control, but did not change the cell length in the zone, and thus this was an increase in the cell number and root elongation. One might hypothesize that such an effect in the B-sensitive genotypes is caused by a reduction in cell division, although further work is required to elucidate more facts about these responses. Indeed, findings in this study are in accordance with those by Liu *et al.* (2000) and Klein and Brown (1981), where it was found that B toxicity resulted in inhibition of cell division activity in the root meristematic zone.

The diameter and surface area of root tips under high B supply was lower in both B-sensitive and tolerant plants in this study. Diameter of roots is generally considered to be related to cell division activity in the root meristematic zone, since tangential division leads to an increase in root diameter while radial division to an increase of the cell number in individual cell layers (Luxova, 1975). However, in this study, the width of the meristematic zone and cell number across the meristematic zone was not related to root diameter of the two barley varieties Clipper and Sahara, and barley grass. High B increased the width of the meristematic zone of Clipper and Sahara, but decreased the root diameter of both varieties. Barley grass decreased the width of the meristematic zone and cell numbers across the meristematic zone at high B supply, but the diameter and surface area was not affected by high B supply. The thinner diameter of the root tip in both sensitive and tolerant plants may therefore not be considered as a reliable indicator of B tolerance or toxicity in crop plants.

One may hypothesize that a reduction of cell division under B toxicity is due to the reduced transport of carbon to the root tip. In general, the growth of a root system depends on the metabolic utilization of sucrose, the main carbon and energy source in root metabolism (Gasparikova, 1992). It is well understood that carbon deficiency causes a reduction in cell division rate and decreasing root elongation (Van't Horf, 1968; Muller *et al.* 1998), symptoms observed with B toxicity in this study. McNairn and Currier (1965) found that supra-optimal concentrations of B resulted in callose formation in the mesophyll cells of the primary leaves, interrupting sugar transport. Stangoulis and Reid (2002) proposed that this effect might be due to the binding of B with sugars, which thereby disrupts cell division and further cellular development in the roots. This alteration of sugar status in the sink organs might subsequently disrupt carbohydrate metabolism in the leaf tissue as well though a feedback mechanism. As shown in this and previous chapters, the dry weight of the root and also the shoot of Clipper significantly decreased during four days of high B supply, whereas a loss of shoot or root dry weight (data in Chapter 4) was not evident in Sahara. In barley grass, although shoot growth was affected much more slowly than root growth (data in Chapter 4) at high B supply, the dry weight was reduced for three or four weeks of high B supply. However, there is still no experimental data on the relationship between reduction of root elongation at high B and sugar status in the sink organs, and this concept will be explored for three barley varieties, Sahara, VB9953, and Clipper in the following chapter.

CHAPTER 6

Effects of Boron Toxicity on Carbohydrate Status and Metabolism in Barley

6.1 Introduction

In Chapter 5, the effect of B-toxicity in Clipper barley and barley grass resulted in a reduced length of the root meristematic zone, including an increase of cell length, and a decrease in cell number. In contrast, the effect of B-toxicity on root morphology was the opposite in the B-tolerant Sahara compared to B-sensitive varieties, implicating the B tolerance mechanism is either directly or indirectly associated with physiological metabolic function.

Root growth depends on sucrose utilization, the main carbon and energy source (Gasparikova, 1992). It is well established that carbon deficiency causes a reduction in the rate of cell division, reduced cell density, and therefore a decrease in root elongation (Van't Horf, 1968; Muller *et al.,* 1998). With this in mind, the data reported in Chapter 5 may indicate an effect of B toxicity on carbohydrate metabolism in the root. There is evidence for a specific effect of B on sugar metabolism. Under B toxicity, glucose declines in both the leaf sap and root of sugar beet, and in addition, N (measured as NO_3^-) accumulates in the leaf sap commensurate with a reduction of nitrate reductase activity under excess B supply (Bonilla 1980). B toxicity also leads to reduced levels of the reducing sugar, α-amylase, during barley seed germination (Jimenez and Barea, 1979). The repression of protein synthesis in germinating seeds at high B (Haba, *et al.,* 1985) may account for such an effect. This lower content of protein was also observed in the root tips of sugarcane grown at high B supply, which resulted in a decrease in the activity of a number of enzymes, specifically aldolase and glyceraldehydes 3-phosphate dehydrogenase, enzymes involved in carbohydrate metabolism (Bowen, 1972 (b)).

However, the relationship between B toxicity and sugar transport, or metabolism, associated with root elongation remains unclear. The aim of this study is to investigate the reasons for the apparent variability in root growth between the barley varieties Sahara (B-tolerant), VB9953 (B-tolerant) and Clipper (B-sensitive) under B toxicity. In particular, the aim was to investigate whether reduced or enhanced root elongation in these varieties results from changes in sugar status in both source and sink organs, and whether a B-tolerance mechanism is associated with sugar status and metabolism.

6.2 Materials and Methods

6.2.1 Experiment 6 (a) Estimation of reducing sugar contents in roots

This experiment was conducted with the same design and growing conditions as Experiment 4 (a) except for the growth duration and barley varieties used. Three barley varieties, Sahara, VB9953, and Clipper were grown for two weeks in 15 μM B (B-adequate) solution and then transferred to their treatments for a further week. Treatments included 15 and 5000 μM B, with the latter a toxic treatment.

6.2.1.1 Harvesting root segments

Roots grown at both 15 μM B and 5000 μM B were harvested one week after the treatments were imposed and sectioned into three root zones. The first zone included the first 2 mm of the root tip (root cap plus meristematic zone), the second up to 10 mm from the root tip (growing zone), and the third, the remainder of the root. The root segments were kept in Eppendorf® tubes within an icebox while they were harvested, and then were dried in a Virtis® automatic freeze drier for 3 days.

6.2.1.2 Sugar Extraction from root segments

Freeze-dried sample was weighed, diluted to 750 μl with nanopure water (>18 MΩ resistivity), shaken on an agitator platform for 10 minutes, and then centrifuged for 10 min at 8000 rpm. The supernatant was collected, placed in fresh 1.5 ml Eppendorf® tubes, and then stored at 4 °C until use.

6.2.1.3 *3,5-Dinitrosalicylic acid* assay

200 µl of supernatant was diluted to 300 µl with nanopure water with the addition of 0.5 ml 2 % DNS reagent (*3,5-Dinitrosalicylic acid*) in 0.7 N NaOH solution. This procedure was repeated for duplicate samples that were then mixed by vortexing for five seconds and then kept on ice for a further 10 minutes prior to transferring to a boiling water bath for 5 minutes, after which samples were immediately transferred to an iced water bath for a further 10 minutes. 2 ml of nanopure water was then added to each samples, and mixed by vortexing. Reducing sugars were observed after measuring spectrophotometrically at 590 nm with comparison to glucose standards.

6.2.2 Experiment 6(b) Soluble carbohydrate contents in root tips

This experiment was conducted with the same design, species and growing conditions as in Experiment 6 (a).

6.2.2.1 Measurement of soluble carbohydrate determination by Dionex

Leaf tissue (the second youngest mature leaf blade) and root tips (0-10 mm from apex) were sampled and extracted for soluble carbohydrate composition using a method reported by Stangoulis (1998). 10 mm of root tip was cut off and kept in 1.5 ml Eppendorf® tubes. The tubes were immediately plunged into liquid nitrogen (within 20 s of excising) to minimise enzymatic activity. The samples were dried for 48h in a Virtis® automatic freeze drier, and then weighed to obtain dry weights. Root tips were extracted in hot 80% EtOH (EtOH: water (80:20 v/v) at 80 °C) to extract sugars. After extraction and collection of supernatant, samples were centrifuged and taken to dryness in a Speed-Vac® vacuum centrifuge. Immediately prior to analysis, samples were resuspended in 900 µl of high purity water.

Simple sugar levels were measured by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex GP40 gradient pump, a Dionex ED 40 electrochemical detector and a Shimadzu SIL-10AD autoinjector. It was fitted with a Dionex CarboPac PA-1 column and PA-1 precolumn. 10 μL of the sample was injected into the column with elution using a linear gradient of sodium hydroxide (100 mM, made from low CO_2, 50 % w/w liquid NaOH) over 13 minutes with a flow rate of 1ml min^{-1}. A standard was prepared (0.256 nmol /μL glucose, 0.107 nmol/μL fructose, 0.064 nmol/μL sucrose) and measured every 10 samples. The Dionex Peaknet software was used to compare the peak areas of the standards to the peak areas of each sample to determine the concentration of each sugar in nmols/L.

6.2.3 Experiment 6 (c) Effect of boron toxicity on invertase activity

This experiment was conducted with the same design, species, and growing conditions as Experiment 6 (a).

6.2.3.1 Sampling and extraction

Leaf tissue (the second youngest mature leaf blade) and root tip (0-10 mm from apex) tissue (30 mg fresh weight) were immediately frozen in liquid N_2 and stored at –80 °C, until used for biochemical analyses. For the assay of neutral invertase activity, extraction buffer [Hepes-NaOH (pH 7.0) 50 mol m^{-3}, $MgCl_2$ 10 mol m^{-3}, Na_2 EDTA 1mol m^{-3}, Dithiotreitol 2.6 mol m^{-3}, Ethylene glycol 10 %, and Triton 0.02 %] (Pelleschi *et al.,* 1997) was added into ground tissues bathed in liquid N_2 to a final volume of 60 μl. For the assay of acidic invertase activity, extraction buffer [50 mM NaH_2PO_4 buffer pH 6.0, 1mM DTT, and 1mM EDTA, 10 % Ethylene glycol] (Shaikh, *et al.,* 2000) was added into ground tissues bathed in liquid N_2 to a final volume of 60 μl. The extracts were centrifuged for 15 minutes at 13200 rpm and 4 °C. Aliquots of the supernatant collected were desalted on G25 Sephadex columns for 3 minutes at 2 rpm.

6.2.3.2 Neutral invertase activity

A 25 µl volume of desalted extract was boiled for five minute, and the other 25 µl volume of desalted extract was not boiled. A 50 µl volume of sucrose 0.9 M was added into both boiled and non-boiled extracted sample. They were incubated for 25 min at 37 °C, and then were boiled for a further five minutes to stop invertase activity. A control reaction was performed with the 25 µl extract sample, which had been boiled for five minutes to inactivate invertase activity. The glucose formed was assayed by the method of Moreno *et al.* (1981) that included the addition of 50 µl 0.1 M $NaPO_4$ (pH 7.0) buffer containing 10 µl of glucose oxidase, 2 µl of peroxidase, 40 µl of *o*-dianisidine, and 325 µl of high purity water. After 30 min incubation, the reaction was stopped with the addition of 100 µl of 3M HCl. The concentration of color produced was measured spectrophotometrically at 540 nm by comparison with glucose standard curve prepared at the same time.

6.2.3.3 Acidic invertase activity

A 30 µl volume of desalted extract was boiled for five minutes, and the other 30 µl volume of desalted extract was not boiled. A 30 µl volume of the leaf extract was incubated in 120 µl 200 mM sodium-acetate buffer pH 5.0, 150 µl 200 mM sucrose for 30 min at 37 °C. The reaction was stopped by the addition of 500 µl DNSA reagent (1% (w/v) 3,5-Dinitrosalicylic acid in 0.7 N NaOH solutions), and cooled on ice for five minutes. The color was developed by boiling for 10 minutes, and cool on ice for 5 minutes. The presence of reducing sugars resulted in the development of a deep orange color, which was assayed spectrophotometrically at 560 nm and compared with glucose standards.

6.3 Results

6.3.1 Experiment 6(a) Estimation of reducing sugar contents in roots

6.3.1.1 Plant growth

Genotypic variation in biomass (assessed by tissue dry weight) was measured between the shoot and roots grown for one week after B treatment applied (Table 6.1). B toxicity significantly (*P<0.001*) reduced the dry weights of the shoot (by 28 %) and root (by 59 %) in Clipper. In contrast, B toxicity did not affect the dry weight of shoot and root in Sahara and VB9953. The root to shoot ratio indicated that root growth was more sensitive than shoot growth under B toxicity, and was related to the tolerance of the genotype tested, with Sahara more tolerant than VB9953, which was more tolerant than Clipper (Table 6.2).

Table 6.1. Effect of B treatment on root and shoot dry weight in three barley varieties Clipper, Sahara, and VB9953. Values represents mean ± standard error for three plants.

B (μM)	Shoot (mg plant^{-1})			Root (mg plant^{-1})		
Variety	Clipper	Sahara	VB9953	Clipper	Sahara	VB9953
15	275±54	175±6	181±8	82±4	42±9	49±3
5000	199±6	187±11	206±10	34±5	40±1	46±2
Variety		17***			5.0***	
Boron		*NS*			4.0***	
Variety *Boron		25***			7.0***	

***, *P<.001*

NS, Not significant

Table 6.2. Effect of B treatment on the root:shoot ratio by dry weight in three barley varieties, Clipper, Sahara, and VB9953. Values represents mean ± standard error for three plants.

B (μM)	Root:Shoot ratio		
Variety	Clipper	Sahara	VB9953
15	0.29±0.02	0.25±0.01	0.27±0.02
5000	0.17±0.02	0.23±0.02	0.22±0.01

6.3.1.2 Boron concentration in shoot and root tissue

The B concentration in the whole shoot grown at 15 μM B was greater in Clipper (23.7 mg kg^{-1}) when compared to Sahara (14.8 mg kg^{-1}) and VB9953 (16.1 mg kg^{-1}) (Table 6.3). At high B supply (5000 μM B), the B concentration in the whole shoot increased in all three barley varieties compared to the control plants. However, B concentration varied between varieties, in the order, Clipper > VB9953 > Sahara. The B concentration in the whole shoot of Clipper was about 6-fold and 2-fold higher than that observed in Sahara and VB9953, respectively. The B concentration in the whole root of Clipper at high B supply was 2.6-fold and 1.4-fold greater than Sahara and VB9953, respectively. These results indicate that the accumulation of toxic B concentration in the shoot and root tissue was greater in Clipper grown at high B supply. Boron accumulated in the shoot and root tissues of VB9953 much more than that of Sahara, however, the growth of VB9953 was not affected which in this case is consistent with a B-tolerance mechanism that does not exclude B from plant tissue, but is able to tolerate it.

Table 6.3. Effect of B treatment on the B concentration in shoots and roots of three barley varieties Clipper, Sahara, and VB9953. Values represent mean ± standard error for three plants.

	B concentration (mg kg^{-1}DM)					
B conc. (μM)	Shoot			Root		
	Clipper	Sahara	VB9953	Clipper	Sahara	VB9953
15	24±0.7	15±0.4	16±1.1	22±1.5	43±2.8	42±1.2
5000	3100±58	517±3.3	1590±12	887±12	333±8.8	620±5.8
Variety	53***			15***		
Boron	43***			12***		
Variety× Boron	74***			21***		

***, *P<.001*

6.3.1.3 Reducing sugar contents in root tips

The reducing sugars in the growing zone (2-10mm from root apex) and meristematic zone (0-2mm from the apex) in root tips of Clipper, Sahara, and VB9953 grown at adequate (15 μM) or high (5000 μM) B supply were measured (Fig 6.1). Reducing sugars were higher in the growing zone (2-10 mm from apex) when compared to the meristematic zone (0-2 mm from apex). In the control, reducing sugar contents (mg g^{-1} root DM) in the growing zone (2-10 mm) was in the order, Clipper (119) > Sahara (26) > VB 9953 (15), however B toxicity led to a significant alteration of this ranking as concentrations in the growing zone changed while levels in the meristematic zone remained constant. B toxicity significantly (*P<0.001*) reduced reducing sugars of Clipper by around 85%, whereas in Sahara and VB9953, reducing sugars increased by 31 % and 119 % respectively. While B concentrations of the whole shoot and root tissue of VB9953 were 3-fold and 2-fold higher than the shoot and root tissues of Sahara, the concentration of reducing sugars in the root tip were higher than Sahara.

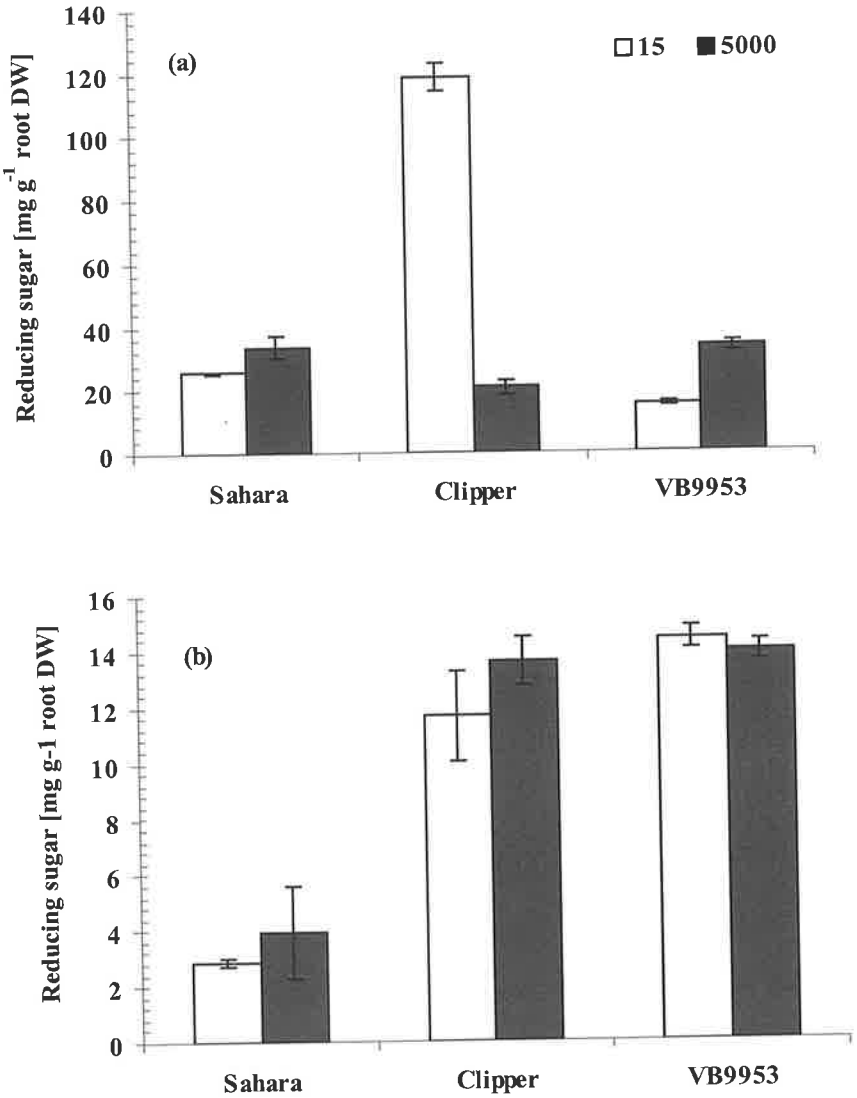


Fig. 6.1. Reducing sugar contents in the root growing zone ((**a**): 2-10mm from root apex) and in the root meristematic zone ((**b**): 0-2mm from the apex) in root tips of two barley varieties, Clipper and Sahara grown at either adequate (15 μM) or high (5000 μM) B supply. Values represent mean ± SE for three plants. LSD (*P<0.001*), Fig 6.1 (A) (B treatment (4.4), B treatment × variety (5.3), variety (7.5)). LSD (*P<0.001*), Fig 6.1 (B) (B treatment (1.9), B treatment × variety (2.331), variety (3.297)).

6.3.2 Experiment 6 (b) Soluble carbohydrate contents in leaf and root tip tissues

6.3.2.1 Soluble carbohydrate contents

The aim of this study was to quantify the levels of total soluble carbohydrates, in particular, glucose, fructose and sucrose in the second youngest matured leaf, and in the root tip (0-10 mm from apex) tissue, in two barley varieties, VB9953 and Clipper grown at adequate and toxic B supply. When comparing the sugar concentrations (Table 6.4), sucrose was greatest in the leaf tissue of the two barley varieties grown at adequate B supply when compared to other sugars, whereas glucose was at the higher concentration in the root tip tissue. Clipper had a three fold higher (27.4 mg $g^{-1} \pm 0.7$) sucrose content in the shoot than VB9953 (8.0 mg $g^{-1} \pm 0.5$) grown at adequate B supply. This is consistent with results presented in Experiment 6(a) and indicates a high demand for carbon (C) in Clipper.

In the second youngest mature leaf, B toxicity (5000 μM) altered the sucrose contents in both B-tolerant and B-sensitive varieties (Table 6.4). A two-fold increase in sucrose content in the leaf tissue of VB9953 was observed, whereas a significant reduction was observed in Clipper. B toxicity significantly increased the hexose sugars (glucose and fructose) in the leaf of Clipper and these B toxicity effects increased the ratio of hexose sugars to sucrose (Fig 6.2). The glucose content of leaf tissue of Clipper was lower than the fructose content, decreasing the glucose to fructose ratio significantly (Fig 6.3). In the leaf tissue of VB9953, glucose and fructose increased as well, but not as high as Clipper.

In the root tips, B toxicity increased the contents of both glucose and fructose of root tips in VB9953 by about 1.4 fold (Table 6.4), so the ratio was maintained the same as the control (Fig. 6.3). By contrast, Clipper reduced the content of glucose and fructose by seven-fold and three-fold, respectively, and the reduction was higher in glucose than fructose, decreasing the glucose to fructose ratio (Fig 6.3). Interestingly, while Clipper root tips demonstrated a great reduction of glucose and fructose

contents at high B supply, sucrose did not accumulate in the root tip.

Table 6.4. Soluble carbohydrates in the second youngest matured leaf, root tips (0-10 mm from apex), and remainder of roots of barley varieties VB9953 and Clipper grown at either 15 μM B (adequate) or 5000 μM B (toxic). Values represent mean ± standard error for three plants.

Soluble carbohydrate content (mg g^{-1} root DM)						
Leaf[y]						
Boron	**Sucrose**		**Glucose**		**Fructose**	
	VB9953	Clipper	VB9953	Clipper	VB9953	Clipper
15	8.0±0.5	27.4±0.7	2.8±0.3	4.5±0.3	1.9±0.3	2.9±0.1
5000	21.9±0.7	19.2±1.3	6.3±0.7	7.8±0.1	4.7±0.5	11.5±0.5
LSD	3.1***	1.3*	2.2*	1.3*	1.6*	1.4*
Vari*Boron	3.0***		1.8*		1.7***	
Root tip[z]						
15	2.0±0.9	6.8±0.7	14.9±3.1	38.8±3.6	9.9±1.3	25.9±1.3
5000	3.1±0.3	5.0±0.4	21.3±2.9	5.1±0.2	14.3±1.4	9.4±0.3
LSD	NS	1.5*	2.1*	9.9***	5.4*	3.6***
Vari*Boron	*1.4		9.0***		3.7***	
Remainder of root						
15	2.9±0.2	3.9±0.05	2.1±0.2	2.8±0.2	4.2±0.4	4.0±0.2
5000	2.7±0.2	3.9±0.07	1.6±0.07	1.2±0.1	2.4±0.08	1.3±0.1
LSD	NS	NS	NS	0.5***	1.2*	0.7***
Vari*Boron	NS		0.5*		NS	

[z]: Root tip, 0-10 mm from the apex
[y]: The second oldest matured leaf
***; $P < 0.001$
*; $P < 0.05$
NS, not significant

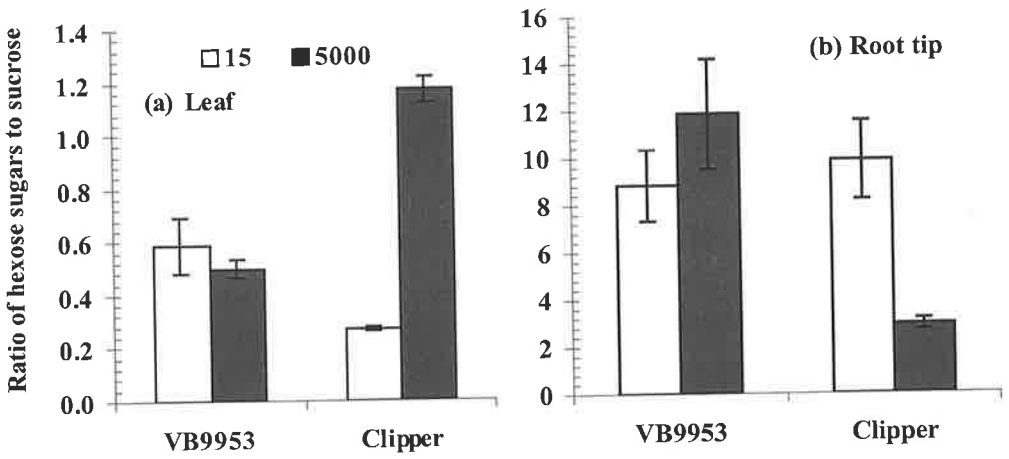


Fig 6.2. Ratio of hexose sugars to sucrose in the leaf (a) and root tips (b) of two barley varieties VB9953 and Clipper grown at either 15 μM B (adequate) or 5000 μM B (toxic). Values represent mean ± standard error for three plants.

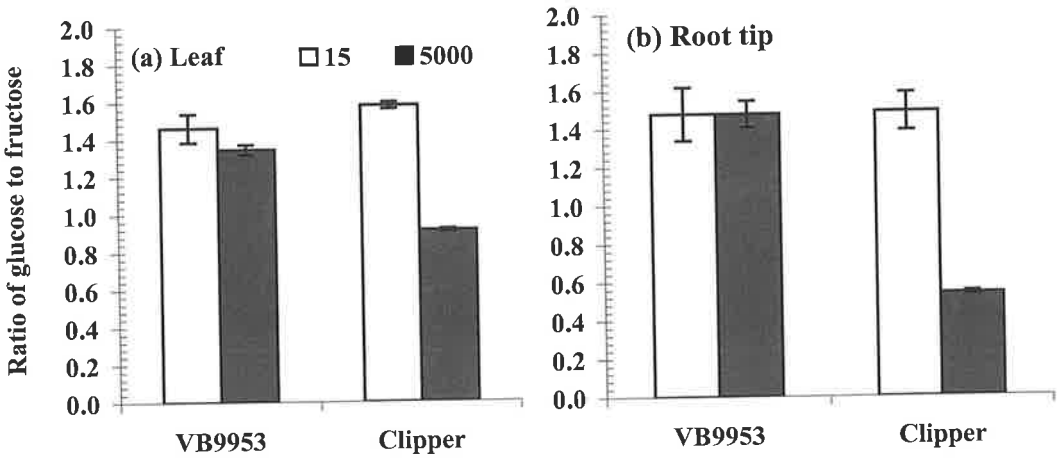


Fig 6.3. Ratio of glucose to fructose in the (a) leaf and (b) root tips of two barley varieties VB9953 and Clipper grown at either 15 μM B (adequate) or 5000 μM B (toxic). Values represent mean ± standard error for three plants.

6.3.3 Experiment 6 (c) Effect of high B supply on invertase activities in leaf and root tip tissues

6.3.3.1 Soluble invertase activity

To investigate whether invertase activity was correlated to the apparent change in reducing sugars both in the second youngest matured leaf (source) and root (sink), the activity of acidic invertase (AI) and neutral invertase (NI) was measured. The two barley varieties, VB9953 and Clipper, displayed different levels of invertase activity when subjected to B toxicity (Table 6.5). The activity of both invertases was higher in the root tips when compared to the youngest mature tissue regardless of B treatment.

AI activity responded to B toxicity in both roots and shoots of Clipper and VB9953 (Table 6.5). B toxicity increased the shoot AI activity by 73 % for Clipper and 68 % for VB9953, and as seen previously this was accompanied by an increase of glucose and fructose contents in the shoots of both varieties (Table 6.4). By contrast, B toxicity decreased the AI activity in roots of Clipper and VB9953, although the reduction in VB9953 roots was not as great as Clipper (Table 6.5). B toxicity reduced AI activity in Clipper by 74 % and in VB9953 by 54 %. This result for VB9953 is opposite to that expected to be observed given the accumulation of reducing sugars in root tips of VB9953 under B toxicity.

NI activity in both roots and shoots of VB9953 did not respond to B toxicity, whereas in Clipper B-toxicity increased NI activity by 30 % in the shoot and decreased it by up to 90 % in the root (Table 6.6). It is likely that there is a correlation between reducing sugar contents and the AI and NI activity in Clipper. Excess B supply (5000 μM B), where the root elongation by Clipper was inhibited, caused a reduction of AI and NI activity indicating a relationship between lack of reducing sugars and root growth under B toxic conditions.

Table 6.5. The effect of B on acidic invertase activity (μmol reducing sugars g^{-1} root FW h^{-1}) in the second youngest mature leaf and roots of two different barley cultivars (VB9953 and Clipper) grown for 3 weeks in adequate (15μM) or high (5000 μM) B. Values represent mean ± SE for three plants of Clipper and six plants of VB9953.

Acidic Invertase Activity				
B conc. (μM)	Shoot		Root	
	Clipper	VB9953	Clipper	VB9953
15	24.4±3.8	20.9±0.7	63.4±7.3	56.0±10
5000	42.2±4.9	35.1±7.1	16.7±1.4	25.7±3.1
Variety	*NS*		*NS*	
Boron	9.9*		14.2***	
Variety* Boron	*NS*		*NS*	

***, *P<0.001*
*, *P<0.05*
NS, Not significant

Table 6.6. The effect of B toxicity on neutral invertase activity (μmol glu g^{-1} F.W. $hour^{-1}$) in the youngest mature leaf and roots of two different barley cultivars (VB9953 and Clipper) applied B (from adequate, 15μM, to toxic level, 5000 μM) for 3 weeks. Values represent mean ± SE for three plants.

Neutral Invertase Activity				
B conc. (μM)	Shoot		Root	
	Clipper	VB9953	Clipper	VB9953
15	7.0±0.6	11.5±1.5	26.7±1.4	12.1±2.5
5000	9.4±0.6	11.5±0.2	2.8±0.1	9.6±0.1
Variety	*NS*		3.3*	
Boron	2.4*		3.3***	
Variety* Boron	*NS*		4.6***	

***, *P<0.001*
*, *P<0.05*
NS, Not significant

6.4 Discussion

The aim of this study was to investigate reasons for variability in root growth under B toxicity between the barley varieties, Sahara (B-tolerant), VB9953 (B-tolerant), and Clipper (B-sensitive). In particular, whether reduced or enhanced root elongation in these varieties result from changes in sugar status in both source and sink organs, and whether a B-tolerance mechanism is associated with sugar status and metabolism.

This study has clearly shown that B toxicity affects the sugar status and soluble invertase activity in the leaf and root tip tissues and that in the B-intolerant variety, Clipper, B-toxicity is associated with sugar starvation as observed by a reduction in reducing sugar contents within the root growing zone, whereas in both the B-tolerant Sahara and VB9953, reducing sugars were enhanced in this zone (Fig 6.1A). After further evaluation of the sugar status in both source (second oldest mature leaf) and sink (root tips) regions of both tolerant and intolerant genotypes, variation in reducing sugar status within the growing zone was observed. In Clipper, B-toxicity lead to reduced levels of sucrose in both source (leaf) and sink (root growing zone) tissue. In contrast, sucrose contents within the leaf tissue of the B-tolerant VB9953 were enhanced under B-toxicity and corresponded to the maintenance of sucrose concentrations within root tip. Elevated levels of sucrose in the shoot of VB9953 may aid in osmoregulation. Enhanced or maintained levels of sucrose in both source and sink regions are obviously associated with the mechanism of B-tolerance in VB9993, enabling it to maintain root elongation when grown under B-toxic conditions.

Lowering of sucrose concentrations in the leaf tissue of Clipper in response to toxic B corresponded to an increase in soluble glucose and fructose, most likely in response to increased demand for the hexose sugars for maintaining metabolic function. In Clipper, the increase in both acidic, and to a lesser degree neutral, invertase activity would lead to a lowering of the sucrose concentration and enhancement of the hexose sugars, as was witnessed. Whilst a reduction in sucrose concentrations in Clipper leaf tissue may be due to a greater demand for the hexose sugars for metabolism, it may also be due to two other factors, including a direct

effect of B on the enzyme, invertase, causing a disruption to normal activity, or due to an effect of excess B on photosynthesis and subsequent sucrose synthesis. B toxicity has been shown to reduce photosynthetic capacity (Lovatt and Bates, 1984; Sotiropoulos, *et al.*, 2002). Further research is required to investigate these hypotheses.

The reduced level of sugars in the root tip of Clipper is an indication that sucrose transport to the root growing zone becomes limited at high B supply. A reduction in sugar transport may lead to 1) reduced respiration rate, 2) reduced organic compound synthesis (sugars, proteins, fatty acids, and adenine nucleotides), 3) reduced activity of enzymes involved in glycolysis, the pentose phosphate pathway, the tricarboxyl cycle, and fermentation, and 4) reduced intracellular osmolarity as reported in maize roots during glucose starvation. (Brouquisse *et al.*, 1991). These effects may lead to reduced sink strength that can explain current observations.

There may also be a physical barrier to sucrose transport and unloading from the phloem into the root growing zone of the Clipper root tip. B toxicity results in callose deposition within the leaf mesophyll (McNairn and Currier, 1965) and one may hypothesize that under B toxicity, a reduction in the loading of sucrose from the leaf veins into the phloem occurs. Under B deficiency, a similar effect is observed when callose is deposited in the sieve tubes and this in turn reduces sucrose transport in the phloem (Van de Venter and Currier, 1977). Against this hypothesis, sucrose concentrations in the whole root (excluding the root tip) were maintained in both Clipper and VB9953 (Table 6.4) and this may indicate reduced sucrose unloading from the phloem into the root growing zone. After phloem unloading, solute transport into the growing zone must occur through plasmodesmata (Pritchard *et al.*, 2000). It is possible that occlusion of the plasmodesmata (by callose deposition) is an effect of B toxicity which in turn would result in reduced sucrose transport to the growing zone. This hypothesis requires further testing.

Results presented in this study indicate that a mechanism of B-tolerance is associated with increased sugar deposition in the root growing zone. The trait appears to be associated with the B-tolerance mechanism on chromosome 2H of the barley genome as VB9953 has only the 2H Quantitative Trait Loci (QTL) from the donor parent Sahara (Jeffries *et al.,* 1999, 2000). Further research should include the mapping of the high sugar trait in the root tips of a Clipper x Sahara double haploid population to see if this new B-tolerance trait maps to the 2H Loci previously reported.

The increased sugar contents in the root growing zone of VB9993 may compensate for sugars complexed to B, which in turn reduces sugar availability for metabolism. B forms complexes with pyridine nucleotide coenzymes (NAD^+), ATP, RNA in the symplasm, and several sugar moieties within the cell wall (Pfeffer *et al.*, 1999; Ralston and Hunt, 2000). Even small structural changes due to complexing with B could result in a loss of function or alteration of enzyme activities and therefore lead to metabolic disruption (Wimmer *et al.*, 2003). Increased sugar deposition in the root growing zone may arise from a need for maintaining root cell turgor and subsequent cellular growth. Growth in the root growing zone is facilitated by a high turgor pressure, low membrane resistance or an increased cell wall extensibility, thus growth requires both the maintenance of turgor to extend the cell wall and a water potential difference between the cell and wall to allow water uptake (Pritchard *et al.*, 2000). Solutes, such as the sugars, are required to generate the turgor pressure but will be diluted by water influx across the membrane thereby leading to cell expansion. A further role of the excess sugar deposited in the root growing zone may be to act as a detoxification mechanism in complexing excess B. This hypothesis is hard to support as glucose and sucrose form very weak bonds with boric acid. One would also expect a higher concentration of B in the root of the B-tolerant variety if such a mechanism were to occur, and this is not the case from results presented in this thesis.

CHAPTER 7

General Discussion

7.1 General conclusions

The work described in this thesis allows the following general conclusions to be made regarding the underlying mechanisms of B toxicity and tolerance, particularly in relation to barley.

1. Based on a glasshouse screening experiment with subsoil B treatments, and on measuring a range of B-tolerant response parameters including shoot toxicity symptoms, shoot dry weight, shoot B concentration, total root dry weight, subsoil root dry weight and root:shoot ratio, the relative order of B tolerance for crop and weed genotypes was: VB9953 (advanced breeding line of barley) and barley grass > fababean (crop), evening primrose and prickly lettuce (weed species) > Clipper (a barley variety) and lincoln weed.

2. A reduction in both root and shoot growth of crop and weed species, with subsequent development of visible toxicity symptoms, was caused by an extractable B concentration of 2.4 mg B kg^{-1} in the subsoil. This data implies that the influence of B on crop growth in agricultural areas of southern Australia may be more widespread than currently believed as reported soil surveys indicate higher B levels in these regions (Cartwright *et al.*, 1984; Sadras *et al.*, 2002), and relatively low concentrations of extractable B in upper regions of the soil profiles could be restricting root growth for some crops.

3. Shoot growth, as well as both topsoil and subsoil root growth, of the sensitive genotypes Clipper and lincoln weed was severely impaired by high subsoil B. This result suggests that an immediate consequence of high shoot B concentrations may be an alteration in carbohydrate status of the plants, and

ultimately reduction in root growth. However, barley grass and VB9953 maintained good shoot growth and sustained root growth in the upper soil depths; in fact, both tap-rooted and fibrous-rooted weed species, including evening primrose, lincoln weed, and barley grass, redistributed more fine roots to the topsoil in response to high subsoil B. These observations suggest the use of an avoidance' strategy that promotes sufficient roots in the upper non-toxic soil depth in order to support shoot growth and thus maintain carbohydrate metabolic functions. Such a strategy should be taken into consideration when field testing breeding lines for B tolerance, although the advantages may be less under water-limited field conditions.

4. Barley grass was unable to demonstrate an avoidance mechanism in response to high B under solution culture conditions where the entire root system was subject to uniform B concentrations. This is opposite to what is observed in a soil situation (as highlighted in point 3 above).

5. The tolerance to toxic concentrations of B in the shoot and root tissue was observed in VB9953, which accumulated high B concentrations in these tissues without a reduction of plant growth. This barley advanced breeding line appears to possess a tolerance mechanism commonly termed 'internal tolerance' (Stangoulis and Reid, 2002).

6. A capacity to reduce B uptake into the plant was observed in the B-tolerant Sahara, and it seems to be associated with a physical barrier or an active/facilitated efflux transport system to reduce B from root tissues. The reduction in B appears to be distinctively developed at the site of xylem loading.

7. B toxicity changes root morphology, and this is associated with a reduction in cell division activity in the root meristematic zone of the B-sensitive variety Clipper and barley grass. The opposite effect in response to B toxicity was observed in the B-tolerant variety Sahara. These results imply that B tolerance can be associated with an increase in length of the meristematic zone as well as in cell number, and thus causes an increase in the root elongation as observed in the B-tolerant variety Sahara.

8. B toxicity reduced the root diameter in both B-tolerant and-sensitive barley varieties, but not in barley grass, suggesting that the parameter of diameter is not a suitable indicator of B tolerance.

9. B toxicity is associated with the occurrence of sugar starvation as identified in the root tip of the B-sensitive Clipper, while the B-tolerant VB9953 significantly enhanced the concentration of reducing sugar at the same B concentration. This result provides some explanation for the typical response of inhibited root elongation by the B-sensitive variety Clipper observed in this study under B toxicity.

10. Further observations identified alteration of carbohydrate status both in the sink (root tip 0-10 mm from apex and remainder of roots) and the source (second oldest matured leaf) in response to B. In Clipper, B toxicity led to reduced levels of sucrose in both source (leaf) and sink (root growth zone) tissue. In contrast, sucrose contents within the leaf tissue of VB9953 were increased under B-toxicity and correspond to the maintenance of sucrose concentrations within the root tip. These results indicate that maintained or enhanced levels of sucrose in both source and sink regions are obviously associated with the mechanism of B-tolerance in VB9953, enabling it to maintain root elongation under B toxicity.

11. The alteration of sugar status under B toxicity was accompanied by an increase in both acidic and neutral invertase activity in the leaf of B-sensitive Clipper, and led to a lowering of the sucrose concentration and increase in hexose sugars. Whilst, a reduction in sucrose concentrations in Clipper leaf tissue may be due to a greater demand for the hexose sugars for metabolism, it may also be due to two other factors, including a direct effect of B on the enzyme, invertase, causing a disruption to normal activity, or due to an effect of excess B on photosynthesis and subsequent sucrose synthesis.

7.2 Boron tolerance mechanisms

From data presented in this thesis, one can hypothesize that up to four tolerance mechanisms may exist and they include: 1) an exclusion mechanism involving a physical barrier to B uptake between the root cortical cells and the xylem, 2) an efflux system (either active or facilitated) operating in the tolerant root to reduce passive B uptake through the root, 3) an internal tolerance mechanism that allows function at high B concentration in the shoot, and 4) an avoidance strategy that operates in response to non-uniform distribution of high B concentration in the rooting medium.

The results presented in this study suggest that passive transport is operating at high B supply, which is in agreement with recent reports of passive uptake across biological membranes at high B supply (Stangoulis *et al.*, 2001; Dordas and Brown, 2001; Dordas *et al.*, 2000; Pfeffer *et al.*, 1999). Yet it is clearly obvious that the B-tolerant Sahara has a mechanism to reduce B uptake that is first manifest as a reduced xylem B concentration, and then within the rest of the root. It implies that the ability to reduce B uptake at the site of xylem loading develops faster than the capacity to decrease B concentration in the rest of roots. The B concentration in the xylem sap was lower (by 47 %) than that in the root at high B supply. Bellaloui and Brown (1998) reported that a tolerant wheat genotype reduced B uptake more than a sensitive genotype, but the transfer of B from root to shoot through xylem was not different between tolerant and sensitive genotypes. However, there is evidence to support the suggestion of decreased B uptake rate at high B supply in this study. Dannel *et al.* (1998) reported a reduced B uptake into the xylem when plants were grown with high (400 to 1600 μM) B that resulted in a 50-60 % lower B concentration in the xylem when compared to the root cell sap. The reduced B uptake could be due to a physical barrier due to the induction of suberin, or increased lignin in the cell wall, to control permeability of boric acid in both the apoplast and symplast of the root cortical cells and in the xylem parenchyma, thereby reducing B

uptake. Recent evidence supports the hypothesis that under B toxicity, both suberin and lignin increase in the cell walls (Ghanati, *et al.*, 2002).

Whilst the reduction of B uptake may be due to a physical barrier, it may also be due to an active or facilitated efflux system. The data presented in Chapter 4 demonstrate that the efflux system appears to slowly develop compared to the passive B transport system at high B supply, and that it is present in the region of the root. This unknown physical barrier or efflux system in Sahara seems to have a key role for B distribution into the root and shoot at high B supply. As identified in this thesis (Chapter 5), B toxic effects on inhibition of root elongation observed in the B-sensitive variety were the opposite in B-tolerant Sahara. It appears that Sahara is isolating the root meristematic zone from high B concentration, maintaining cell division and also regulating the sugar status in the root growing zone, as identified in this study (Chapter 6).

Further B tolerance mechanism was identified in this study from the observation that the B tolerant barley advanced breeding line VB9953 had high B concentration in the shoot (2000 mg kg^{-1} dry weight) but this did not impact negatively on shoot growth (Chapter 3). This feature is commonly termed 'internal tolerance' (Stangoulis and Reid, 2002). Such observations reinforce the view that reduced B uptake is unlikely to be the only tolerance mechanism. The high tissue tolerance to B is associated with enhanced sucrose contents within the leaf tissue and this corresponds to increased sugar deposition in the root growing zone (Chapter 6). This may compensate for sugars complexed to B that reduces sugar availability for further metabolism. Increased sugar deposition may also have a role in osmotic adjustment.

The fourth potential tolerance mechanism observed in barley grass, fababean, and the fine-roots of tap-rooted weed plants grown in soil (Chapter 3) has been less widely discussed. These plants were clearly able to compensate for root sensitivity to subsoil B by maintaining or increasing root biomass in the upper soil depths where

the concentrations of B were not toxic, thus they can continue to maintain shoot and root growth. This avoidance strategy, as an adaptive aspect to avoid B toxicity, may be sufficient to maintain healthy shoot growth where toxic B is only present in the subsoil. It is a tolerance mechanism that clearly requires consideration when field testing breeding lines for B tolerance, although it should be remembered that the advantage of such an avoidance strategy may be less under water-limited (drought) conditions experienced in the field.

In summary, plant tolerance to high concentrations of B in the growth medium may therefore comprise one or more of the following components:

- Reduction in passive B transport into the root by a potential physical barrier between the root cortex and xylem,
- Reduction in B within the root by induction of an active or facilitated efflux system.
- Tolerance of tissues to high B concentrations, possibly by expression of 2H Quantitative Trait Loci (QTL) that has a capacity to regulate carbohydrate metabolism in the source and sink regions and is linked to osmotic adjustment under B toxicity.
- An avoidance mechanism to cope with subsoil B is to redistribute root biomass in the upper soil depth.
- All the above hypotheses require further elucidation

7.3 Boron toxicity mechanism

Whilst attempting to understand the B tolerance mechanism, this study also observed that toxic B concentration negatively affect carbohydrate metabolism, including soluble sugar status and invertase activity, as well as cell division activity in the root meristematic zone. Although further work is required, the data indicates that B toxicity may not only be a direct effect on root morphology and function.

When the B-sensitive Clipper was challenged by high subsoil B concentration (Chapter 3), marked reductions in shoot and root growth in both topsoil and subsoil were observed. The same responses have been reported for another sensitive barley line where there was 21 mg kg^{-1} extractable B in the subsoil (Yau, 2001, 2002). In further evaluation of root morphology, a reduction of cell division in the root meristematic zone and effects on root elongation were observed in Clipper grown for 4 days at high B supply (Chapter 5). An investigation of the reducing sugar status in the root growing zone, where sucrose is unloaded from the phloem, indicated that B toxicity caused sugar starvation in the root zone of Clipper (Chapter 6). These results indicate that B toxicity has an effect on carbohydrate metabolism in the root tip of Clipper. Indeed, Picchioni *et al.* (1991) proposed that a direct consequence of high leaf B concentrations in pistachio seedlings is alteration in carbohydrate status of the root, and that ultimately reduces root growth. McNairn and Currier (1965) suggest that excessive callose accumulation in the leaf tissue with excess B causes impaired export of leaf sugars to root. The process of transporting B from root into the shoot at high B supply must be very quick as observed in this study (Chapter 4), and thus the excess B translocated into the shoot may complex with many sugar moieties that may act as a physical barrier to phloem loading of sucrose. However, the results presented in the study of Picchioni *et al*, 1991 and in this study (Chapter 6) demonstrate that the sucrose content itself did not significantly decrease in the root and rest of root, but only in the root tip at the site of phloem unloading (Chapter 6). In addition, in Chapter 3 (Expt. 3(b)), a significant reduction of subsoil root caused

by subsoil B toxicity was observed before any serious effect on the shoot. These results may therefore suggest that a site of B toxicity is at phloem unloading. This is a logical hypothesis as phloem unloading of solutes occurs through plasmodesmata (Pritchard *et al.*, 2000). Therefore it appears that the effect of B toxicity on root tips may be both direct and indirect.

The drastic decrease in reducing sugars in the root tips of Clipper at high B supply was accompanied by a decrease of acidic and neutral invertase activity in the root tip at which sucrose is unloaded. This lowering of activity in response to high B was also observed in the B-tolerant VB9953, but it was not as great as in Clipper. The negative affect of B on invertase activity in the Clipper root tip may cause a decrease of sucrose supply from the source leaf. It has been suggested that sucrose transport from source into sink tissues is controlled by 'sink strength' (i.e the ability to attract sucrose)(Sturm and Tang, 1999). The B stressed root of Clipper may gradually feedback the signal to decrease sucrose synthesis in the shoot tissues, and this may be one reason for a reduction in sucrose and increase in the reducing sugars within Clipper leaf tissue. It is known that released hexose sugars (glucose and fructose) play an important role in stress signaling. The accumulation of genes responding to a stressed condition requires a certain threshold concentration of hexose sugars (Sturm and Tang, 1999). It may also be due to three other factors. As was witnessed, the increase in both acidic and neutral invertase activity in the leaf of Clipper would lead to a lowering of the sucrose concentration and enhancement of the hexose sugars. While a reduction in sucrose concentrations in Clipper leaf tissue may be due to a greater demand for the hexose sugars for metabolism, it may also be due to a direct effect of B on the enzyme, invertase, or due to an effect of excess B on photosynthesis and subsequent sucrose synthesis.

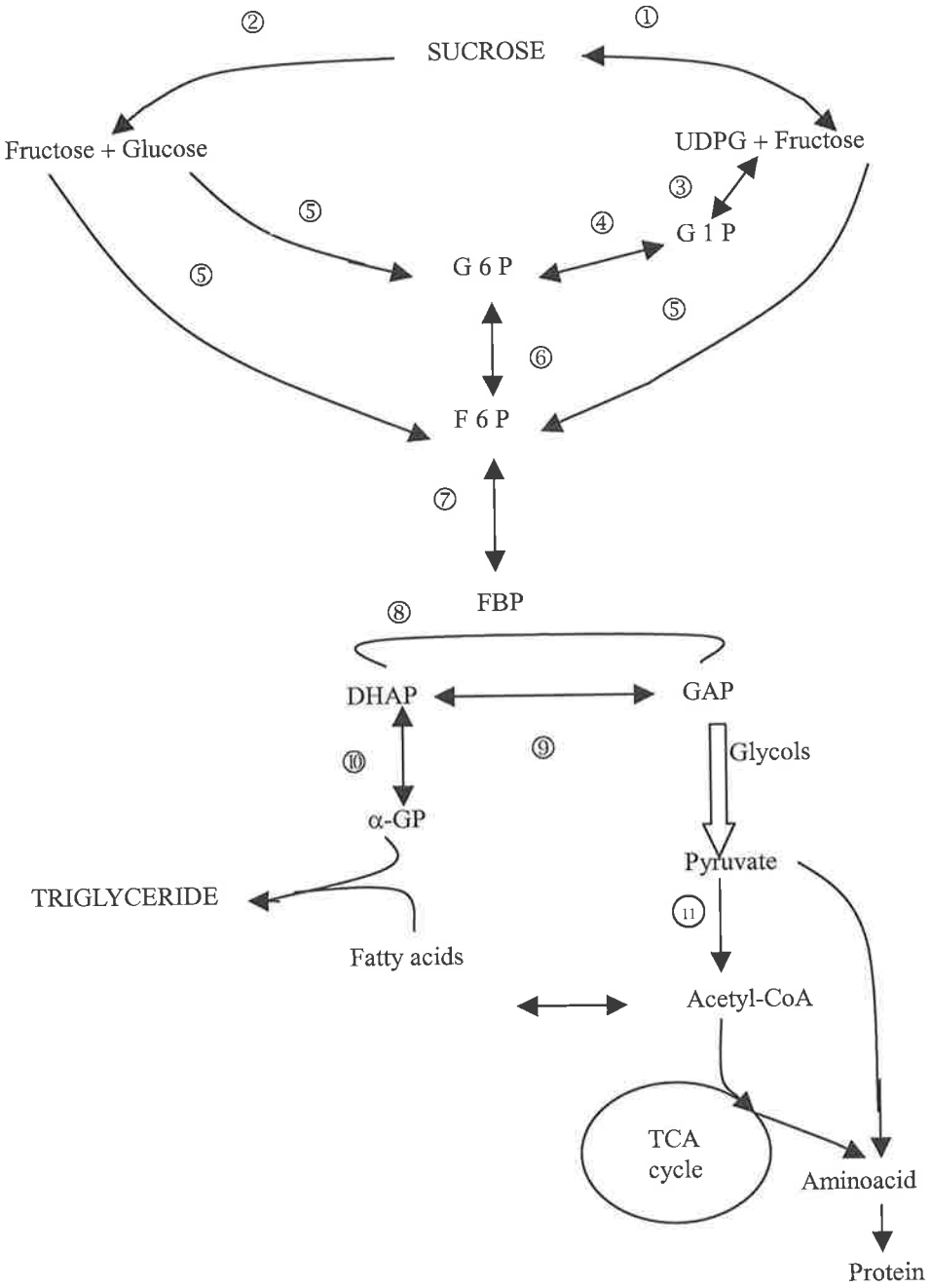
7.4 Physiological implications of this research

The data presented in Chapter 5 and 6 of this study suggest that a mechanism of B-tolerance is associated with increased sugar deposition in the root growing zone in B-tolerant VB9953 and Sahara, which maintains root elongation under B toxic conditions. This may arise from a need for maintaining root cell turgor and subsequent cellular growth. There are some reports to support this hypothesis. Cosgrove (1999) suggested that cell elongation requires cell wall loosening, and accumulation of intracellular solutes (i.e. soluble sugars). The increase of sugars may have a role in osmotic adjustment at the expense of cell wall synthesis and also inhibit cellulose synthesis in roots (Zhong and Lauchli, 1993). Growth in the root growing zone is facilitated by a high turgor pressure, low membrane resistance or an increased cell wall extensibility, thus growth requires both the maintenance of turgor to extend the cell wall and a water potential difference between the cell and wall to allow water uptake (Pritchard et al., 2000). Thus, solutes, such as the sugars, are required to generate the turgor pressure but will be diluted by water influx across the membrane thereby leading to root elongation in VB9953 and Sahara under B toxicity. VB9953 also enhanced sucrose in the shoot at the same time as enhancing glucose and fructose concentration in the root tips at high B supply, although the acidic invertase activity was increased in the shoot tissue (Chapter 6). This may suggest that elevated levels of sucrose in the shoot of the B-tolerant VB9953 may aid in osmoregulation. This adaptive mechanism would allows plants to maintain a positive cell turgor by continuing water uptake so that plants can grow under B stress situation. The elevated levels of sugar in VB9953 mature leaf tissue may also result from a need to supply more sugar to the root tip as part of the B-tolerance mechanism. Further physiological studies are required to investigate the relationship between mature leaf sugar status and the B-tolerance mechanism in the root growing zone.

7.5 Biochemical implications of this research

This study identified that B toxicity is associated with a starvation of sugars observed in the root growing zone of the B-sensitive Clipper. The reduced sugars in the root tips may lead to a 1) reduced respiration rate, 2) reduced organic compound synthesis (sugars, proteins, fatty acids, and adenine nucleotides), 3) reduced activity of enzymes involved in glycolysis, the pentose phosphate pathway, the tricarboxyl cycle, and fermentation, and 4) reduced intracellular osmolarity as reported in maize roots during glucose starvation (Brouquisse *et al.*, 1991).

At the same time when the root tip is starved of sugar as a result of B-toxicity, a decrease of sucrose and increase of hexose sugars in the leaf tissue was observed. While this may be due to a greater demand for the hexose sugars in metabolism, or due to a lower sink strength, it may also be due to a direct effect of B on the enzyme, invertase. As deficient B inhibits enzymes, such as 6-phosphogluconate (Dugger, 1973) and phosphoglucomutase (Loughman, 1961), B toxicity may also affect enzymes involved in carbohydrate metabolism. Theoretically, excess B may combines with glucose-1- phosphate or 6-phosphogluconate, reducing the utilization of glucose-1-phosphate, followed by inhibiting the formation of glucose-6 phosphate, or fructose-6-phosphate, hence reducing fructose available for the synthesis of sucrose (Diagram 2). Overall, the glycolytic pathway and pentose phosphate pathway could be interrupted (Loughman pers. comm.). There is some prior only evidence for excess B affecting enzymes in carbohydrate metabolism. Specifically the activity of 'aldolase' and GAPD (glyceraldehydes-3-phosphate dehydrogenase) in the glycolytic pathway was specifically decreased (Bowen, 1972 (b)). More biochemical research is required to elucidate the site where excess B may affect plant metabolism.



(Sites most likely affected by B toxicity are ③, ④, ⑥, ⑦, ⑧, ⑩)

Diagram 2. The conversion of sucrose to protein and lipid (Duffus, 1984).

DHAP	-dihydroxyacetone phosphate	FBP	-fructosebisphosphate
F6P	-fructose-6-phosphate	α-GP	-glycerol-3-phosphate
G1P	-glucose-1-phosphate	G6P	-glucose-6-phosphate
GAP	-glyceraldehyde-3-phosphate	UDPG	-uridinediphosphate glucose

(1) UDP-dependant sucrose synthase; (2) invertase; (3) UDP-glucose pyrophosphorylase; (4) phosphoglucomutase; (5) hexokinase; (6) phosphohexoisomerase; (7) phosphofructokinase; (8) aldolase; (9) triosephosphate isomerase; (10) glycerol-3-phosphate dehydrogenase; (11) pyruvate dehydrogenase

7.5 Suggestions for future research

The outcomes of this thesis can be viewed as another important step towards the development of B tolerant varieties of barley. The work provides detailed comparisons of the shoot and root response to B for a number of crop and weed species. In particular, the morphological and physiological nature of B tolerance in barley is investigated, and several tolerance mechanisms proposed. The scientific community is encouraged to consider the following suggestions for future research.

1. Investigating crop plant root development in relation to the temporal and spatial dynamic of extractable B in the soil profile, particularly as influenced by seasonal factors such as rainfall (Yau, 2002, Sadras *et al.*, 2003).
2. Testing the response of roots to the complex of edaphic constraints that often occur in conjunction with B (Nuttall *et al.*, 2003a), and thus attempt to identify, at the farm level, where and when B may be a limitation to wheat yield (Nuttall *et al.*, 2003b). Likewise, at the physiological level, there is a need to investigate the effect of excess B on accumulation of lignin and suberin in root cell walls, which may act to reduce B uptake rate in B tolerant Sahara.
3. Determine whether there are differences among cultivars in major sterol composition the plasma-membrane that may influence B uptake.
4. Elucidating more facts about the B toxic effect on the reduction of cell division.
5. Mapping the high sugar trait in the root tips of a Clipper × Sahara double haploid population to see if this new B-tolerance trait maps to the 2H QTL, which may have patterns of developmental regulation for carbohydrate metabolism, suggesting specialized functions for their response classes.

6. Determine phytohormone (i.e. auxin and cytokine in shoot, root and xylem and phloem exudates) that may induce root elongation under B stressed conditions and act to regulate carbohydrate metabolism in the B tolerant genotype.

7. Measure respiration rates, gas exchange, stomatal activity, and pectin contents of both tolerant and sensitive shoots and root under high B condition to determine the capacity of carbon partitioning to facilitate osmotic adjustment and cellulose synthesis in roots at the expense of cell wall synthesis.

8. Test the effect of B toxicity on plasmodesmata occlusion, which in turn would result in reduced sucrose transport to the root growing zone.

9. Determine whether organic acid or other compounds are binding to B in Sahara with their subsequent secretion. Sahara has the capacity to prevent excessive B uptake, and this may be due to the production of a compound that can complex the excess B and given it's water soluble, release it to the external growth medium. Although there is no evidence for the relationship between B tolerance and organic acids, secretion of organic acids from roots has been shown to play an important role in the external and internal Al-tolerance mechanism (Silva *et al.,* 2001). Some organic acids form a stable complex with ionic Al^{3+} and act as a detoxification agent (Ma, 2000).

10. Investigate the effect of excess B on carbohydrate metabolism, identifying the enzymes in the glycolytic pathway that B may inhibit.

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