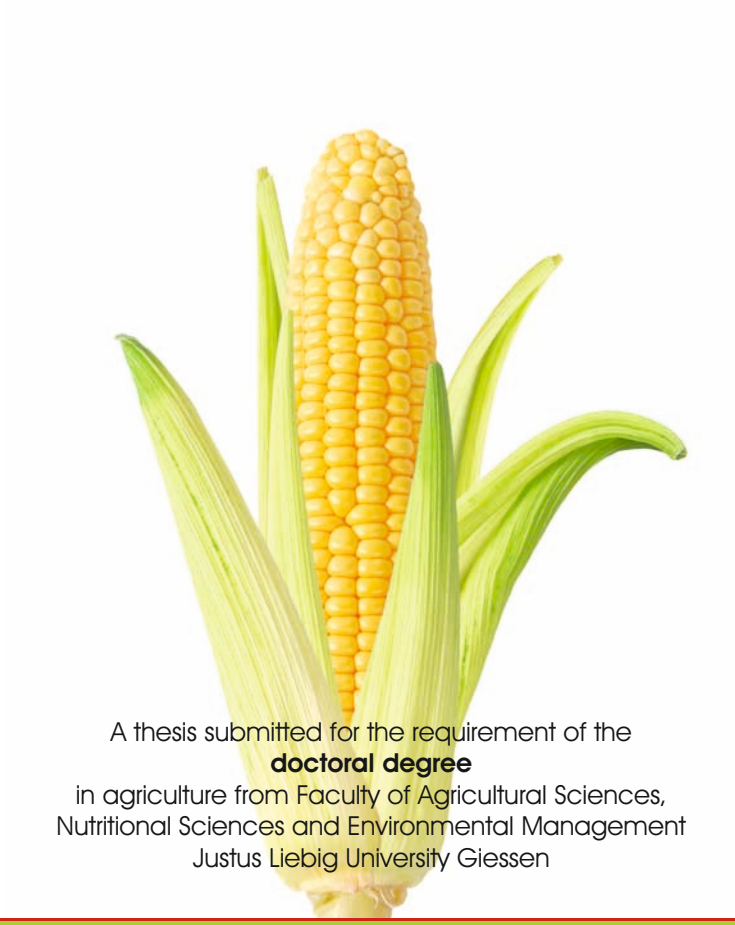
Role of Boron in Plasma Membrane H^+-ATPase Hydrolytic and Pumping Activity in Maize (*Zea mays* L.)

Ammara Fatima



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This thesis is dedicated to my parents, my husband, Sajid and finally my beloved sons, Haris and Saad, without their love and support; I would not have pursued my education to this level.

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1 INTRODUCTION

1.1 Boron in soil and plant

Boron (B) is member of the metalloid group of elements and has intermediate properties between metals and non-metals (Marschner 1995). The abundance of B in the universe is very low: about 10^{-9} times that of hydrogen and about 10^{-6} that of carbon, nitrogen, or oxygen (Kot 2009). In spite of its inadequacy, B is broadly distributed in both, lithosphere and hydrosphere, B concentrations ranging from 10 to 20 mg kg^{-1} in rocks, 3-30 µg kg^{-1} in rivers and about 1-10 mg kg^{-1} in oceans (Power and Woods 1997).

The B requirement for plant growth was first established in the early 1920s; nowadays it is well known that B is an essential micronutrient for all vascular plants. Boron deficiency or toxicity causes impairments in various biochemical and physiological processes (Blevins and Lukaszewski 1998; Bolanos et al. 2004). Soils with a B concentration less than 10 mg kg^{-1} are considered to be poor in B (Woods 1994). Moreover, the majority of this B is immobilized in rocks and not readily available for plants. During rock weathering, B goes easily into soil solution mainly as boric acid (Nable et al. 1997) and is readily available for plant uptake, but this is usually about 10% of total soil boron content (Power and Woods 1997).

Boron availability can be affected by several soil factors such as pH, texture, temperature, and organic matter, among others, soil pH being one of the most important parameters (Goldberg 1997). In fact, boric acid is a very weak acid and when the pH is below 7 appears in its undissociated form; at alkaline pH, boric acid dissociates to form the borate anion:

$$B(OH)_3 + H_2O \rightleftharpoons B(OH)_4^- + H^+ \qquad (pK_a\ 9.25)$$

Therefore, at common soil pH values (5.5-7.5), B exists mainly as soluble uncharged boric acid ($B(OH)_3$) having a pK_a value 9.25 , and in this form B is absorbed by plant roots (Hu and Brown 1997; Power and Woods 1997). Boron is mobile and easily lost by leaching under high rainfall conditions leading to B deficiency in plants that grow there.

Boron requirement for plant growth considerably varies among the plant species, the optimum quantity of B for one species could be either toxic or insufficient for other species (Blevins and Lukaszewski 1998). According to the B requirement, plants are separated into three groups: graminaceous species, the remaining monocots and most of the dicots and lactifers (latex-forming plants), having lowest, intermediate and the highest boron demand among plant species, respectively (Goldbach 1997; Blevins and Lukaszewski 1998).

Boron deficiency causes major disorder that can limit plant growth on soil with high rainfall (Nable et al. 1997). Therefore, it is important to understand the mechanisms that take part in B transport and distribution in plants in order to improve agricultural production. There are three different molecular mechanisms for transport of boric acid from soil solution into root cells and xylem loading of B. Thus, depending on B availability, B transport can be carried out by: (i) passive transport across plasma membrane mediated by simple diffusion. This system operates mainly when adequate or excessive boron is available in the soil; (ii) energy-dependent high-affinity transport that is induced in response to low B supply, and it is mediated via BOR transporters; and (iii) facilitated transport carried out by channels (NIP) belonging to the superfamily of major intrinsic protein (Tanaka and Fujiwara 2008).

After root cells have absorbed B, it must be loaded into xylem. When plants grow in media with enough B availability, uptake of B involves passive diffusion across lipid bilayer as well as facilitated transport of boric acid via the MIPs channel (Dannel et al. 2002; Miwa and Fujiwara 2010). Nevertheless, under limited B availability an energy-dependent high-affinity transport system mediated via BOR transporters is required to facilitate the transport of B towards xylem. Under B limitation, such B transporter involved in the process of xylem loading was identified as BOR1 in *Arabidopsis* (Takano et al. 2002). Afterwards, another BOR1-like gene has been identified in *Eucalyptus* (Domingues et al. 2005) and rice (Nakagawa et al. 2007), which is involved in both xylem loading of B and its absorption into the root cells under B limitation. Under sufficient B supply, expression of both NIP5;1 and BOR1 is decreased by transcriptional and post-translational regulations, respectively (Miwa and Fujiwara 2010).

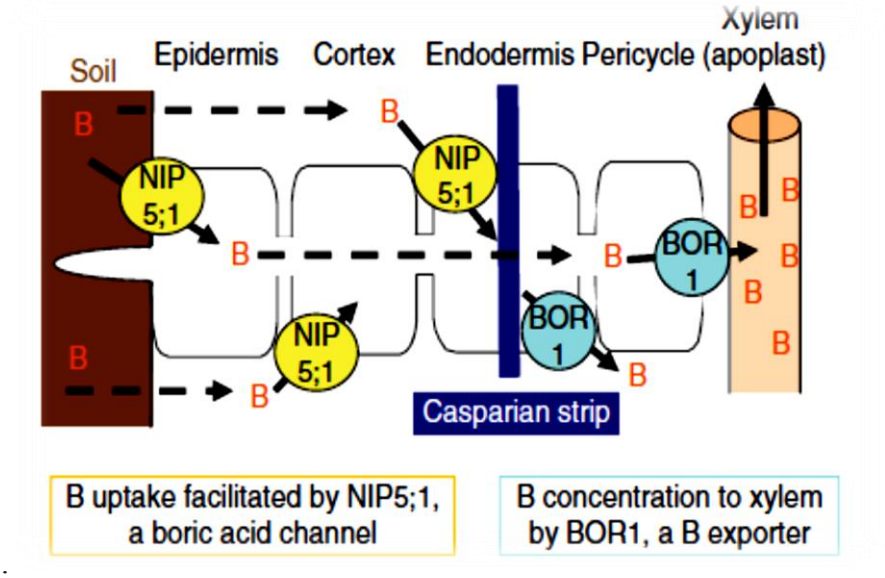

Figure 1. A schematic model of B transport in *A. thaliana* roots under B limitation (Miwa and Fujiwara 2010)

Once B entered the xylem, it is transported from root towards shoot via the transpiration stream (Shelp et al. 1995). Although the B mobility through phloem drastically varies among species (Brown and Hu 1996; Brown and Shelp 1997), there is some evidence that showed B can also be translocated via phloem to both vegetative and reproductive tissues (Matoh and Ochiai 2005). Theses authors suggested that the mechanism for B transport through phloem involves the formation of boron-diol complexes with sugar alcohols as transport molecules (Brown and Hu 1996; Hu et al. 1997). It was reported that transgenic tobacco and rice plants with enhanced sorbitol concentration had higher ability to transport B by phloem towards the young tissues (Bellaloui et al. 2003; Brown et al 1999). Although B transport via phloem, especially to growing tissues, also occurs in plant species that are not able to produce such types of carbohydrates, this translocation is not as efficient as in those plants that produce sugar alcohols (Stangoulis et al. 2001; Takano et al. 2001; Matoh and Ochiai 2005).

1.2 Role of boron in plant growth

Since the beginning of last century, B has been convincingly demonstrated to be an essential microelement for normal plant growth. However, its biochemical role is not well understood. Among the plant micronutrients, B deficiencies occur widely and have a significant agronomic impact throughout the world (Gupta 1979). Boron deficiency occurs mostly in light-textured soils, where B is readily leached down the soil profile with the percolatory water and becomes unavailable for the plants (Blevins and Lukaszewski 1998). Adequate B nutrition is crucial for high yields as well as for high quality of crops. Boron deficiency induces many anatomical, biochemical, and physiological aberrations. Because of the rapidity and the wide variety of symptoms followed by B deficiency, determining the primary function of B in plants has been one of the greatest challenges in plant nutrition.

Boron is directly or indirectly involved in many physiological and biochemical processes during plant growth. In recent years, considerable research activities have been carried out to demonstrate the function of B in plant physiology, including a structural role for boron in cell walls as well as in membrane function, and boron involvement in metabolic activities (Bolanos et al. 2004).

To date the only established physiological role of B in plants is based on the formation of borate esters with apiose residues of rhamnogalacturonan II (RG II) in the cell wall (Kobayashi et al. 1996), which is essential to its structure and function and contributes to the cell wall porosity and strength (Fleischer et al. 1999; Ryden et al. 2003). Furthermore, B deficiency led to a decrease of gene transcription of various hydrolytic enzymes such as XTHs, expansins, pectin methylesterases, and pectin lyases in *Arabidopsis* roots (Camacho-Cristobal et al. 2002). These enzymes play key a role in cell-wall loosening necessary for cell elongation (Cosgrove 1999). This indicates that B not only stabilizes cell wall by cross-linking of two RG-II molecules but regulates the transcription level of genes involved in the cell-wall synthesis and modification (Camacho-Cristobal et al. 2011).

The nutritional status of higher plants greatly affects the structural and functional integrity of cell membranes. There is considerable evidence that indicates a role of B in the

functioning of enzymes as well as other proteins of plasma membrane, transport processes across the membrane, and the membrane integrity by cross-linking the membrane molecules containing hydroxlated ligands such as glycoproteins and glycolipids (Cakmak and Römheld 1997; Goldbach et al. 2001; Brown et al. 2002, and Wimmer et al. 2009). Furthermore, B deficiency changed the membrane potential in *Daucus carota* (Blaser-Grill et al. 1989) and reduced the activity of proton-pumping ATPase in *Helianthus annuus* roots (Ferrol and Donaire 1992). Similarly, it has been also reported that B deficiency alters plasma membrane permeability for ions and other solutes (Cakmak et al. 1995; Wang et al. 1999). The influence of B on the ion flux can be mediated by direct or indirect effects of B on plasma membrane-bound proton-pumping ATPase (Cara et al. 2002). Pollard et al. (1977) also observed that the activity of the K^+-stimulated ATPase in B-deficient maize roots was considerably lower than in control plants. These results indicate that the action of B could be associated with membrane components. It is unclear, however, whether B directly interacts with membrane proteins or indirectly modifies membrane properties with subsequent changes in enzymatic activities.

The literature indicates the possible roles of B in several metabolic functions. For instance, it has been shown that B deficiency causes qualitative and quantitative changes in the phenolic metabolism (Camcho-Cristobel et al. 2002), affects the photosynthesis by reducing photosynthetic oxygen evolution rate and efficiency of photosystem II (Kastori et al. 1995; El-Shintinawy 1999), and affects nitrogen metabolism in vascular plants (Bolanos et al. 1994). Furthermore, B-deficient plants showed lower nitrate reductase activity and enhanced accumulation of nitrate, these effects indicating a role of B in the *de novo* synthesis of the nitrate reductase (NR) protein or facilitation of nitrate absorption (Ruiz et al. 1998).

1.3 Plasma membrane H^+-ATPase, nutrient uptake and plant growth

The plant plasma membrane H^+-ATPase is an important functional protein which plays a central role in plant physiology. Plasma membrane H^+-ATPase involves in ATP hydrolysis to transport protons out of the cytosol into apoplast and establishes an electrochemical gradient across the plasma membrane (Duby and Boutry 2009). This enzyme controls the major transport processes in the plant, such as root nutrient uptake and xylem or phloem loading. Moreover, this pump is involved in other physiological processes, such as stomata opening, expansion growth,

and cytosolic pH regulation. Generation of an electrochemical gradient across the membrane results in a proton-motive force wich is used by secondary transport for assimilation of various nutrients (Briskin and Hanson 1992; Morsomme and Boutry 2000; Palmgren 2001). A number of studies demonstrated that the plasma membrane H^+-ATPase is involved in the uptake of various nutrients such as nitrogen (Schubert and Yan 1997), phosphorus (Yan et al. 2002), potassium (Schachtman and Schroeder 1994), and iron (Schmidt 2003; Dell'Orto et al. 2000).

According to the acid-growth theory, protons extruded by an activated H^+-ATPase decrease the apoplastic pH and activate enzymes involved in cell-wall loosening (Hager 2003). It is well established that auxin activates the proton pump, resulting in loosening of the cell wall (Hager 2003). A higher concentration of H^+ in the apoplast may also activate cell-wall proteins such as expansins (Cosgrove and Li 1993; Cosgrove 2000) contributing to increase the cell-wall extensibility by breaking the load bearing bonds (Keller and Cosgrove 1995; Purugganan et al. 1997).

Plasma membrane H^+-ATPase is encoded by a multigene family and contains several isoforms, 9-12 isoforms of plasma membrane H^+-ATPase have been identified in various plant species. Isoforms related to nutrient transport and cell growth are widely expressed in most plant parts (Arango et al. 2003; Gaxiola et al. 2007). Isoforms present in a single cell or tissue type may have different enzyme characteristics, such as substrate affinity, $V_{max,}$ and pumping efficiency (Luo et al. 1999). The activity of plasma membrane H^+-ATPase is controlled by an auto-inhibitory domain at the C-terminus (Palmgren et al. 1991) and modifications in this domain can change the pumping efficiency of the enzyme (H^+ transport /ATP coupling).

Several reports in literature demonstrated that the presence of B in the root medium increased plant growth. B seems to be involved in cell wall expansion. As B is mainly localized in the cell wall (Hu and Brown 1994; Hu et al. 1996) and cross-links with rhamnogalacturonan II Kobayashi et al. 1996; O'Neill et al. 2004), it can be considered to be an important factor of cell-wall extensibility (Hu and Brown 1994; Findeklee and Goldbach 1996). It is well known that plasma membrane H^+-ATPase is involved in expansion growth by changing the cell-wall acidification. It may be assumed that the B-induced stimulation of plant growth is caused by changes in plasma membrane H^+-ATPase activity.

To elucidate the role of B on maize growth and its contribution in the regulation of plasma membrane H^+-ATPase in roots and shoots the following hypotheses were tested:

1. Exogenously B supply to the nutrient medium improves maize growth under normal growth conditions.
2. Changes in plasma membrane H^+-ATPase are responsible for the B-induced maize growth.
3. Boron modifies plasma membrane H^+-ATPase by direct interaction of B with ATPase molecule.
4. Boron-induced transcriptional modifications in H^+-ATPase contribute to enhance the activity of plasma membrane H^+-ATPase.

2 MATERIAL AND METHODS

2.1 Effect of boron supply in nutrient solution on maize growth under controlled conditions

2.1.1 Plant cultivation

Maize seeds (*Zea mays* L. cv. Amadeo) were soaked in aerated 1 mM $CaSO_4$ solution for 1 d and germinated on filter paper moistened with 1 mM $CaSO_4$ at 25°C for 4 d in the dark and then seedlings were exposed to light for 1 d. After 5 d, seedlings were transferred to 4.5 L plastic containers (three plants per container) containing ½ full-strength nutrient solution. After 2 d the concentration of nutrient solution was increased to full-strength concentration. With the transfer to nutrient solution plants were divided into two groups: one group was provided with boron-deficient (0 μM B) medium and the other with boron-sufficient (10 μM B) medium. To avoid any external boron concentration, all the nutrient solutions were prepared in Milli-Q ultra pure water and plastic wares were used during the whole experiment. The experiment was replicated four times per treatment. Plants were grown in a growth chamber under controlled conditions. Conditions in the climate chamber were: 50% relative humidity and a day/night temperature 26°C/ 16°C under a 16 h period with a light intensity of 150 Wm^{-2} (Philips Master HPI-T Plus, 400 W). The complete nutrient solution was changed after every 2 d. The concentration of nutrient solution had the same composition as described by Zörb et al. (2004, Table1).

Table 2.1 Composition of the full-strength nutrient solution.

Salt	Concentrations (mM)	Salt	Concentrations (μM)
$Ca(NO_3)_2$	2.5	H_3BO_3	10.0
K_2SO_4	1.0	$MnSO_4$	2.0
KH_2PO_4	0.2	$ZnSO_4$	0.5
$MgSO_4$	0.6	$CuSO_4$	0.3
$CaCl_2$	5.0	$(NH_4)_6Mo_7O_{24}$	0.005
Na_2SiO_3	1.0	Fe-EDTA	200

2.1.2 Plant height and leaf-area measurement

To study the effects of B supply in nutrient solution on maize expansion growth, plant height and leaf area of different leaves were measured using a ruler. Plant height was measured as the distance from plant base to youngest leaf tip. For leaf area calculation, length of each leaf was multiplied with its width and divided by two.

2.1.3 Plant fresh and dry mass measurement

21 d after germination, plants were harvested and separated into three parts: root, old shoot (portion below 4th leaf blade) and young shoot (portion with 4th leaf blade and above) Plant roots were thoroughly washed with deionized water thrice and blotted dry with tissue paper. After determination of fresh weights, different plants parts were oven-dried at 78^{o}C for 72 h and dry weight of plants was measured.

2.1.4 Boron analysis

Boron contents the plant tissues were determined using azomethene-H colorimetric method. Finely ground oven-dried plant material was ashed at 550 ^{o}C overnight. The cooled ash was carefully moistened with 2 mL of deionized water and then 2.5 mL of 5M HNO_3 were added to it. The samples were heated prior to boiling followed by cooling and then filtered through white band 589 filter paper to 25 mL volumetric flasks and filled up to mark. Then an aliquot of the sample was added to the reaction mixture containing:

2 mL buffer solution
2 mL color reagent
1 mL sample

Absorption was measured after 30 min using a spectrophotometer (Spektralphototmeter PM7) at 420 nm wavelength. A standard curve was prepared by measuring the absorption for each standard (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}) proceeding in the same way as for samples. A blank was also measured by adding 1 mL de-ionized water (Ryan et al. 2001). The composition of color reagent and buffer solution was as follows:

Buffer solution:
250 g ammonium acetate
15 g Na-EDTA
400 mL de-ionized water
125 mL glacial acetic acid
The solution was filtered and pH was adjusted to 5.1 with H_2SO_4.

Color reagent
0.45 g azomethine-H
100 mL 1% ascorbic acid

The reagent was filtered.

2.2 Effect of boron on plasma membrane H^+-ATPase hydrolytic and pumping activity isolated from maize root and shoot

2.2.1 Plant cultivation

Seeds of *Zea mays* L. cv. Amadeo were soaked in aerated 1 mM $CaSO_4$ for 1 d and germinated for 4 in dark between two layers of filter paper. Then seedlings were transferred to a climate chamber with a light intensity approximately 400 µE $m^{-2}s^{-1}$, a day/night cycle of 16 h/8 h at 26°C/18°C and a relative humidity of 50%. After 1 d, seedlings were transferred to 50 L

plastic containers (70 plants per container) with half-strength nutrient solution. After 2 d, plants were transferred to full-strength nutrient solution and boron treatment was started (as described in 2.1.1). Nutrient solution was renewed after each second day. The full-strength nutrient solution was as in Table 2.1.

2.2.2 Plasma membrane isolation

To investigate the effect of boron on plasma membrane H^+-ATPase, plasma membrane vesicles from the lower 10 cm of roots and from young leaves were isolated according to Yan et al. (2002). Shoots were cut above the fourth leaf and immediately placed in a beaker with the cut end immersed in cold water. The lower segment of about 10 cm length was cut and the mid-rib was removed. Root and shoot material was washed three times with chilled, deionized water, and then ground in ice-cold homogenization buffer (4 mL of buffer / g fresh weight).

The composition of homogenization buffer was:

250 mM sucrose
250 mM KI
2 mM EGTA
10% (v/v) glycerol
0.5% (w/v) BSA
2 mM DTT
1 mM PMSF
5 mM 2-mercaptoethanol,
50 mM BTP (adjusted to pH 7.8 with MES)

KI was not used for preparation of root vesicles. Homogenate was then filtered through two layers of Miracloth (Calbiochem-Novabiochem, San Diego) and was centrifuged in a swinging bucket rotor at 11,500 *g* (AH 629 rotor, 36 mL, Sorvall Products, Newtown, CT) for 10 min at 0°C. The supernatants were again centrifuged at 87,000 *g* for 35 min to yield the microsomal pellet which was re-suspended in phosphate buffer.

The composition of phosphate buffer was as follow:

250 mM sucrose

3 mM KCl

5 mM KH_2PO_4 (pH 7.8)

The microsomal membrane was fractionated with two-phase partitioning in aqueous dextran T-500 and polyethylene glycol according to the method of Larsson (1985). Stock solutions of polymers were prepared with concentrations of 20% and 40% (w/w) for dextran and polyethylene glycol, respectively. The concentration of the dextran stock solution was determined using optical rotation.

Phase separation was carried out in a series of 32-g phase system which contained:

For roots:

6.3% (w/w) dextran T-500

6.3% (w/w) PEG 3350

250 mM sucrose

3 mM KCl

5 mM KH_2PO_4 (pH 7.8)

For shoot:

6.1% (w/w) dextran T-500

6.1% (w/w) PEG 3350

250 mM Sucrose

3 mM KCl

5 mM KH_2PO_4 (pH 7.8)

The phase stock was diluted to 6.3% (w/w, each polymer) for roots and 6.1% for shoots with phase buffer to a final weight of 32 g. In the start tubes, polymers were diluted to 26 g. Six grams of microsomal suspension were added to the upper phase of each start tube. The tubes were sealed with Parafilm and then mixed by inversion (30 times). The phase system was centrifuged at 4°C and 720 *g* (Sorvall AH-629 rotor, 36 mL) for 23 min. The resulting upper

phase which contained plasma membrane was collected and centrifuged again in two identical separation steps. The centrifugation times for washing steps were 15 and 10 min, respectively. The third upper phase was diluted with phosphate buffer and centrifuged at 134,600 *g* for 40 min. The pellet was resuspeded and centrifuged again at 134,600 *g* for 40 min.

The resuspension buffer contained:
250 mM sucrose
3 mM KCl
5 mM BTP/MES (pH 7.8)
1 mM DTT
The resulting pellet was resuspended in resuspension buffer, divided into aliquots, and immediately stored in liquid nitrogen.

2.2.3 Protein quantification

Protein was quantified according to the method of Bradford (1976) using bovine serum albumin (Sigma) as a standard. 20 µL resuspended membrane protein were mixed with 2.375 mL reagent and incubated for 40 min at room temperature. The absorption was measured at 595 nm using a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia).

The composition of Bradford reagent was:
0.01% (w/v) Coomassie Brilliant Blue G-250
4.7% (w/v) alcohol
8.5 % (w/v) phosphoric acid

2.2.4 Hydrolytic activity of plasma membrane H^+ ATPase

Activity of plasma membrane H^+-ATPase was determined by measuring the amount of inorganic phosphate released by ATPase through ATP hydrolysis per unit time. Plasma membrane purity was estimated by measuring the inhibitor-sensitive ATPase activity of the various membranes. Vanadate (0.3 mM), nitrate (100 mM) and azide (1 mM) were used to inhibit the activities of plasma membrane, tonoplast and mitochondrial ATPases, respectively. 1 mM molybdate was used to measure the phosphate release due to activity of unspecific acid

phosphatases. Hydrolytic activity of plasma membrane ATPase was measured by incubating 3 µg of membrane protein at 30°C in 0.5 mL of reaction medium.

The reaction medium was composed of:
30 mM BTP/MES (pH 6.5)
5 mM MgSO4
50 mM KCl
100 mM KNO3
1 mM Na2MoO4
1 mM NaN3
0.02% (w/v) Brij 58
5 mM Na_2-ATP

ADP formation during ATP hydrolysis can inhibit the ATPase activity in the assay medium. Accumulation of ADP and decrease of ATP was prevented by an ATP-regenerating system which included 5 units of pyruvate kinase (Fluka) and 5 mM K-PEP The reaction was stopped after 30 min by adding 1 mL of stopping reagent containing:

2% (v/v) conc. H_2SO_4
5% (w/v) SDS
0.7% (w/v) $(NH_4)_2MoO_4$

Then 100 µL of 10% (w/v) ascorbic acid were added immediately after the stop reagent. To avoid the phosphate release by ATP hydrolysis under acidic conditions (Baginski et al. 1967), 1.45 mL of arsenite citrate reagent (2% [w/v] sodium citrate, 2% [w/v] sodium m-arsenite, and 2% [w/v] glacial acetic acid) were added after 15 min. Color development was completed after 30 min, and ΔA_{820} was measured by means of a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia). ATPase activity was calculated as phosphate liberated in excess of a boiled-membrane control. All enzyme assays were performed with two chemical replicates. Five vesicle preparations from parallel plant cultivations (biological replicates) were used to determine mean values of ATPase activity and standard errors (SE).

To determine the *in vivo* effect of boron on the hydrolytic activity of ATPase, 10 µM B were added to the assay medium. The kinetic characteristics (V_{max} and K_m) of plasma membrane ATPase were determined by means of a non-linear regression analysis with Dynafit (Kuzmic, 1996). Activation energy of ATPase was calculated, using the Arrhenius equation (given below), from V_{max} values determined at 20°C and 30°C, respectively.

$$E_a = \frac{RT_1T_2}{(T_1-T_2)} [\ln k_1 - \ln k_2]$$

2.2.5 Proton pumping

The pumping activity of plasma membrane H^+-ATPase in inside-out vesicles was measured as the quenching of A_{492} of acridine orange (AO) using a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia, Figure 2.1).

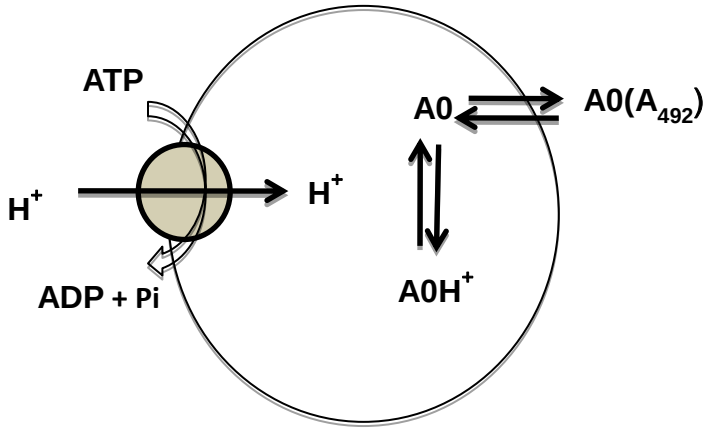

Figure 2.1: Principle of measurement of proton pumping.

The assay mixture contained:

5 mM BTP/MES (pH 6.5)

7.5 μM acridine orange

100 mM KCl

1 mM sucrose

1 mM NaN_3

1 mM Na_2MoO_4

100 mM KNO_3

0.05% (w/v) Brij 58

50 μg membrane protein in a final volume of 1.5 mL

To create *inside-out* vesicles Brij 58 was used (Johansson et al. 1995). After equilibration of the membrane vesicles with the reaction medium (about 15 min), the reaction was initiated by the addition of 5 mM Mg-ATP (mixture of $MgSO_4$ and Na_2-ATP, adjusted to pH 6.5 with BTP). The reaction temperature was 25°C. Change in absorbance during first min was calculated as the initial rate (IR) of active proton pumping and maximum pH gradient, respectively. Maximum quenching was achieved 70 min after initiation of the H^+ pump. At equilibrium, net H^+ transport across the plasma membrane was zero and active H^+ influx and passive H^+ efflux reached equilibrium. To determine passive H^+ transport, Na_3VO_4 (500 μM) was added after pH gradients of plasma-membrane vesicles had reached identical levels. The established pH gradient was completely collapsed by 5 μM gramicidine.

2.3 Gel electrophoresis and immunodetection of plasma membrane H^+-ATPase

Plasma membrane proteins were separated with SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) using the system of Laemmli (1970). Membrane vesicles containing 10 μg membrane proteins were solubilized in SDS-loading buffer for 30 min at room temperature to denature the proteins.

SDS-loading buffer contained:
0.125 mM TRIS-HCl, pH 7.4
10% (w/v) SDS
10% (v/v) glycerol
0.2 M dithiothreitol
0.002% (w/v) bromocresol blue
5 mM phenylmethylsulfonyl fluoride
0.05% (w/v) trasylol

The addition of the SDS detergent to these samples gives the proteins the same electrical charge. After 30 min shaking at room temperature (22°C), samples and standard marker (Sigma) were loaded on a discontinuous SDS-polyacrylamide gel (6% [w/v] acrylamide stacking gel and 10% [w/v] acrylamide resolving gel) and gel was run at 200 V until the blue band reached the bottom of the gel (approx. 1 h).

Composition of the stacking gel:

1.25 mL H_2O bidest.
0.625 mL 0.5 M TRIS-HCl, pH 6.8; 0.4% SDS
0.5 mL acrylamide solution
10 µL 10% APS (w/v)
10 µL TEMED

Composition of the resolving gel:
1.5 mL H_2O bidest.
1.5 mL 1.5 M TRIS-HCl, pH 8.8; 0.4% SDS
3 mL acrylamide solution
50 µL 10% APS (w/v)
5 µL TEMED

For Western blotting, the gels were incubated in blotting buffer for 15 min at room temperature. The buffer was prepared as under:

0.025 M TRIS base

0.192 M glycine and,

10% methanol with a pH of 8.3.

After incubation, the separated membrane proteins were electrophoretically transferred to polyvinylidene diflouride (PVDF) membrane filters (0.2 μm, Pall) as described by Zörb et al. (2005) using a semi-dry blotting system with a buffer containing 10 mM 3-cyclonexylamino-1-propane sulfonic acid (pH 11, adjusted with NaOH) and 20% (v/v) methanol for 1 h at room temperature and at a current intensity of 0.8 mA cm^{-2}. After transfer, the membrane filter was washed with H_2O bidest and incubated for 2 h in blocking buffer which was prepared by adding 2.5 g of milk powder in 50 mL of TRIS-bufferd saline (TBS).

The TBS solution was prepared as follows;

1 mM TRIS-HCl (pH 8.0)

15 mM NaCl

After 2 h, the blocking buffer attached to the membrane was removed by washing it three times with TBS-T. For the identification and quantification of plasma membrane H^+-ATPase, the PVDF membrane filter with plasma membrane proteins was incubated with a polyclonal antibody specific for the central part of plant H^+-ATPase (amino acids 340-650 of AHA2). The anti-serum was diluted 1: 3,000 in TBS-T buffer that has 1 mM TRIS-HCl (pH adjusted to 8.0 with NaOH), 15 mM NaCl, and 0.1% [v/v] Tween 20) and incubation was carried out for 1 h at room temperature followed by incubation at 4°C overnight. Next day, the membrane was rinsed to remove the antibody with TBS-T twice for 10 min. After washing, the membrane was incubated with secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma) for 2 h and was rinsed in TBS-T. After rinsing the filter was incubated for 5 min in a buffer containing 100 mM TRIS-HCl (pH 9.5, adjusted with NaOH), 100 mM NaCl, and 5 mM $MgCl_2$. After several washing steps in TBS-T or TBS buffers, filters were incubated for 5 min in AP buffer and Western Blots were developed using a buffer containing the substrates BCIP and NBT.

The buffer solutions were prepared as described below:

AP-buffer:

100 mM TRIS-HCl, pH 9.5

100 mM NaCl

5 mM $MgCl_2$

Developing buffer:

66 µL NBT (100 mg NBT in 1.9 mL 70% (v/v) dimethylformamid)

32 µL BCIP (100 mg BCIP in 1.9 mL dimethylformamid)

10 mL AP-buffer

For quantification of plasma membrane H^+-ATPase, the blots were scanned, and the H^+-ATPase immuno-reactive bands were quantified densitometrically (software TINA, Raytest Isotopenmessgeräte, Straubenhardt, Germany).

2.4 Effect of boron on the transcription of plasma membrane H^+-ATPase isoforms.

2.4.1 Isolation and purification of total RNA

To determine the effect of boron on mRNA transcription of plasma membrane H^+-ATPase isoforms, total RNAs were extracted from shoot tissues of maize. Shoots were cut above the fourth leaf and immediately placed in liquid nitrogen. The frozen shoots were ground in liquid nitrogen using a pre-cooled mortar and pestle. Total RNA was isolated from the powdered shoot material with TRIZOL reagent. This phenol-based reagent contains guanidine isothiocyanate and phenol. Guanidine isothiocyanate denatures RNAases and proteins while the phenol dissolves the proteins. Lipids are dissolved in chloroform which is added after the homogenization of samples. 100-150 mg plant material was mixed with 1 mL of TRIZOL reagent by vigorously vortexing. After 5 min of incubation at room temperature to permit the complete dissociation of nucleoprotein complexes, 200 µL of chloroform were added and samples were shaken for 15s then incubated at room temperature for 3 min and centrifuged at 12,000 *g* for 15 min at 4°C. Following centrifugation, the mixture was separated into three phases: upper colorless phase, an inter-phase and a lower phenol-chloroform phase (organic phase). The upper colorless aqueous phase contained RNA while DNA and protein remained in

the inter and organic phase, respectively. The volume of the aqueous phase was about 60% of the volume of TRIZOL reagent used for homogenization. The aqueous phase was collected and mixed with 0.5 ml isopropanol to precipitate the RNA. RNA were subsequently pelleted with 10 min centrifugation (13 000 rpm) at 4°C, washed with chilled 75% (v/v) ethanol to remove the isopropanol. The supernatant was removed and the pellets were air-dried to evaporate the residual ethanol. The samples were dissolved in 40 µL of DEPC-water and incubated at 60°C for 10 min. The RNA samples were shock-frozen in liquid nitrogen and stored at -80°C for further analysis.

2.4.2 Quantification of RNA

The amount of RNA was quantified using a Nano Drop spectrophotometer (ND 1000, Thermo Scientific. RNA has its maximum absorption at a wavelength of 260 nm. An OD_{260} of 1.0 is equivalent to about 40 µg/mL of RNA. The ratio of absorbance at 260 nm to 280 nm (OD_{260}/OD_{280}) provides an estimate for the purity of the RNA. Pure samples of RNA have an OD_{260}/OD_{280} ratio of 2.0 or more. If the ratio is below 2.0, the sample is contaminated with protein or phenol and cannot be used for further analysis.

2.4.3 Determination of RNA integrity

RNA integrity and DNA contamination were checked by using the 1% agarose gel electrophoresis. The integrity of the RNA can be assessed by the band intensity. A good-quality RNA shows two distinct bands of 18 S and 28S ribosomal RNA. The sharpness of the ribosomal RNA bands provides a rough indication of whether the mRNA is degraded. If the bands appear like a smear it is likely that the RNA was degraded during preparation. The gel was prepared by adding 1 g of agarose in 100 mL of TBE buffer. For the dissolution of agarose, the mixture was heated in a microwave for 3 min. The solution was allowed to cool down to 60°C and then ethidium bromide (6 µL) was mixed in agarose solution and poured onto the gel tray and allowed to solidify for 1 h. Prior to loading of RNA sample to the gel, 3 µg of RNA sample were mixed with RNA loading-buffer. The gel was run at 120 V for 1 h. The buffer used for electrophoresis purpose contained 400 mM TRIS-borate and 10 mM EDTA (pH 8.0), dissolved in bidest. H2O. RNA bound with ethidium bromide shows increased fluorescence compared to the unbound dye

in the background. The gel was illuminated under UV light and analyzed to check the integrity of RNA.

2.4.4 cDNA synthesis

Eukaryotic mRNA contains a 3‘sequence of polyadenylic acid, which is different from other RNAs (rRNA, tRNA and prokaryotic RNAs). With an oligo-dT primer, mRNA can be used as a template for reverse transcriptase to synthesize a complementary DNA (cDNA) on it. Compared with genomic DNAs, cDNAs have no introns or non-transcriptable regions.

The cDNA was synthesized following the manufacturer's instructions in the Verso™ cDNA kit from Thermo Fisher Scientific. 5 µg total RNA were diluted up to 10 µL with DEPC-Water and incubated at 70°C for 5 min to remove any RNA secondary structure. The reaction tubes were placed immediately on ice at least for 5 min and then the following reagents were added:

4 µL 5 × cDNA synthesis buffer
2 µL dNTP mix
1 µL RNA primer (Oligo-dT)
1 µL RT enhancer
1 µL Verso enzyme mix
1 µL DEPC water

The reaction was carried out in 20 µL of total volume. The reverse transcription was carried out at 42ºC for 50 min followed by inactivation of reverse transcriptase at 70ºC for 10 min. The cDNA was aliquotated and stored at -20 ºC for further use.

2.4.5 PCR amplification of cDNA

The polymerase chain reaction (PCR) is an enzymatic technique that facilitates the production of millions of copies of specific DNA. An enzyme called DNA polymerase carries out the amplification of cDNA, previously reverse-transcribed from RNA.

Principally, each PCR cycle consists of three steps:

Denaturation: separation of double-stranded DNA into single strands,
Annealing: primer binding to the appropriate sequence of single DNA strands,
Elongation: synthesis of a new DNA strand by DNA polymerase.

Amplification of cDNA was performed according to the manufacturer's instructions provided with Dream taq^{TM} DNA polymerase (Fermentas). All reactions were carried out in a T-Gradient Thermocycler (Biometra, Germany). The reaction mixtures were prepared for 10 µL reaction volume containing:

1.0 µL 10x PCR buffer (20 mM $MgCl_2$)
0.4 µL 25 mM $MgCl_2$
0.2 µL dNTPs (10 mM)
0.2 µL primer pair (100 p mol/ µL)
6.1µL DEPC H_2O
0.1µL *Taq* DNA polymerase (5 units/ µL)
2.0 µL cDNA synthesis (1:10)

The PCR reaction components were mixed on ice and reaction tubes were put into a T-Gradient Thermocycler (Biometra, Germany). List of primers used with target genes and annealing temperature is given on Tab 2.2. Negative (NTC) controls were used to control the specificity and reliability of the PCR.

The PCR was performed with the following profile:

Step	**Temperature**	**Time**
Initial denaturation	95ºC	3 min
Denaturation	95ºC	30 s
Primer annealing	50-60ºC	30 s
Extension	72ºC	1 min
Final elongation	72ºC	5 min

Annealing temperatures varied depending on the primers used in the experiment.

These steps were repeated for 35 cycles to produce the sufficient number of copies of amplicon.

Table 2.2: Primers of plasma membrane H^+ -ATPase isoforms from maize shoots designed for real-time PCR.

Primer name	**Primer sequence (5'-3')**	**Amplicon size (bp)**	**Gene accession no.**
sMHA1 asMHA1	TTTGGAAGTTTGACTTCCCA AAGAAGTCGGTCTTGTACGC	215	U09989
sMHA2 asMHA2	AAGACCTTCGGAAAGGAGAGA AAGACGGGTACCCAACCATA	385	X85805
sMHA3 asMHa3	GAGAACAAGACCGCCTTCAC AAGACGGGTACCCAACCATA	436	AJ441084.1
sMHA4 asMHA4	GAGAACAAGACCGCCTTCAC CTTGTTGTTCTTGCGACGAC	230, 380	AJ539534
sMHAfam asMHAfam	ATCGTCAGCCAGGCTCTGAT CGAAGCGGATGAAGAACTTG	231	
sZmActin asZmActin	GAGCTCCGTGTTTCGCCTGA CAGTTGTTCGCCCACTAGCG	172	J0238

2.4.6 Real-time PCR:

Quantitative real-time PCR was used to quantify mRNA. The procedure follows the PCR strategy but after each amplification round, the DNA is quantified. Quantification is performed by means of the fluorescent dye ‘‘SYBR® Green’’ that directly binds to double-stranded DNA. The bound dye generates a signal that is proportional to the DNA concentration. RT-PCR was done using RNA-based cDNA templates extracted from shoot tissues.

The reaction mixtures had the following composition (in a final volume of 10 µL):

2 µL cDNA (1: 10)

5 µL SYBR Green Mix

0.2 µL primer pair (10 nmol/ µL each)

2.8 µL sterile water

The real-time PCR reaction was initiated with activation of taq polymerase at 95ºC for 5 min. The cycling protocol of real-time PCR consisted of 35 cycles, each including:

Step	Temperature	Time
Denaturation	95ºC	30s
Primer annealing	50-60ºC	30s
Extension	72ºC	30s

After every elongation step, the fluorescence of SYBR Green was measured at 470 nm. As intercalating dyes bind nonspecifically to any double-stranded DNA, a melting curve analysis of amplification products allowed the differentiation at the end of the run. At the end, a melting curve was run from 72 to 99ºC. The melting curve prepared using SYBR Green fluorescence of obtained PCR-sequences detected no hairpin or loop formation. Single specific bands of the amplification products were checked using DNA gel electrophoresis. Negative controls with no templates (NTC) were performed with each run.

2.4.7 Relative quantification of the real-time PCR data

The relative transcription of H^+-ATPase at mRNA level was determined with the relative quantification of a target gene in comparison to a reference gene. The relative expression ratio (*R*) of a target gene was calculated based on *E* and the *CT* value presented by Pfaffle (2001):

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_{\text{T target}}(\text{control - sample})}}{(E_{\text{ref}})^{\Delta C_{\text{T ref}}(\text{control - sample})}}$$

Where,

$\Delta C_{Ttarget}$ = difference in the Ct values for the target gene between control and treated samples

ΔC_{Tref} = difference in the Ct values for the reference gene between control and treated samples

E_{target} = real-time PCR amplification efficiency of the target gene.

E_{ref} = real-time PCR amplification efficiency of the reference gene.

The equation shows a mathematical model of relative expression ratio in real-time PCR. The ratio of a target gene is expressed in a sample versus a control in comparison to a reference gene. E_{target} is the real-time PCR efficiency of target gene transcript and E_{ref} is the real-time PCR efficiency of a reference gene. In this study, actin was used as a reference gene according to Zörb et al. (2005). The expression of the reference (house-keeping gene) was not affected by the treatments. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of template concentration in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). Each sample was separately analyzed with actin as well as with other primers listed in Tab 2.2. In each case, standard curves were generated from dilution series of a single DNA sample. The values of the expression in each sample relative to the standard curve were calculated.

2.5 Statistical analysis

All the treatments were set up with at least four replicates and arithmetic means $\pm$ standard errors were calculated. The experimental data were subjected to stastical analysis. The significant differences among the treatments were determined using Microsoft Excel (2007) t-test. For all analyses, a P-value of less than 5% was interpreted as statistically significant.

2.6 Chemicals

Agar (Agar Agar Kobe I): Serva 11392

Ammonium-Molybdate ($(NH_4)_6Mo_7O_{24}$): 82.3 %; Sigma

AO = Acridine Orange (3, 6-Bis[Dimethylamine] Acridine-Base): ca. 95% purity; Sigma

L(+)-Ascorbic acid: min. 99.7% purity (idodometrisch); Merck

Brij 58 (Polyoxyethylen-20-cetyl ether): Sigma

BSA (Bovines serum albumin): fatty acid free ≥ 96% Albumin; Sigma

BTP (1, 3-Bis [tris (hydroxymethyl) methylamino] propane: min. 99 % purity (Titration); Sigma

Coomassie Brilliant Blue G-250: Calbiochem Corp., La Jolla

Dextran T 500: Average molecular weight = 485 000 g/mol; Sigma

Di-potassium hydrogen phosphate (K_2HPO_4): p.a.; Merck

DTT (DL-Dithiothreitol): 99 % purity (Titration); Sigma

EGTA (Ethylene glycol-bis (ß-aminoethylether) N,N,N`,N-Tetraacitic acid): 97 % purity; Sigma

Glycerin: 99 % purity; Sigma

Gramicidine D: from *Bacillus brevis*, 1080 µg Gramicidin mg-1; Sigma

2-Mercaptoethanol: min. 99% purity (GC); Serva

MES (2-[N-Morpholino]ethanesulfonic acid): Free acid, min. 99.5% purity (Titration).; Serva

Magnesium sulfate ($MgSO_4$): p.a.; Fluka

Na_2ATP (adenosin 5´-Triphosphate, Na2-Salz): 98 % purity; Merck

PEG 3350 (Polyethylene glycol): Average molecular weight = 3350 g/mol; Sigma

PEP (Phosphoenol pyruvate): Boehringer Mannheim GmbH

PMSF (Phenylmethylsulfonylfluoride): > 99 % purity (GC); Sigma

Potassium chloride (KCl): p.a.; Fluka

Potassium di-hydrogen phosphate (KH_2PO_4): p.a.; Fluka

Potassium iodide (KI): ≥ 99.5% purity (argentometrische Titration); Fluka

Potassium nitrate (KNO_3): p.a.; Merck

Potassium sulfate (K_2SO_4): p.a.; Fluka

Pyruvate Kinase: 1 000 U; Sigma

D (+)-Sucrose: For biochemical use; Merck

Sodium dodecylsulfate (SDS): 99 % purity; Sigma

Sodium azide (NaN_3): p.a.; Merck

Sodium citrate-Dihydrate: p.a.; Merck

Sodium-meta-arsenite ($NaAsO_2$): min.99 % purity; Sigma

Sodium molybdate (Na_2MoO_4): p.a.; Merck

Sodium orthovanadate (Na_3VO_4): min. 95 % purity; Sigma

Sodium silicate (Na_2SiO_3); Merck

3 RESULTS

3.1 Effect of boron on growth of maize

In order to investigate the effect of B on plant growth, plants were grown in B-deficient and B-sufficient nutrient medium in different treatments. After 21 d of growth plants were harvested. Before harvesting, plant height and leaf area of various leaves were measured. Plants were separated into three different parts: root, old shoot (portion below 4th leaf blade) and young shoot (portion with 4th leaf blade and above), and fresh weight of plant root and shoot was recorded. Different plants parts were oven-dried and dried mass of plants was measured. The results show that application of B in nutrient medium affected the plant growth (Figure 3.1). The plants grown in boron-deficient nutrient medium showed B deficiency symptoms in young growing leaves as shown in Figure 3.1. These include narrow white stripes along the length of the younger leaves.

Figure 3.1: Effect of boron on growth of *Zea mays* cv. Amadeo. Plants had been grown in nutrient solution without boron (B-) and with 10 µM boron (B+).

3.1.1 Effects of boron on maize plant height and leaf area of young growing leaves

Boron supply in the nutrient medium increased all plant growth parameters. Plants supplied with B in nutrient medium significantly increased the plant height in comparison to the

plants grown in B-deficient medium(Figure 3.2A). Boron did not show any effect on the expansion of older leaves (leaves no. 2,3,4 and 5). The leaf area of sixth and seventh leaf was significantly increased by application of boron in nutrient solution (Figure 3.2B).

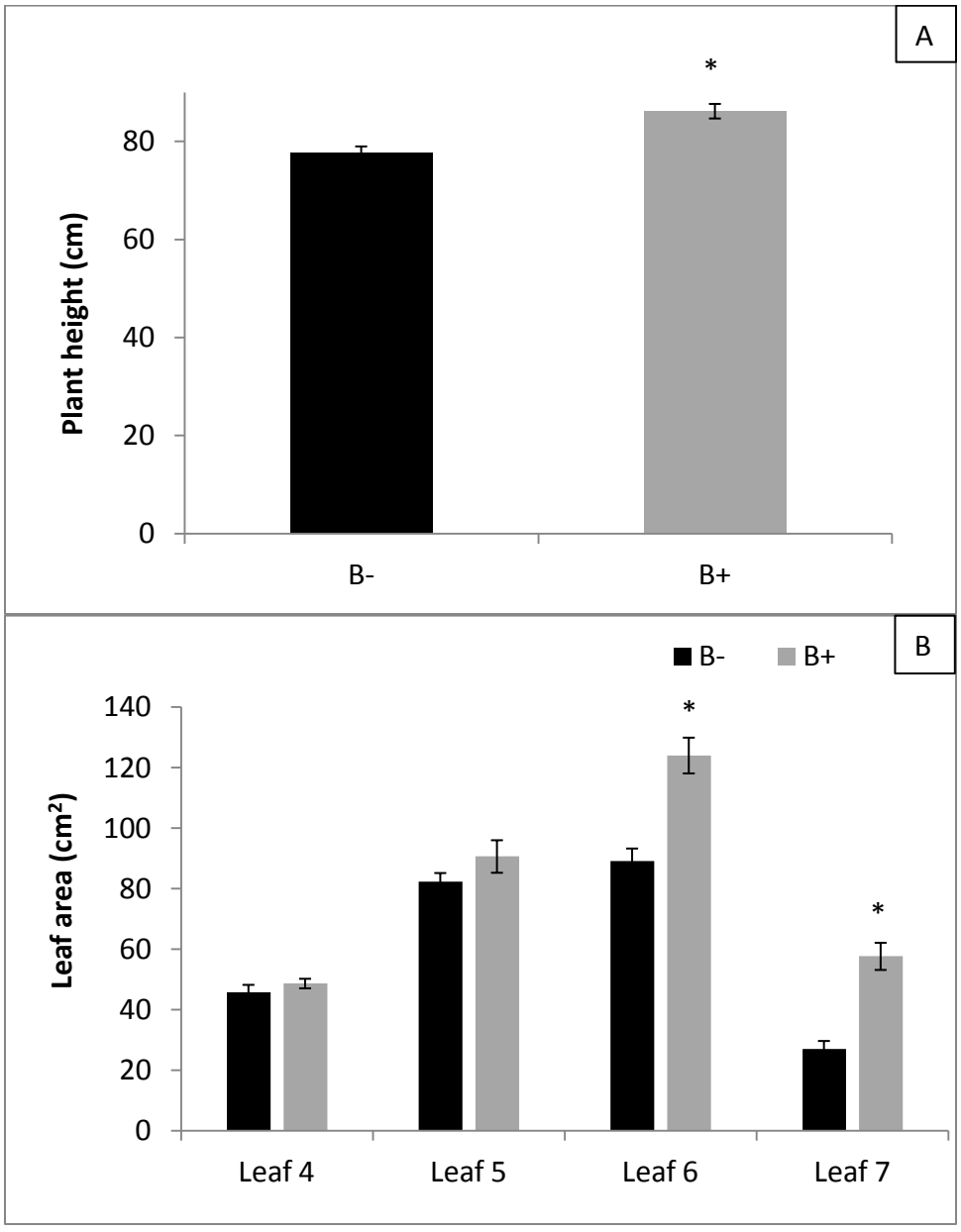

Figure 3.2: Effect of boron on plant height and leaf area of *Zea mays* cv. Amadeo. Plants had been grown in nutrient solution with boron (B+) and without boron (B-). Values are the means of four replicates $\pm$ standard error.

3.1.2 Effects of boron on fresh and dry mass of maize shoot and root

The results show that B application in the nutrient soution increased the root and shoot biomass. The plants supplied with B in nutrient medium produced higher biomass as compared to the plants grown without B supply in nutrient medium and this increase was significant in young shoot part (Figure 3A and B).

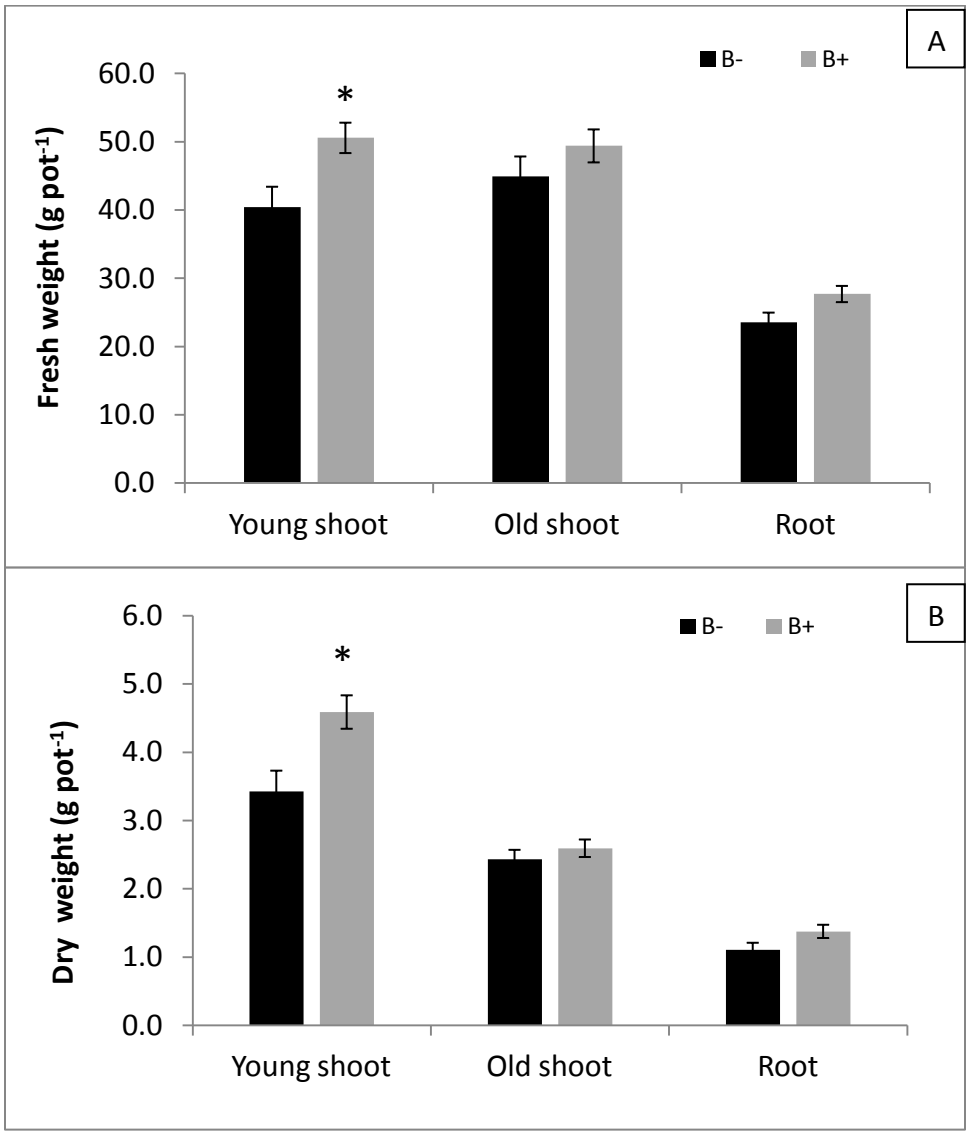

Figure 3.3: Effect of boron on plant fresh weight (A) and dry weight (B) of *Zea mays* cv. Amadeo. Plants had been grown in nutrient solution with boron (B+) and without boron (B-). Values are the means of four replicates ± standard error.

3.1.3 Effects of boron in nutrient solution on boron concentration in maize

To determine the B concentration the azomethine-H colorimetric method was used. The data presented in Figure 3.4 show that B supply in the nutrient medium resulted in higher B concentration in the shoot, and this increase was more pronounced in the young shoot. However, plants grown without exogenous supply of B in nutrient medium also contained substantial amount of B in the plant tissues but this concentration was below the critical concentration of B required for maize shoot. In case of root tissues no differences were found in both treatments (Figure 3.4).

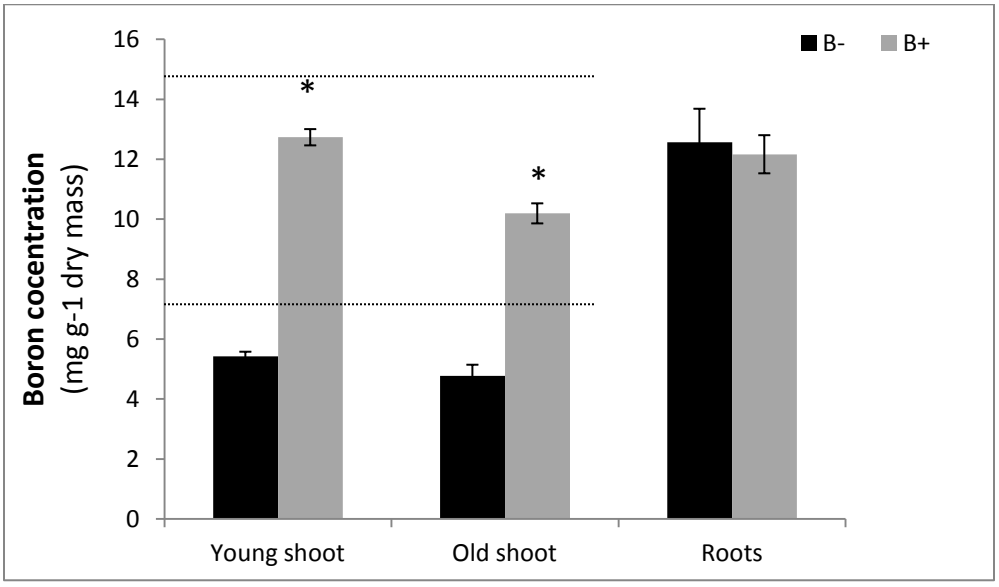

Figure 3.4: Effect of boron supply in nutrient medium on boron concentration in maize shoots (young and old). Plants had been grown in nutrient solution with boron (B+) and without boron (B-). Dotted line shows the critical range of boron concentration for maize shoots. Values are the means of four replicates ± standard error.

3.2 Role of boron in plasma membrane H^+-ATPase characteristics

3.2.1 Effects of boron supply in nutrient solution on the purity of plasma membrane vesicles isolated from maize shoots and roots

In order to investigate the role of B in H^+-ATPase activity, plasma membranes were isolated with two phase-partitioning from maize roots and shoots grown under boron-deficient (B-) and boron-sufficient (B+) conditions. To avoid an underestimation of the enzyme activity due to contamination by other endoplasmic membrane phosphatases, we analyzed the ATPase hydrolytic activity at pH 6.5 in the presence of nitrate, azide, molybdate, and vanadate, the inhibitors of tonoplast H^+-ATPase, mitochondrial H^+-ATPase, nonspecific phosphatases, and plasma membrane H^+-ATPase (Yan et al., 1998). The inhibitor-sensitive ATPase hydrolytic activity of each membrane fraction was calculated by subtracting the ATPase hydrolytic activity in the presence of specific inhibitors from the activity without any inhibitor. The summary of ATPase specific activities is presented in Figure 3.5. The ATPase activity showed a negligible inhibitory effect to azide in all membrane fractions, while the nitrate-sensitive activity was slightly increased in shoot vesicles due to the presence of extra potassium in the form of KNO_3. On the other hand, the membrane fraction showed about 80% (in roots) and 85% (in shoots) sensitivity to vanadate. Boron supply in the nutrient solution during the plant cultivation did not affect the membrane purity significantly. However, the membrane fractions showed a slight sensitivity to molybdate, which indicates the presence of unspecific acid phasphatases (Widell and Larsson, 1990). Therefore, to exclude the contamination effect of all non-specific phosphatases, all analysis of ATPase activity were carried out in the presence of 1 mM molybdate along with 100 mM nitrate and 1 mM azide.

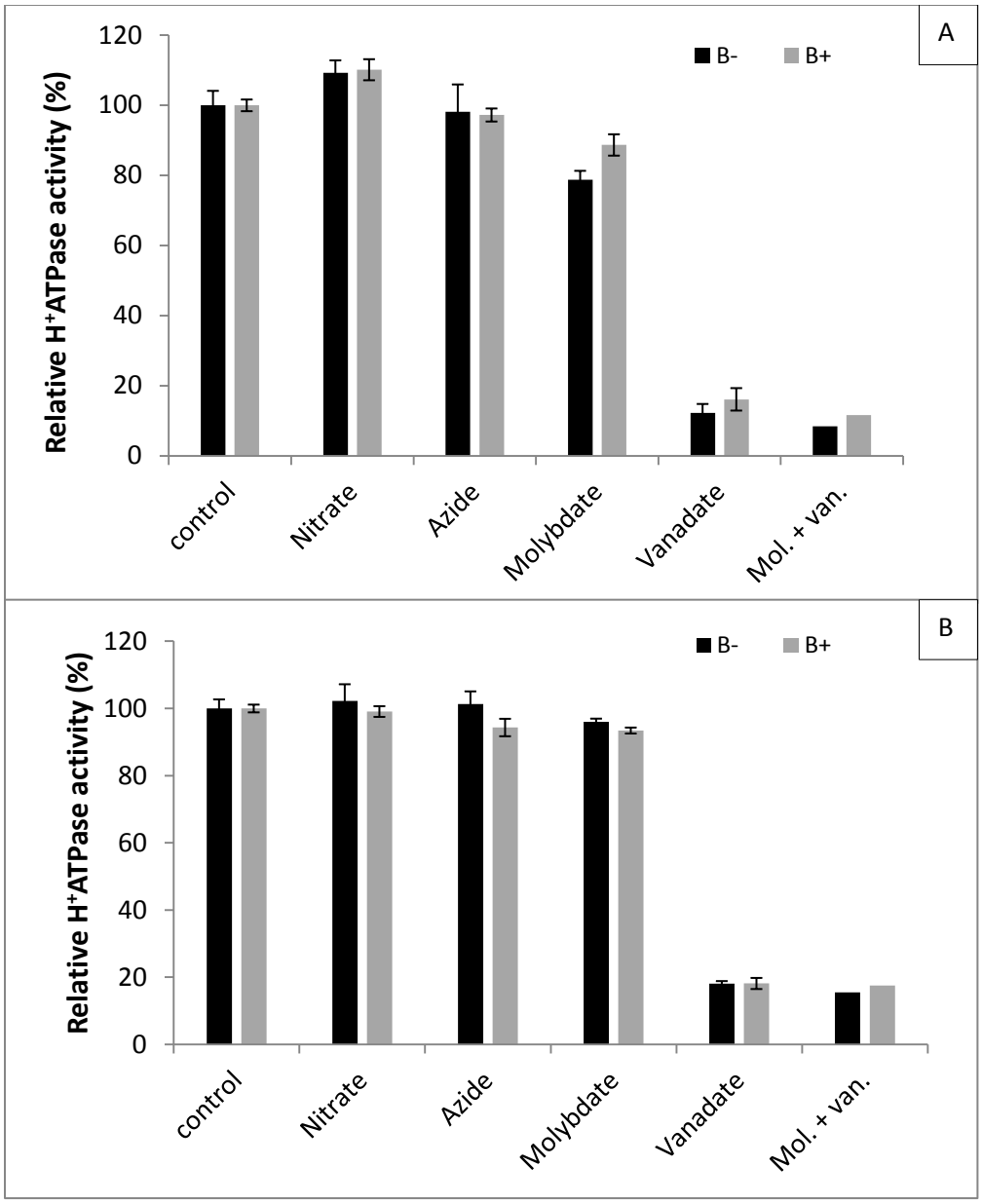

Figure 3.5: Inhibitor-sensitive ATPase hydrolytic activity associated with plasma membranes isolated from maize shoots (A) and roots (B). Specific inhibitors were used as markers of tonoplast (nitrate), mitochondrial (azide), acid phosphatase (molybdate), and plasmalemma (vanadate) origin. Assays were conducted at 30°C. The inhibitor-sensitive activity was calculated by subtracting the ATP hydrolytic activity in the presence of inhibitor from the activity of the control. The values represent means ± SE (percentage relative to control) of four independent experiments.

3.3.2 Effect of boron nutrition on hydrolytic activity of plasma membrane H^+-ATPase isolated from roots and shoots

To determine the effect of B on plasma membrane H^+-ATPase, the hydrolytic activity was measured at pH 6.5. Hydrolytic activity of plasma membrane H^+-ATPase was determined as hydrolysis of ATP and subsequent release of inorganic phosphate per unit time. Boron application in nutrient medium did not change the hydrolytic activity in vesicles isolated from maize root significantly (Figure 3.6B). However, slightly increased hydrolytic activity in vesicles isolated from shoot treated with boron was observed, compared with plants grown without B (Figure 3.6A). This difference was not statistically significant.

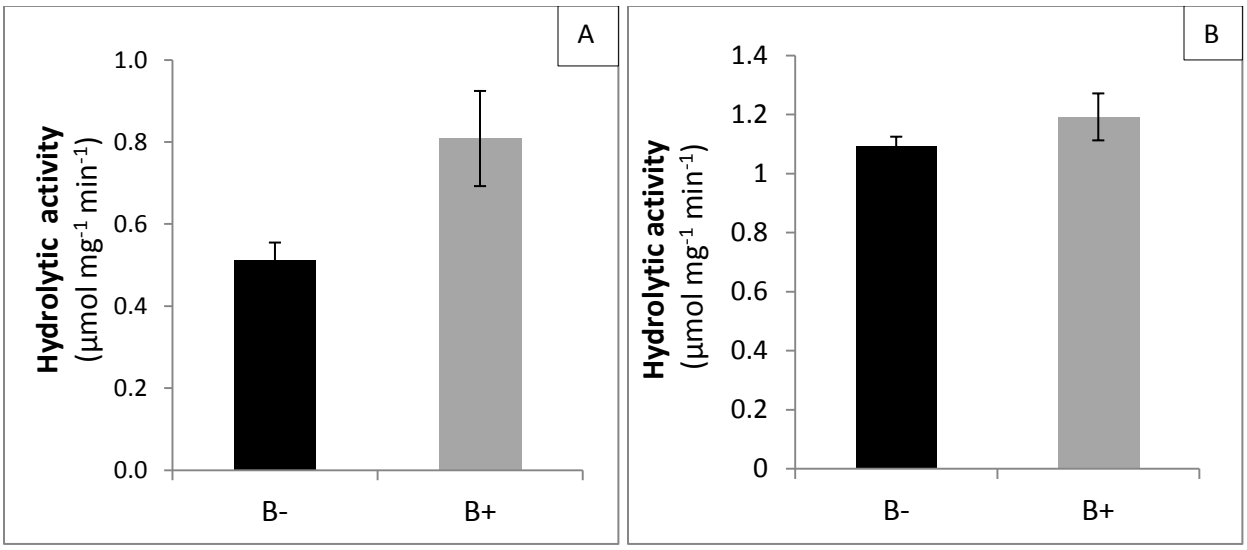

Figure 3.6: H^+-ATPase hydrolytic activity of plasma membranes isolated from maize shoots (A) and roots (B). Plants were grown in nutrient solution with boron (B+) and without boron (B-). Plasma membrane vesicles were isolated and purified from the young, developing shoots and from roots by two-phase partitioning. Plasma membrane ATPases activities were analyzed in 30 mM BTP-MES buffer (pH 6.5) in the presence of 1 mM molybdate, 1 mM azide, and 50 mM nitrate at 30°C. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentrations (5 mM) in the assay medium. Values represent means ± SE of four independent experiments.

3.2.3 Effect of boron on H^+ transport activity of plasma membrane ATPase isolated from roots and shoots

In order to investigate the role of boron supply in nutrient medium on plasma membrane H^+-ATPase transport activity, active and passive transport of H^+ across the membrane were measured. Active transport can be characterized as initial rate of H^+ pumping and maximum pH gradient, whereas passive transport was measured as passive efflux of H^+ from vesicles.

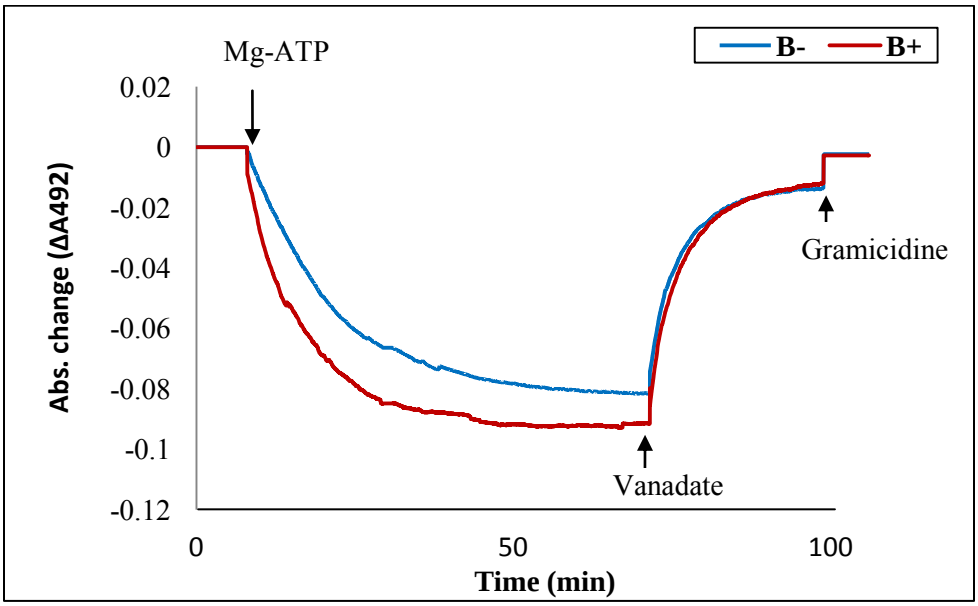

Figure 3.7: Comparison of active H^+ transport across the plasma membrane of inside–out vesicles isolated from maize shoots grown in nutrient solution with boron (B+) and without boron (B-). Formation of the pH gradient was monitored as quenching of acridine orange at 492 nm. The assay medium contained 5 rnM BTB/MES (pH 6.5), 7.5 µM acridine orange, 100 mM KCI, 0.05% (w/v) Brij 58 and 50 mg membrane protein in a final volume of 1.5 mL. After equilibration of the membrane vesicles with the reaction medium (at least 15 min) the reaction was initiated by the addition of Mg-ATP to give a final concentration of 5 mM. The reaction temperature was 25°C. For passive H^+ transport, Na_3VO_4 (500 µM) was added after pH gradients of plasma-membrane vesicles had reached on equilibrium. The established pH gradient was completely collapsed by 5 µM gramicidin (Gram.).

Plasma membrane H^+ transport was monitored by A_{492} quenching of acridine orange (AO). Acridine orange is a weak base, which accumulates inside the vesicles on acidification and

consequently absorbance at 492 nm of acridine orange is decreased. After initiation of H^+ pumping by adding 5 mM Mg-ATP to the reaction medium containing 50 µg plasma membrane protein, there was rapid quenching indicating rapid transport of H^+, eventually reaching at a constant level. To determine the passive transport of H^+, the pumping activity was completely inhibited by adding 500 µM vanadate. Furthermore, the established pH gradient was completely reversed by adding 5 µM gramicidin (Figure 3.7). The initial rate of H^+ pumping was the quenching of absorbance within the first minute after activation of H^+-ATPase (Figure 3.7) which shows active H^+ influx into plasma membrane vesicles (Yan et al., 1998). Maximum quenching was attained 60 min after initiation of the proton pump. At this point, equilibrium was reached between H^+ influx due to active pumping and passive efflux because of leakage, and the net H^+ transport across the plasma membrane was zero. This parameter indicates the maximum pH gradient that can be created by H^+-pumping activity. At assay pH 6.5, initial rate, maximum pH gradient and passive efflux of H^+-ATPase isolated from maize roots remained the same in both treatments. These results show that boron supplied in the nutrient solution did not affect the pumping activity of plasma membrane H^+-ATPase isolated from maize roots as shown in Table 3.1.

Table 3.1: Effect of boron on the initial rate of H^+ transport into vesicles, maximum pH gradient, and passive H^+ transport across the membrane of vesicles isolated from maize roots grown in nutrient solution under controlled conditions. The assay was conducted at 25 °C, pH 6.5, with 5 mM Mg-ATP concentrations, using 50 mg of membrane protein. The values represent means ±SE of five independent experiments.

Treatments	**Active H^+ transport**		**Passive H^+ transport**
	Initial rate of H^+ pumping ($1000\Delta A_{492}$ min^{-1})	pH gradient (ΔA_{492})	($1000\Delta A_{492}$ min^{-1})
B-	8.71 ± 1.02	0.075 ± 0.004	6.16 ± 0.53
B+	9.18 ± 0.95	0.077 ± 0.006	6.27 ± 0.80

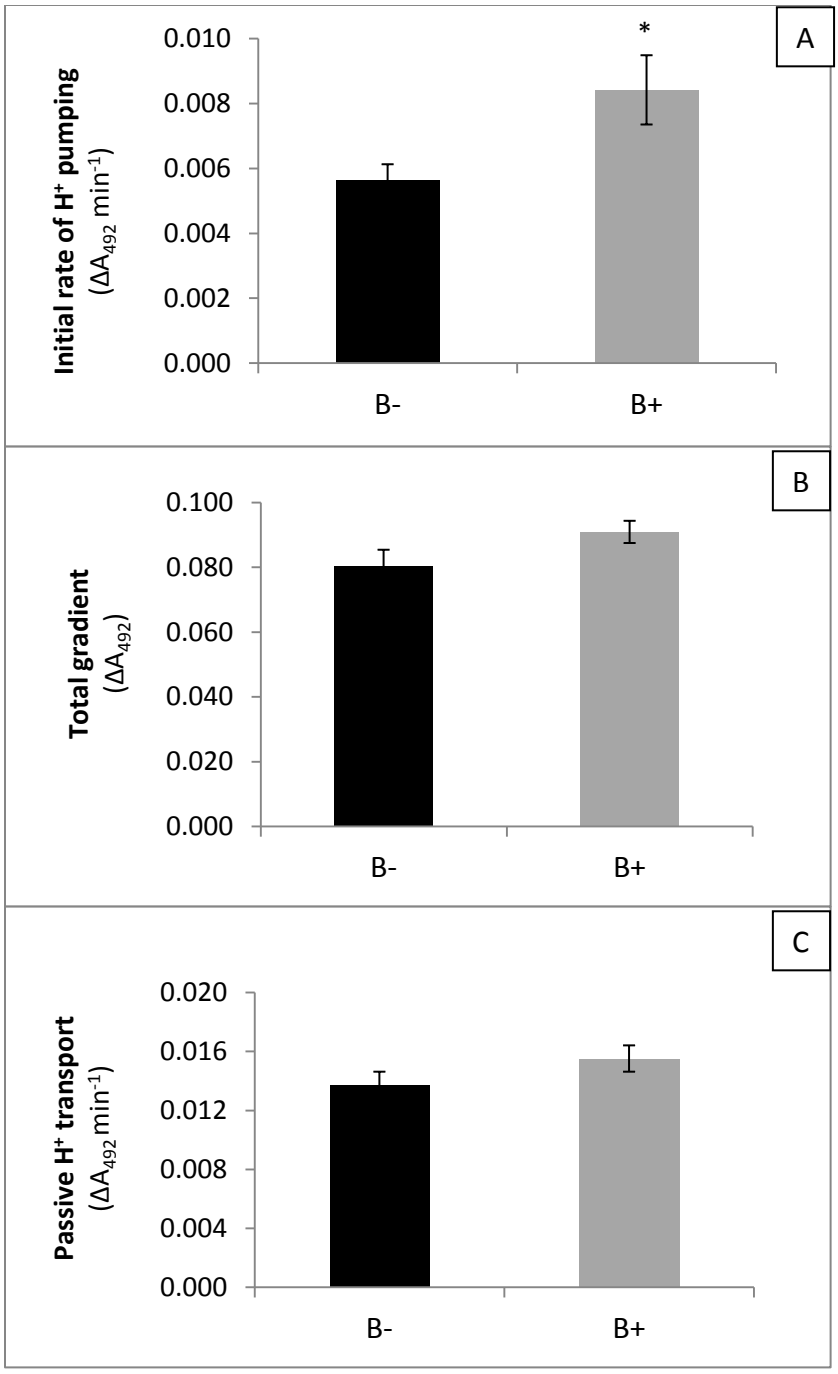

Figure 3.8: Effect of boron on pH gradient, initial rate of H^+ pumping, and passive efflux of H^+ -ATPase shown in A,B and C, respectively. Membrane vesicles were isolated from maize shoots treated with and without B in nutrient solution. Assay conditions were described in Figure 2 .Values are the means of five replicates $\pm$ SE.

In case of shoot vesicles, active H^+ transport rate of H^+-ATPase was significantly increased in boron-treated plants as compared to the plants grown without boron (Figure 3.8A

and 3.8B), while the passive H^+ transport rate was not affected by the boron supply in nutrient medium (Figure 3.8C).

To conclude the overall effect of B on plasma membrane H^+-ATPase, pumping efficiency was calculated. Pumping efficiency is ratio of active proton pumping to hydrolytic activity. This ratio reflects the decrease in absorbance of AO due to number of proton pumped by H^+-ATPase per unit of ATP utilized. No significant differences were observed in pumping efficiency of plasma membrane H^+-ATPase derived from shoots as well as roots of maize (Figure 3.9Aand B).

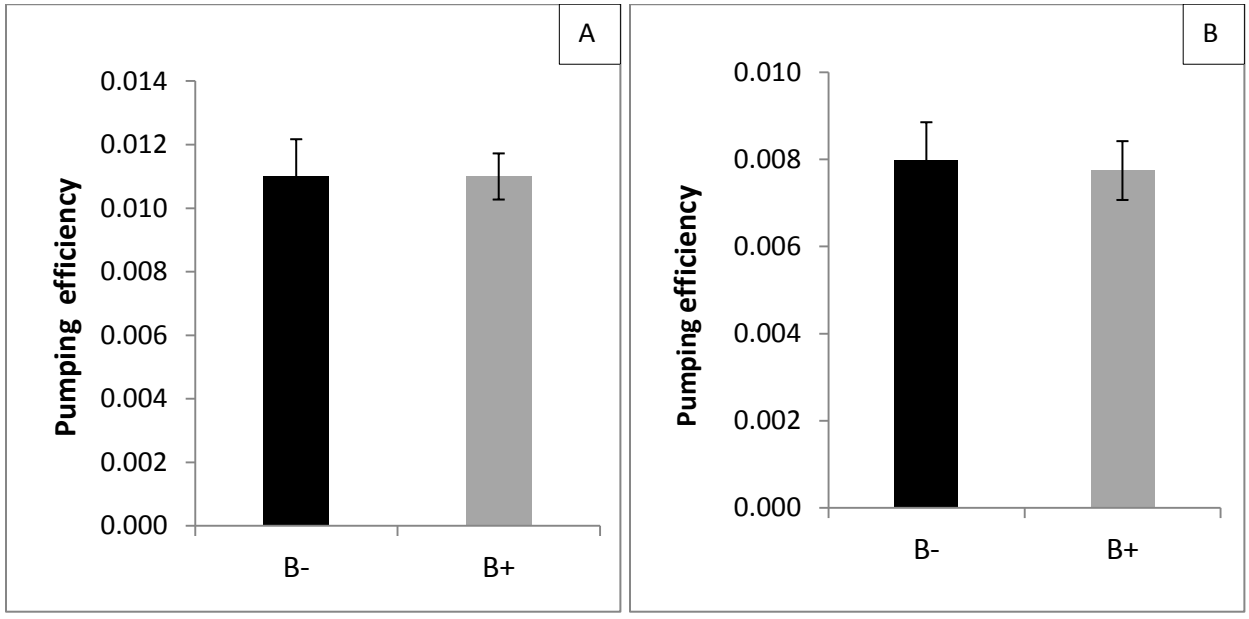

Figure 3.9: Effect of boron on proton pumping efficiency of plasma membrane H^+-ATPase isolated from maize shoots (A) and roots (B). Pumping efficiency was calculated as ratio of initial rate of H^+ pumping to the hydrolytic activity.

3.2.4 Effect of *in vitro* boron addition on plasma membrane H^+-ATPase hydrolytic activity isolated from maize shoot and root

The results in previous experiments showed that *in vivo* application of B in nutrient medium enhanced the pumping activity of plasma membrane H^+-ATPase. In order to elucidate the direct effect of boron on plasma membrane H^+-ATPase *in vitro*, hydrolytic activity was determined by adding boric acid (pH 6.5) in the reaction medium. The results presented here showed that B addition in assay medium slightly decreased the hydrolytic activity of plasma membrane H^+-ATPase (Figure 3.10). This decrease was more pronounced in the plants grown in the presence of boron in nutrient medium but the results are statistically non significant.

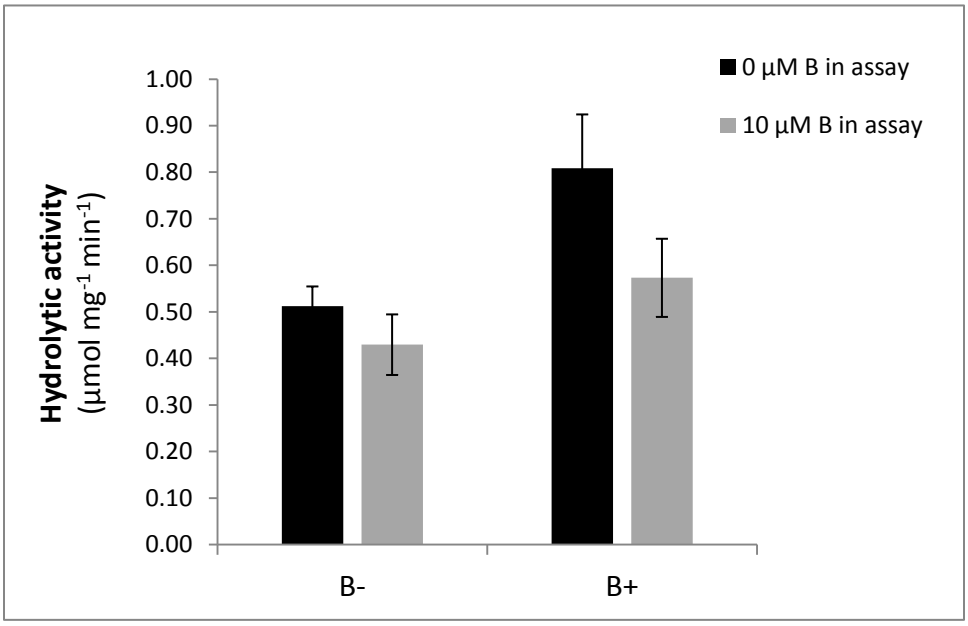

Figure 3.10: *In vitro* effect of boron (10 µM) on the hydrolytic activity of the plasma membrane ATPase. Plasma membrane vesicles were isolated from young growing maize shoots grown with and without 10 µM boron supply in nutrient solution. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentration (5 mM). The values represent means ± SE of four independent experiments.

In case of root vesicles, the results showed that *in vitro* application of B did not affect the plasma membrane H^+-ATPase hydrolytic activity (Figure 3.11).

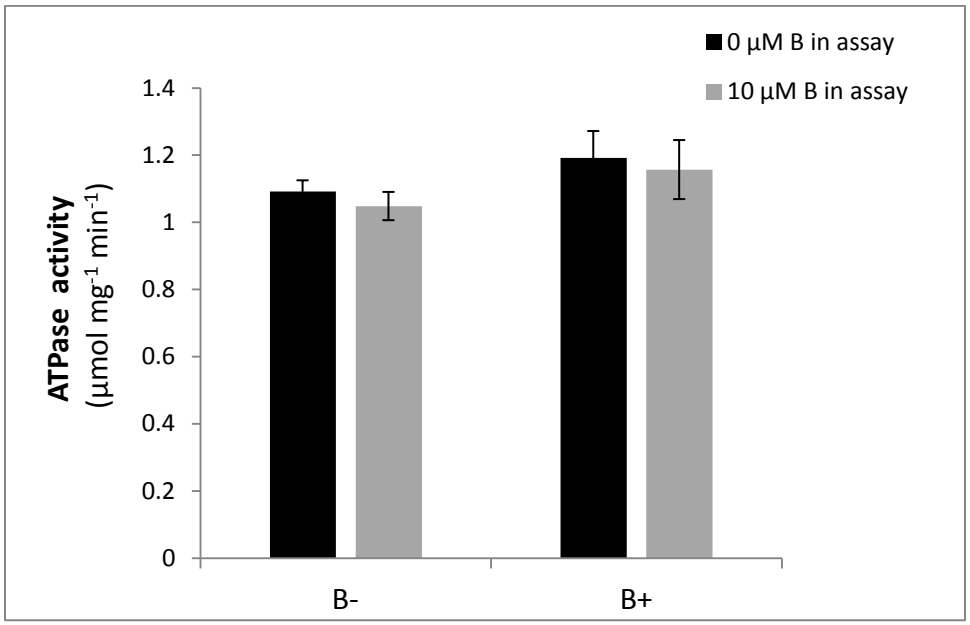

Figure 3.11: *In vitro* effect of boron (10 µM) on the hydrolytic activity of the plasma membrane ATPase. Plasma membrane vesicles were isolated from young growing maize roots grown with and without 10 µM boron supply in nutrient solution. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentration (5 mM). The values represent means ± SE of five independent experiments.

3.2.5 Effect of *in vitro* boron addition on plasma membrane H^+-ATPase transport activity isolated from maize shoot and root

Effects of *in vitro* application of B on the initial rate of active proton pumping, passive efflux and pH gradient developed by H^+-ATPase in membrane vesicles of both treatments are shown in Tab. 3.2. In this assay same plant material as for the results resented in Figure 3.8. Addition of B in the assay medium did not affect the proton extrusion by plasma membrane H^+-ATPase, which indicates that B does not directly inhibit H^+-ATPase activity.

Table 3.2: *In vitro* effect of boron (10 μM) on H^+-transport activities of the plasma membrane ATPase. Plasma-membrane vesicles were isolated from young growing maize shoots and roots grown with and without 10 μM boron supply in nutrient solution. The assay was conducted at 25 °C, pH 6.5, with 5 mM Mg-ATP concentrations, using 50 mg of membrane protein. Values represent means ± SE of five independent experiments.

Treatment	B in assay medium	Initial rate ($1000\Delta A_{492}$ min^{-1})	pH gradient (ΔA_{492})	Passive efflux ($1000\Delta A_{492}$ min^{-1})
Root (-B)	**0**	8.71 ± 1.02	0.075 ± 0.004	6.16 ± 0.53
	10	9.47 ± 0.71	0.080 ± 0.005	7.00 ± 0.33
Root (+B)	**0**	9.18 ± 0.95	0.077 ± 0.006	6.27 ± 0.80
	10	7.69 ± 0.43	0.077 ± 0.007	6.22 ± 0.97
Shoot (-B)	**0**	5.60 ± 0.51	0.081 ± 0.004	13.7 ± 0.94
	10	5.99 ± 0.88	0.081 ± 0.004	14.7 ± 1.63
Shoot (+B)	**0**	8.42 ± 1.07	0.091 ± 0.003	15.5 ± 1.55
	10	8.67 ± 1.46	0.089 ± 0.003	16.8 ± 0.73

3.2.6 Effect of boron on kinetic parameters of plasma membrane H^+-ATPase isolated from maize leaves

Kinetic parameters of an enzyme provide an ideal tool to study the enzyme properties**.** To study the enzyme characteristics of H^+-ATPase isolated from maize leaves, hydrolytic activity was measured at various ATP concentrations ranging from 0 to 15 mM in the presence of a regeneration system at 20 and 30˚C (Figure 3.12). Kinetic parameters, K_m and V_{max} were calculated using the regression analysis with DynaFit (Hanstein *et al.,* 2011). Plasma membrane H^+-ATPase derived from the maize leaves grown under boron-sufficient condition showed almost the same values for *km* and V_{max} as those grown under boron-deficient conditions. The result showed that boron supply in nutrient medium did not produce a significant change in kinetic parameters at both temperatures.

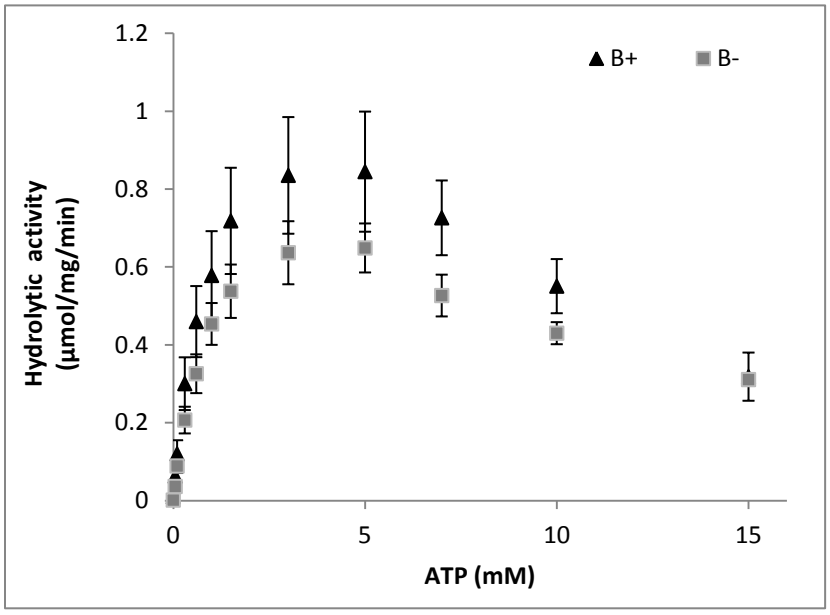

Figure 3.12: Comparison of the kinetic characteristics of plasma membrane H^+-ATPase from maize, Dependence of ATPase activity on ATP concentration. Plants were grown in nutrient solution with (B+) and without (-B) boron for 3 weeks. ATPase activity was analyzed in the presence of 1 mM molybdate, 1 mM azide, and 100 mM nitrate at 30°C. The concentration of ATP was kept constant in the range of 0 to 15 mM. Values represent means ± SE of four independent experiments.

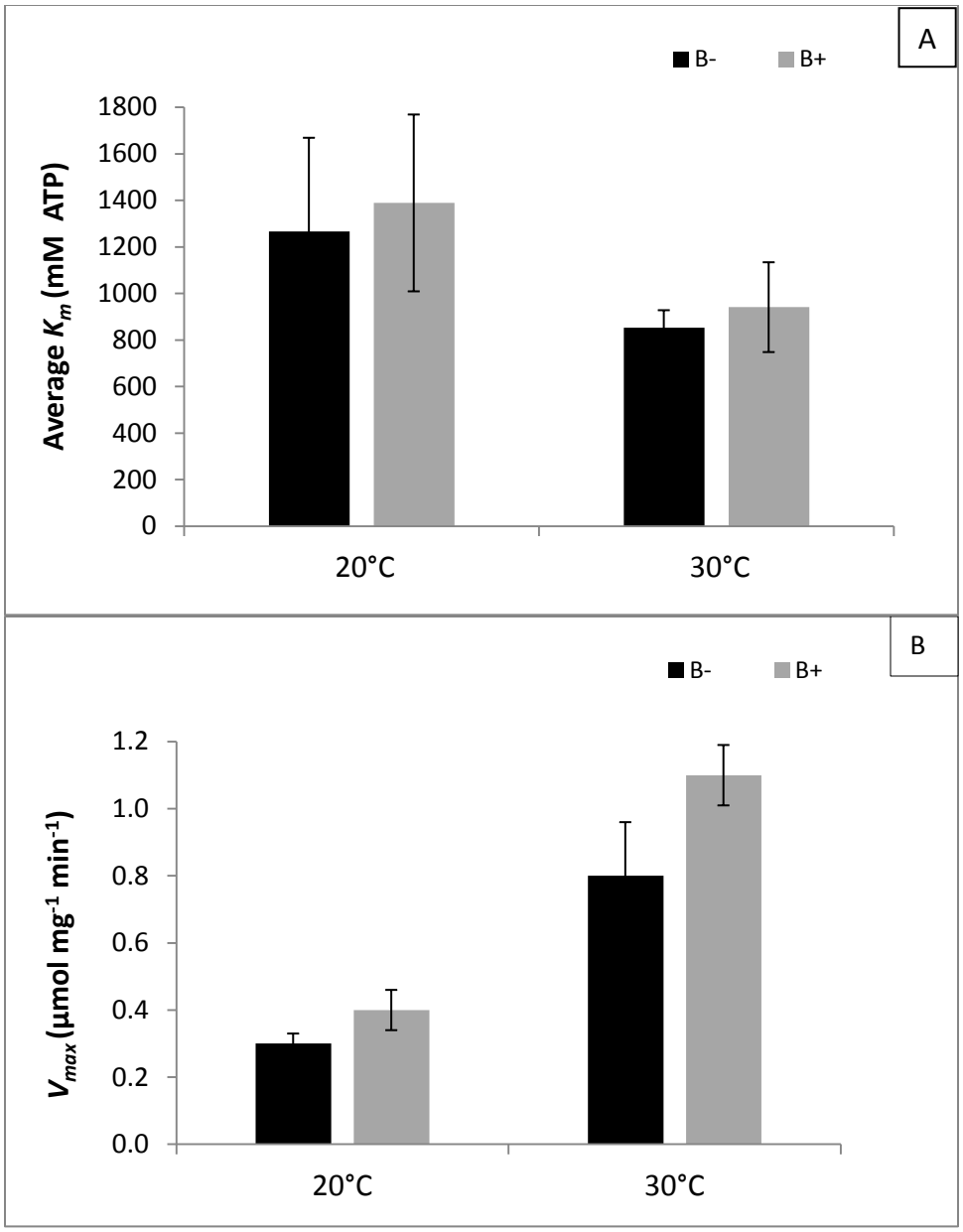

Figure 3.13: Effect of boron on the kinetic characteristics of plasma membrane ATPase derived from maize leaves. Plants were grown in nutrient solution with (B+) and without (-B) boron for 3 weeks. V_{max} and K_m were determined using ATP concentrations from 0 to 15 mM at 30°C and 30°C. An ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was used to keep constant ATP concentrations. The values represent means ± SE of five independent experiments.

3.2.7 Effect of boron on turnover rate of ATP hydrolysis in plasma membrane H^+-ATPase isolated from maize leaves

Various environmental conditions have been reported which can change the activity of plasma membrane but in many cases it was not clear whether the observed change in H^+-ATPase show change in the amount or the turn-over rate of hydrolysis of the enzyme (Serrano,1989). To understand the plant response to environmental conditions, these two components should be identified. This study was conducted to solve this problem by determining the activation energy, which is related to turnover rate of hydrolysis of the enzyme and by quantifying the concentration of enzyme. For the determination of activation energy of plasma membrane H^+-ATPase according to Arrhenius equation, V_{max} was analyzed at two different assay temperatures (20°C and 30°C) at optimum pH level (6.5) for each membrane fractions. In both membrane factions isolated from boron-deficient and boron-sufficient plants, V_{max} increased by about 2.7 times as temperature increased from 20°C to 30°C (Figure 3.13).

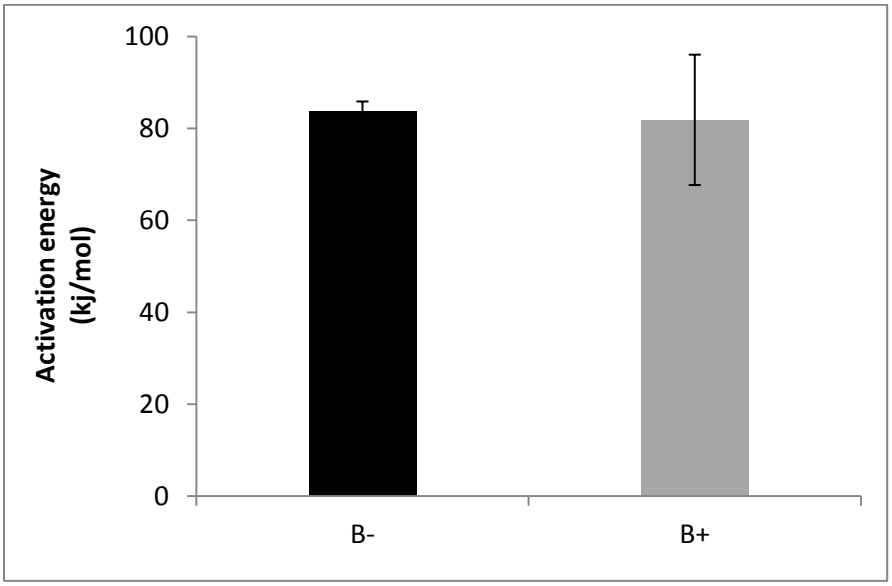

Figure 3.14: Effect of boron on the activation energy of H^+-ATPase calculated according to the Arrhenius equation. V_{max} was determined at 20°C and 30° C. Membrane vesicles were isolated from maize leaves treated with and without B in nutrient solution. Values are the means of four replicates ± SE.

As a consequence, boron application did not change the activation energy (Figure3.14). These results indicate that plasma membrane H^+- ATPase has a comparable turnover rate of ATP hydrolysis in boron-deficient and boron-sufficient plants.

3.3 Effect of boron on the relative transcription of H^+ATPase isoforms

In order to understand the mechanism responsible for the change in H^+ pumping activity of plasma membrane in maize shoot grown in the presence of boron, various isoforms of the *MHA* gene were analyzed. The total RNA was extracted from the young leaves maize cultivated in boron-sufficient medium and boron-deficient medium. The purified mRNA was reversely transcribed into cDNA and the relative gene transcription of plasma membrane H^+-ATPase was quantified by means of Real Time-PCR using isoforms-specific primers, *MHA1, MHA2, MHA3* and *MHA4*. In addition, to investigate changes in transcription of all isoforms of H^+-ATPase in total, family-specific primers were also used. The transcription of mRNA for control treatment was defined as 1 and mRNA transcription in boron-treated plants was compared with that of control and defined as relative transcription. Primer-specific amplification was confirmed with melting curve analysis and was further verified by running the product on agarose gel after RT-PCR. In the present study, we were not able to detect the amplification for *MHA2*. There were single-peak melting curves for actin and *MHA1*, *MHA3*nd *MHAfam* as shown in Figure 3.15, indicating the amplification of single-gene products that were further confirmed using gel electrophoresis. *MHA4* showed double-constant peak (data not shown), indicating the contamination or amplification of more than one product due to non-specificity of primers. However, a single distinct band for *MHA4* can be seen on gel that clearly means primers were highly specific for the gene of interest and that the nature of the product itself responsible for the double peak signal. Similarly, no product was amplified in the NTC excluding the possibility of contamination. This double peak persists even when different annealing temperature, different primers and different cDNA concentrations were tested (data not shown). It has been suggested by Weis et al. (2010) that the annealing of sense/sense or antisense/antisense primer strands or the formation of hairpins could be responsible for the observation. These products/hairpins may have lower melting temperatures than the antisense/sense hybrids and may have contributed to the second peak. As the presence of a double peak in the melting-curve analysis would

compromise the accuracy of results, the data for *MHA4* were not subjected to further analysis. The results or RT-PCR analysis show that plants supplied with boron in nutrient solution increased transcription of *MHAfam* gene and *MHA3*. The transcription of *MHA3* significantly increased and *MHAfam* also showed a similar increasing trend in shoot tissues. However, the transcription level of *MHA1* remained un-changed.

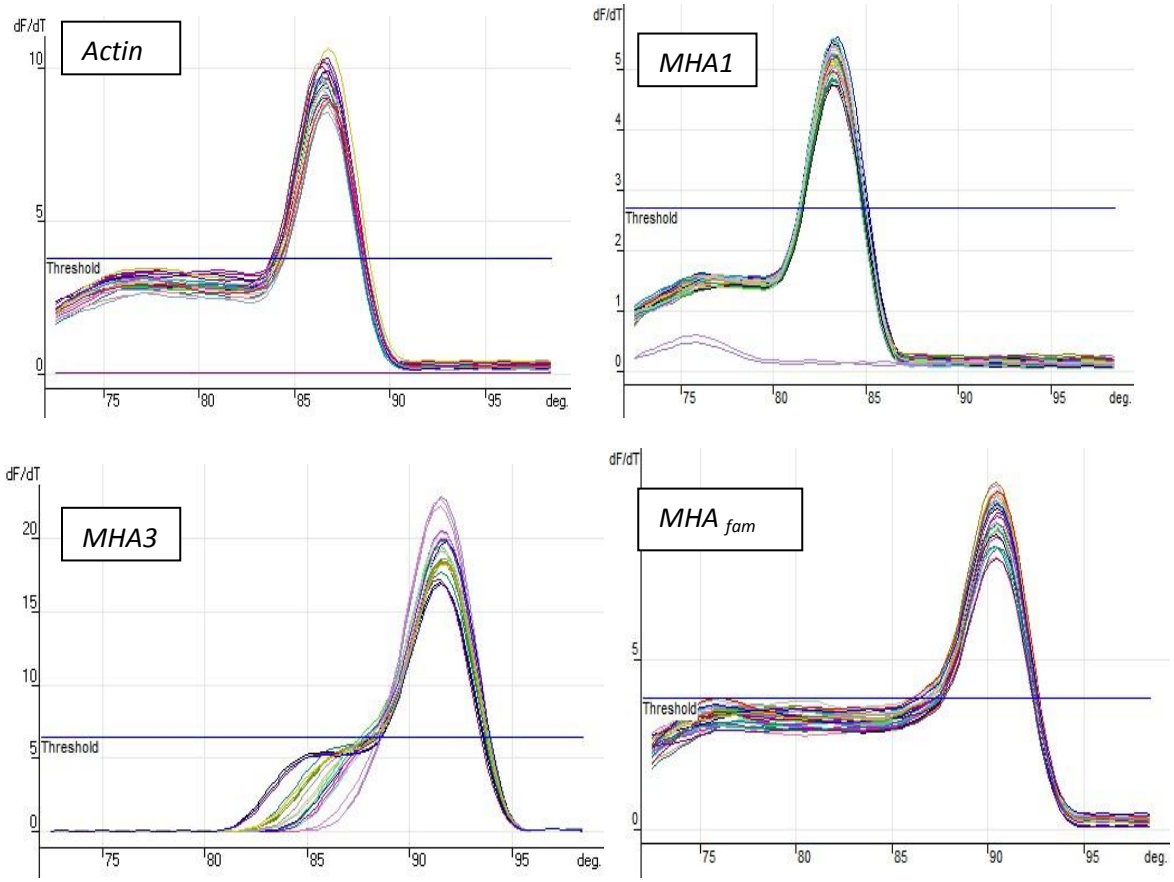

Figure 3.15: Representative melting-curve analysis for the amplicons of *actin*, *MHA1*, *MHA3* and *MHAfam*. Melting curves were obtained in a Light Cycler using SYBR Green fluorescence (dF/dT) versus temperature (°C).

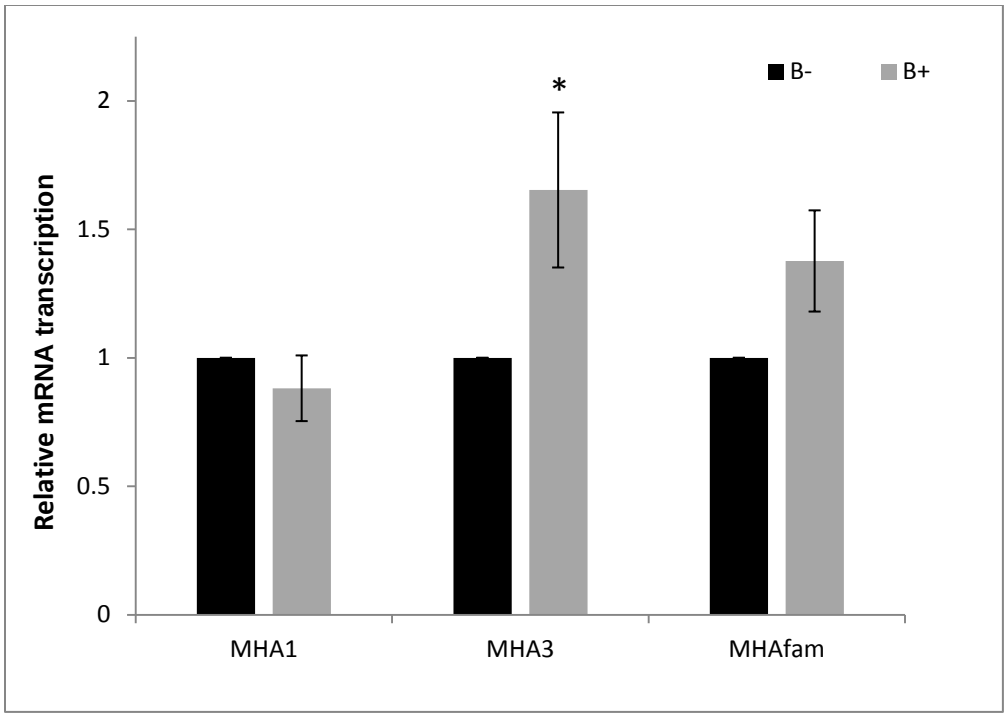

Figure 3.16: Relative change in transcription of plasma membrane H^+-ATPases (*MHAfam*) and specific isoforms (*MHA1* and *MHA3*) from maize young shoots. Plants were grown with and without 10 µM Boron supply in nutrient solution for 21 d. RNA was isolated from young leaves and used to prepare cDNA that was further used as a template for real-time PCR. SYBR Green was used as fluorescence probe in Rotor Gene cycler. Relative expression intensity was calculated using the Pfaffl equation in comparison to the actin as internal control. Values are the means of four replicates ± SE.

3.4 Effect of boron on concentration of plasma membrane H^+-ATPase in maize leaves

The concentration of plasma membrane H^+-ATPase was measured using the western blot technique. 10 g of membrane protein were separated by means of SDS-PAGE on 10% acrylamide gel. Immunoblotting with a polyclonal antibody raised against the central part of plant H^+-ATPase was used to determine the concentration of ATPase enzyme. For the quantitative comparison, the intensity and band area of signals was measured by setting control as 100% in four independent experiments. The band intensity of plasma membrane H^+-ATPase isolated from maize shoot cultivated in the presence of boron was increased by 45% as compared to maize shoot grown in boron deficient medium boron deficient medium.

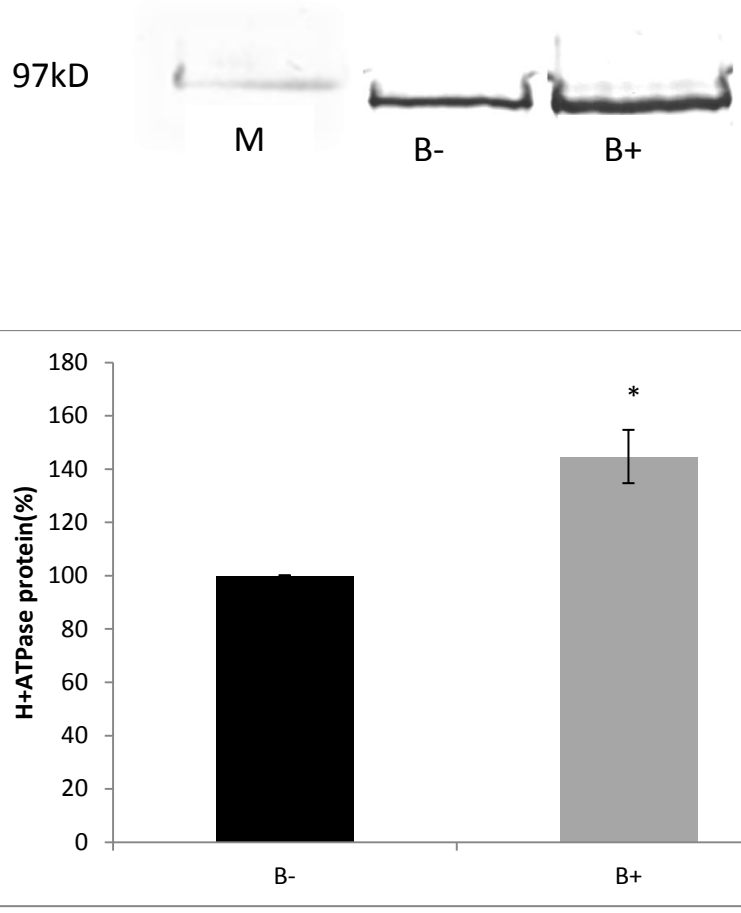

Figure 3.17: Immuno-detection of plasma membrane H^+-ATPase (97 kD) by Western-blotting. M abbreviates for standard marker of a known molecular mass. For separation of plasmalemma proteins, membrane vesicles (10 µg membrane proteins) were loaded onto polyacrylamide gel. For Western-blot analysis, after separation on the gel the membrane proteins were transferred to polyvinylidene difluoride (PVDF) membrane filter. Membrane blots were incubated with a polyclonal antibody raised against the central portion of AHA2 (amino acids 340-650) and visualized with a secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma).

4 DISCUSSION

4.1 Boron supply in nutrient medium improves plant growth

Boron (B) is one of the seven micronutrients required for normal growth of plants. In earth's crust, boron is usually present at an average concentration of 10 ppm. However, the range of B concentrations in the soil solution, in which plants suffer neither toxicities nor deficiencies, is very narrow. The essentiality of B for plant growth was first described in the beginning of the 20th century, and nowadays it is widely accepted that B is an essential nutrient for all vascular plants (Blevins and Lukaszewski 1998). As reviewed by Marschner (1995), Cakmak and Römheld (1997), and Brown et al. (2002), intensive research activities have been done to characterize the physiological and biological role of B in plant growth and development. Various authors have proposed that B is involved in several processes including cell-wall structure and function, maintaining the membrane function and integrity, and supporting the metabolic activities in the plants (Brown et al. 2002). To the date, the only well described physiological role for B in plants involves cross-linking of the pectin rhamnogalacturonan II (RGII) in the cell wall (O'Neill et al. 2004). Due to the rapidity and the variety of symptoms caused by B deficiency, determining the primary function of B in plants is a great challenge in plant biology (Blevins and Lukaszewski 1998).

Several reports in the literature indicate that under B-deficient conditions plants showed several biochemical, anatomical, and physiological aberrations (Marschner 1995). Boron deficiency is the most common and widespread micronutrient problem globally (Alloway 2008). One significant feature of B deficiency that contributes to its importance in agricultural production is that a deficiency of B inhibits growth of tissues, specifically reproductive structures, which represent 80% of the world's agricultural product. Boron deficiency causes a wide range of anatomical symptoms including the retardation of apical and extension growth, cracking of stems and petioles, necrosis of epical buds, abortion of flower initials, and shedding of fruits (Goldbach 1997; Li et al. 2001; Brown et al. 2002; Silve et al. 2008). Deficiency symptoms of B vary among crop species, but normally occur in the young growing points or flower and fruiting parts of the plant. Symptoms are characterized by reduced or retarded elongation of apical meristems (Saleem et al. 2011). In the present study, B deficiency symptoms

were also observed in the young growing leaves of the plant grown under B deficient medium (Figure 3.1). This includes narrow transparent stripes along the length of the leaves. Furthermore, Lordkaew et al. (2010) reported that maize plants grown in B-deficient medium exhibited similar B deficiency symptoms of "narrow white to transparent lengthwise streaks on young leaves" and Saleem et al. (2011) also reported similar symptoms in maize plants. Several other studies reported that B deficiency symptoms in some other plant species also occurred in young growing tissues. For instance in canola, the young leaves of B-deficient plants were stunted and curled upwards at the margins, but the older leaves remain green at the early vegetative stage (Asad et al. 2000) and Blamey et al. (1987) also showed B deficiency symptoms in sunflower appeared in younger leaf blades which become hardened, necrotic and brownish in color.

These results are consistent with the findings of the current study that B application in nutrient medium significantly increased the growth of only young growing plant tissues (Figure 3.2 and 3.3). One of the possible explanations for these specific effects of B on young growing tissues could be that the endogenous B of seeds was depleted with the passage of time, and B present in nutrient solution as a contaminant might not be enough to maintain the plant growth. Therefore, these new plant parts were obviously not able to acquire sufficient B for growth, because no external B sources were present, on which the apical growth in plants is mainly dependent. As B is immobile in the phloem, a lack of movement from old to young tissues could explain symptoms specially occurring in young tissues.

In addition, the results of the present study show that exogenous B supply in the nutrient medium increased B concentration in shoots, whereas the B concentration in root tissues of the B-deficient plants remained unchanged. From these results, it suggested that in the plants grown under B-deficient conditions the root retained most of the limited B reserves, while the B availability for the above-ground part was limited. Some other studies in the literature have demonstrated that during the short-term B deficiency, the concentration of B in roots was less affected than that in shoots (Li et al. 2001; Dannel et al. 1998). These authors suggested that a priority was given to the below-ground plant parts concerning maintenance and growth under limited nutrient conditions. Therefore, the limited amount of nutrients absorbed from the soil is mostly allocated to the root tissues to meet growth requirements. Similarly, Camacho-Christobal

et al. (2002) showed that B concentration in the leaves decreased drastically under B deficiency, whereas there was no significant decrease observed in the B concentration of roots during the B-deficient treatment in tobacco plants.

Although a considerable amount of B is tightly bound to the cell wall as an insoluble B pool, there is some B in the cell sap as a soluble B pool, which could be directly available for possible physiological functions in the cell (Dannel et al. 1998). Li et al. (2001) reported that B concentration in cell sap was affected by B deficiency. From this the authors assumed that this bound B plays a structural role in the cell wall and does not function as a storage pool for further use in the plant. Moreover, transport of B to the shoot seems to be dependent on B concentration in root symplasm (Pfeffer et al., 1999). Therefore, low B concentrations in the symplasm may also lead to impairment of plant growth, especially in the shoot apex.

According to Dell and Huang (1997) in vascular plants, B is transported from the roots to the shoots and leaves through the xylem with the transpiration flow, and accumulates in growing apical tissues. Once B reaches the leaves it becomes fixed in the apoplast. Because its re-translocation is restricted, B is usually termed phloem-immobile (Blevins and Lukaszewski 1998).

A number of reviews describe that higher plants showed inhibition or cessation in the elongation rate of growing shoot in response to the limited external B supply (Shelp 1993; Marschner 1995; Dell and Haung 1997). As shown in this study, B supply in the nutrient medium enhanced the plant biomass as well as expansion growth characterized as plant height and leaf area of the young growing (Figure 3.2). In agreement with these results, various studies showed that B deficiency inhibits the vegetative growth and yield in plant species such as maize (Lordkaew et al. 2010), castor bean (Silva et al. 2008), and pea (Li et al. 2001).

The mechanism how B can contribute to plant growth is poorly understood, although it has been proposed that inadequate B supply seems to inhibit cell wall expansion. Plant cell walls consist of rigid microfibrils with highly tensile strength of cellulose molecules embedded in a gel-like matrix composed of hemicellulose, pectin, lignin and protein (Becker and Deamer1991; Brett and Waldron 1996). The primary cell wall has properties of reversible elasticity and

irreversible plasticity, which contribute to the extensibility and allowing the wall to expand in a plastic manner in response to cell growth. Boron is mainly localized in the cell wall (Hu and Brown 1994; Hu et al. 1996) and cross-links with rhamnogalacturonan II Kobayashi et al. 1996; O'Neill et al. 2001), and considered to be an important factor of cell wall extensibility (Hu and Brown 1994; Findeklee and Goldbach 1996). The rapid breaking and reformation of these acid-labile, borate-ester bonds might facilitate the cell growth (Hu and Brown 1994). Furthermore, a short-term B deprivation down-regulates the transcription of several cell wall-modifying enzymes (Camacho-Cristobal et al. 2008), which influences the arrangement of the xyloglucan microfibrillar network and, consequently alters the tensile properties of cell wall (Ryden et al. 2003).

Boron deficiency affects the concentration of plant growth regulators in the plant cell, which could be responsible for the decline in elongation growth and apical dominance under B-deficient conditions. For instance, B deprivation reduced cytokinin level as well as the indole acetic acid export out of the shoot apex (Li et al. 2001).

4.2 Effect of boron on plasma membrane H^+-ATPase in maize shoots and roots

In accordance with the acid growth theory, auxin activates the plasma membrane H^+-ATPase, which lowers the apoplastic pH. The apoplast acidification induces cell wall loosening which contributes to the cellular expansion (Rayle and Cleland 1992; Cleland 1977). Cell-wall extensibility is an important factor responsible for expansion growth, which suggested that increased shoot growth may be attributed to a change in cell-wall extensibility (Cosgrove 2005). Furthermore, lower apoplastic pH activates some of secondary active transporters that are involved in nutrient uptake such as potassium and chloride. These solutes act as osmotica and trigger water uptake. The resulting turgor pressure exerts pressure on the cell wall leads to increase in expansion growth.

In the current investigations, it was hypothesized that the B-mediated increase in maize growth was attributed to an increase in the activity of plasma membrane H^+-ATPase. To characterize the activities of the plasma membrane ATPase, the plasma membrane vesicles were isolated from maize shoots and roots grown with or without B supply in nutrient solution. To

study the characteristics of plasma membrane H^+-ATPase, it is very important to have pure membrane vesicles. In the current study, our results (Figure 3.5) showed that application of B in nutrient solution had no affect on the purity of the membrane vesicles. The membrane vesicles isolated from B-deficient (B-) and B-sufficient (B+) maize shoots showed a similar vanadate-sensitive activity (almost 85%) and almost no sensitivity to nitrate and azide. Similar results were shown for root vesicles. However, 15-20% inhibition in ATPase hydrolytic activity was observed by the addition of molybdate in the assay medium which indicate the presence of some non-specific acid phosphatases in plasma membrane vesicles (Briskin and Poole 1983; Yan et al. 2002). It was concluded from the results that the isolated membrane fractions were free of vacuolar, mitochondrial, and chloroplastic membranes with very small contamination of acid phosphatases and highly enriched with plasma membrane vesicles, and can be used for further characterization of plasma membrane H^+-ATPases.

4.2.1 Direct interaction of boron with the ATPase molecule

It is well known that boric acid has the ability to form borate-diester bond with the plasma membrane-localized hydroxyl-containing compounds such as glycoproteins and glycolipid (Goldbach and Wimmer 2007). It was suggested that, based on formation of B cross links with membrane molecules, B could play a specific role in membrane physical satiability which in turns can influence the conformation of membrane-localized enzymes and ultimately change the activity of enzymes (Brown et al. 2002). Wimmer et al. (2009) have demonstrated B binding to the β-subunit of mitochondrial ATP synthase. As plasma membrane ATPases fulfill several physiological functions in the plant cell and are high ATP consumers they need a tight regulation of activity. The literature shows that phosphorylation of plasma membrane H^+-ATPase may activate or may deactivate the enzyme (Trofimova et al. 1997; Fuglsang et al 2007). Dephosphorylation may also activate the enzyme (Desbrosses et al. 1998). It was therefore hypothesized that B directly interacting with enzymes cause conformational changes in the H^+-ATPase, which affects the enzyme activity.

In order to investigate the direct effect of B on the plasma membrane H^+-ATPase, hydrolytic and pumping activities of enzyme were determined under *in vitro* conditions by adding the boric acid to the assay medium. The results in the current study show that B addition

to the assay medium did not influence the hydrolytic activity as well as pumping activity of plasma membrane H^+-ATPase isolated from maize shoot (Figure 3.10). Similar results were found in the case of root vesicles (Figure 3.11). Little information is available in literature related to the *in vitro* effect of B on the plasma membrane ATPase activity. Roldan et al. (1992) showed *in vitro* B addition did not affect the hydrolytic ATPase activity in sunflower root. However, they found inhibitory effects of B on vanadate-sensitive ATP-dependent H^+ transport. The lack of an *in vitro* effect of B on hydrolytic and proton pumping activity of plasma membrane ATPase suggests that plasma membrane H^+-ATPase activity was not affected by direct interaction of B with the plasma membrane ATPase molecule.

4.2.2 Boron application in nutrient medium enhances the plasma membrane H^+-ATPase activity by a change in expression of an efficient isoform

These experiments were performed to investigate the role of B supply in the nutrient medium on plasma membrane H^+-ATPase. The electrochemical gradient created by plasma membrane H^+-ATPase is responsible to drive ion and metabolite transport across the membrane (Sondergaard et al. 2004). Plasma membrane ATPase activity was characterized on the basis of ATP hydrolytic activity and proton pumping activity. Plasma membrane H^+-ATPase characteristics were studied by investigating the changes in enzyme properties such as affinity towards the substrate, turnover rate, initial rate of H^+ pumping and maintenance of the pH gradient across the vesicles membrane, transcription of different isoforms and changes in the enzyme concentrations.

In this study, the results show that B supply in the nutrient medium did not change the hydrolytic activity of plasma membrane H^+-ATPase isolated from maize roots (Figure 3.6B). These results are in contrast to the observations of Pollard et al. (1977) who showed that activity of the K^+-stimulated ATPase in B-deficient maize roots was considerably lower than in control plants and Roldan et al. (1992) reported that inadequate boron concentration in the growth medium led to inhibition of ATP-dependent proton pumping in sunflower root vesicles. Furthermore, in the present study results show similar values for the initial rate of H^+ pumping, maximum pH gradient, and passive efflux of proton in the plasma-membrane vesicles isolated from roots of the plants treated with and without B in nutrient medium. From these results it can

be concluded that B supply in nutrient medium had no effect on hydrolytic and pumping activity of plasma membrane H^+-ATPase in root vesicles (Figure 3.6B and Table 3.1) because B concentration in roots did not differ (Figure 3.4).

For the maize shoots, the results showed that B supply in the nutrient medium significantly increased the initial rate of proton pumping of plasma membrane H^+-ATPase (Figure 3.8). However, B did not significantly affect the plasma membrane H^+-ATPase hydrolytic activity of the shoot vesicles (Figure 3.6A). These observations are in line with the findings of Obermeyer et al. (1996) that B stimulated the plasma membrane H^+-ATPase hydrolysis and H^+ transport activity in ungerminated pollen grains of lily. However, pumping efficiency of H^+-ATPase remained unchanged in B treated and non-treated plants (Figure 3.9A) which is contradictory to the previous results. The possible reason for this could be that hydrolytic activity showed increasing trend in the B-treated plants but this increase was not statistically significant. Moreover, to gain deeper insight to properties of plasma membrane ATPase of maize shoot, the kinetic parameters (K_m and V_{max}) of the enzyme were analyzed. It was found that B supply in the nutrient solution had no effect on the K_m and V_{max} of hydrolytic activity of the plasma membrane H^+-ATPase.

The question arises by which mechanism B enhances the H^+-ATPase pumping in maize shoots vesicles. The possible mechanisms involved in plasma membrane H^+-ATPase regulation may include modulation of turnover rate of the enzyme molecule or increased number of enzymes per unit membrane. To clarify, whether the observed increase in pumping activity of plasma membrane H^+-ATPase isolates from maize leaves cultivated in boron-sufficient medium reflects the modulation of either amount of enzyme or the turnover rate of hydrolysis of enzyme, the activation energy of the plasma membrane was measured. The results show that no change was observed in activation energy of the plasma membrane H^+-ATPase under B- and B+ treatments (Figure 3.14). The increase in plasma membrane pumping activity in B-treated plants may thus be attributed to higher abundance of H^+-ATPase molecules in the membrane vesicles, not to higher substrate turnover rate per mole of enzyme. This hypothesis was further confirmed by immune-detection of the enzyme concentration. The result found that membrane fractions isolated from the B-supplied shoots showed 40% more enzyme.

Plants supplied with B in nutrient medium showed an increase in enzyme concentration in membrane fractions, which may by be due to higher transcription of plasma membrane H^+-ATPase or increased synthesis of membrane protein. As described earlier auxin treatment may increase the H^+-ATPase protein in plasma membrane by increasing the membrane flow from endoplasmic reticulum to plasma membranes (Hager et al. 1991) and may induce a higher H^+-ATPase mRNA transcription (Frias et al. 1996). It was reported in literature that B is involved in auxin metabolism (Bohnsack and Albert 1977; Blevins and Lukaszewski 1998) which may affect the transcription level of plasma membrane H^+-ATPase. Several studies reported that B nutrition induced changes at the transcriptional level of various genes involved in various physiological processes (Camacho-Cristobel et al. 2011). Thus B deficiency led to a decline in the level of plasma membrane H^+-ATPase (*PMA2)* transcript in tobacco roots (Camacho-Cristobel et al. 2007). The increase in the initial rate of proton pumping activity (Figure 3.8A) of ATPase may be due to a change in the expression of some specific H^+-ATPase isoform.

Plant plasma membrane H^+-ATPases is encoded by a multigene family and on the basis of gene structure, it can be furthered divided into five subfamilies (Oufattole et al. 2000; Gaxiola et al. 2007). To date 9-12 genes have been identified in different plant species as in tobacco 9 genes (Oufattole et al. 2000), in *Arabidopsis* 12 genes (Palmgren 2001), and in rice 10 genes (Baxter et al. 2003). However, in maize only four H^+-ATPase isoforms (*MHA1, MHA2*, *MHA3* and *MHA4)* have been identified (Jin and Bennetzen 1994; Frias et al. 1996; Santi et al. 2003; Zörb et al. 2005). Among these isoforms, *MHA1* placed in subfamily I while *MHA2*, *MHA3*, *MHA4* belong to subfamily II (Santi et al. 2003).

The expressed isoforms can vary according to cell or tissue type and by developmental stage or environmental stimuli. The literature showed that isoforms may differ in their enzymatic characteristics such as affinity towards substrate, turnover rate, optimum pH, and sensitivity to vanadate (Palmgren and Christensen 1994; Luo et al. 1999). For example, comparison of three *Arabidopsis* isoforms namely *AHA1*, *AHA2*, and *AHA3* revealed that *AHA3* had a ten-fold higher *Km* value for ATP hydrolysis and three-fold higher sensitivity to vanadate (Palmgren and Christensen 1994).

The transcriptional regulation of plasma membrane H^+-ATPases in young growing shoots tissues by B treatment was determined using isoform-specific primers namely *MHA1*, *MHA2*, *MHA3* and *MHA4* as well as family-specific primers for H^+-ATPase (MHA_{fam}), which should cover the transcription for the entire multigene family for the plasma membrane H^+-ATPase (Santi et al. 2003; Zörb et al. 2005). The data show that the relative transcription of *MHA1* was not changed in maize leaves. On the other hand, transcription of *MHA3* was increased significantly in B treated plants (Figure 3.16). Moreover, the relative transcription of the H^+-ATPase family showed a similar trend as *MHA3*. These results are in line with the findings of Shahzad (2011) who described that down-regulation of *MHA3* caused a significant decrease in proton pumping. These results indicate that *MHA3* is a major contributor in expression of the MHA_{fam} gene. The up-regulation of *MHA3* coincided with increase in proton pumping of plasma membrane H^+-ATPase in maize leaves cultivated in the presence of B in the nutrient medium (Figure 3.8A).

5 SUMMARY

The essentiality of boron (B) for plant growth was first described in the beginning of the 20th century, and nowadays it is widely known that B is an essential nutrient for all vascular plants (Blevins and Lukaszewski 1998). Intensive research activities have been done to characterize the physiological role of B in plant growth and development. Various authors have proposed that B is implicated in several processes including cell-wall structure and function, maintaining the membrane function and integrity, and supporting the metabolic activities in the plants (Brown et al. 2002). To date, the only well described physiological role for B in plants involves cross-linking of the pectin rhamnogalacturonan II (RGII) in the cell wall (O'Neill et al. 2004). Due to the rapidity and the variety of symptoms caused by B deficiency, determining the primary function of B in plants is a great challenge in plant biology (Blevins and Lukaszewski 1998). Several reports in literature demonstrated that the presence B in the root medium increased plant growth. B seems to be involved in cell-wall expansion. As B is mainly localized in the cell wall (Hu and Brown 1994; Hu et al. 1996) and cross-links with rhamnogalacturonan II Kobayashi et al. 1996; O'Neill et al. 2001), and it is considered an important factor of cell-wall extensibility (Hu and Brown 1994; Findeklee and Goldbach 1996). Plasma membrane H^+-ATPase is a master enzyme and it extrudes H^+ out of the cytosol and creates an electrochemical H^+ gradient. The plasma membrane H^+-ATPase-generated H^+ gradient is responsible for cell-wall extensibility and expansion growth. Moreover, the electrochemical H^+ gradient energizes various proteins involved in nutrient and solute uptake and translocation. It is likely to assume that the B-induced stimulation of plant growth is caused by changes in plasma membrane H^+-ATPase activity.

To elucidate the role of B in maize growth and its contribution to the control of plasma membrane H^+-ATPase in roots and shoots the following hypotheses were tested. **1)** Exogenously B supply to the nutrient medium improves maize (*Zea mays* L cv. Amadeo) growth under normal growth conditions. **2)** Changes in plasma membrane H^+-ATPase are responsible for the B-induced maize growth. **3)** Boron modifies plasma membrane H^+-ATPase by direct interaction of B with the ATPase molecule. **4)** Boron-induced transcriptional modification of H^+-ATPase may contribute to enhance the activity of plasma membrane H^+-ATPase.

The results are summarized as:

1. Boron application in the nutrient medium had a positive influence on plant growth. It improved the plant biomass as well as expansion growth. The results showed that the effect of B is more pronounced in young growing leaves. Furthermore, B concentration in shoot of maize plants was increased by the addition in nutrient medium.

2. Boron addition in the nutrition solution significantly increased the initial rate of proton pumping of the plasma membrane H^+-ATPase isolated from maize shoots. Boron nutrition also showed increasing trend in hydrolytic activity of plasma membrane H^+-ATPase. Moreover, B addition in nutrition medium enhanced the transcription as well as translation of plasma membrane H^+-ATPase. Boron-treated plants showed 40% increased enzyme concentration in shoots and higher transcription on *MHA3* and MHA_{fam} as compared to the B-deficient plants.

3. *In vitro* addition of B did not change the hydrolytic and pumping activities of plasma membrane H^+-ATPase, which indicates that B had no direct interaction of B with the plasma membrane ATPase molecule.

6 ZUSAMMENFASSUNG

Die Essentialität von Bor (B) für das Pflanzenwachstum wurde erstmalig zu Beginn des 20. Jahrhunderts beschrieben und heutzutage ist das Wissen weit verbreitet, dass B ein essentieller Nährstoff für alle Gefäßpflanzen ist (Blevins und Lukaszewski 1998). Intensive Forschungsarbeit wurde durchgeführt, um die physiologische Rolle von B beim Pflanzenwachstum und bei der Pflanzenentwicklung zu charakterisieren. Verschiedene Autoren haben die Behauptung aufgestellt, dass B in verschiedenen Prozessen beteiligt ist, darunter der Aufbau der Zellwand, das Aufrechterhalten der Membranfunktion und –integrität, sowie unterstützend bei metabolischen Aktivitäten in Pflanzen wirkt (Brown et al. 2002). Bisher ist nur eine physiologische Rolle von B in Pflanzen gut beschrieben, die Beteiligung an der Quervernetzung des Pektins Rhamnogalacturonsäure II (RGII) in der Zellwand (O´Neill et al. 2004). Wegen der Schnelligkeit und der Vielfältigkeit von Symptomen, die durch B-Mangel verursacht werden, ist es eine große Herausforderung für die Pflanzenbiologie die Primärfunktion von B zu bestimmen (Blevins und Lukaszewski 1998). Verschieden Berichte in der Literatur zeigen, dass die Präsenz von B im Wurzelmedium zu einem erhöhten Wachstum führt. B scheint außerdem an der Zellwandexpansion beteiligt zu sein. Da B hauptsächlich in der Zellwand (Hu und Brown 1994; Hu et al. 1996) und den Vernetzungen mit RGII (Kobayashi et al. 1996; O´Neill et al. 2001) zu finden ist, wird erwogen, dass B einen wichtigen Faktor für die Zellwandextensibilität darstellt (Hu und Brown 1994; Findeklee und Goldbach 1996). Die Plasmamembran H^+-ATPase ist ein Masterenzym im Hinblick auf Nährstoffaufnahme und Wachstum. Es extrudiert H^+ aus dem Cytosol und baut einen elektrochemischen H^+-Gradienten auf. Der von der H^+-ATPase generierte H^+-Gradient ist verantwortlich für die Zellwandextensibilität und das Expansionswachstum. Des Weiteren führt der elektrochemische H^+-Gradient verschiedenen Transportern Energie zu, die an der Aufnahme von Nähr- und gelösten Stoffen und deren Translokation beteiligt sind. Es ist anzunehmen, dass die B-induzierte Stimulation von Pflanzenwachstum durch Änderungen der Plasmamembran H^+-ATPase-Aktivität verursacht wird.

Um die Rolle von B für das Wachstum von Mais und seine Beteiligung an der Kontrolle der Plasmamembran H^+-ATPase in der Wurzel und im Spross aufzuklären, wurden folgende Hypothesen getestet. **1)** Exogene Zuführung von B in die Nährlösung verbessert das Wachstum

von Mais (*Zea mays* L cv. Amadeo) unter normalen Wachstumsbedingungen. **2)** Änderungen der Plasmamembran H^+-ATPase-Aktivität sind verantwortlich für B-induziertes Maiswachstums. **3)** Bor modifiziert die Plasmamembran H^+-ATPase durch direkte Interaktion von B mit dem ATPase-Molekül. 4) Bor-induzierte transkriptionale Modifikation der H^+-ATPase könnte dazu beitragen, die Aktivität der Plasmamembran H^+-ATPase zu verstärken.

Die Ergebnisse werden wie folgt zusammengefasst:

1. Die Applikation von Bor in das Nährstoffmedium hatte einen positiven Einfluss auf das Pflanzenwachstum. Es verbesserte sowohl die Pflanzenbiomasse als auch das Streckungswachstum. Die Ergebnisse zeigen, dass der Effekt von B ausgeprägter in den jungen Blättern ist. Des Weiteren wurde die B-Konzentration im Spross der Maispflanze durch die Zugabe in die Nährlösung erhöht.
2. Die Zugabe von Bor in die Nährlösung erhöhte signifikant die Pumpaktivität der Plasmamembran H^+-ATPase, die aus dem Maisspross isoliert wurde. Die Borernährung bewirkte außerdem einen ansteigenden Trend in der hydrolytischen Aktivität der Plasmamembran H^+-ATPase. Des Weiteren verstärkte die Borgabe in die Nährlösung die Transkription sowie die Konzentration der Plasmamembran H^+-ATPase. Die mit Bor behandelten Pflanzen zeigten eine 40% erhöhte Enzymkonzentration im Spross und eine erhöhte Transkription von *MHA3* und MHA_{fam} im Vergleich zu den B-Mangel Pflanzen.
3. *In vitro* Zugabe von B änderte die hydrolytische und die Pumpaktivität der Plasmamembran H^+-ATPase nicht, was zeigt, dass die Erhöhung der Plasmamembran H^+-ATPase-Aktivität nicht auf einer direkten Interaktion zwischen B und dem Enzym beruht.

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Erklärung

"Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis"niedergelegt sind, eingehalten".

Giessen 18-03-2013 Ammara Fatima

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I am finally to admit that errors that remain are mine

Ammara Fatima

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