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Boron foliar fertilization: Impacts on absorption and subsequent translocation of foliar applied Boron

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Kühner als das Unbekannte zu erforschen, kann es sein,
das Bekannte zu bezweifeln.
(*Alexander von Humboldt*)

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LIST OF ABBREVIATIONS

AIC: based model selection approach

B: Boron

$^{11}B(OH)_3$: isotopic enriched (99.8%) boric acid

BA: Boric acid

BaSol: Basic solution

BCa: BaSol with 0.5 mM $CaCl_2$

BCaS1: BaSol with 50 mM sorbitol and 0.5 mM $CaCl_2$

BCaS2: BaSol 500 mM sorbitol and 0.5 mM $CaCl_2$

Be: Beryllium

BM: foliar formulation containing boric acid and mannitol

BS: foliar formulation containing boric acid and sorbitol

BS1: BaSol with 50 mM sorbitol

BS2: BaSol with 500 mM sorbitol

$Ca(NO_3)_2$: Calcium nitrate

$CaCl_2$: Calcium chloride

$CaSO_4$: Calcium sulfate

Ctr.: De-ionized water with 0.3% (v/v) surfactant

$CuSO_4$: Copper sulfate

DRH: deliquescence humidity

DW: dry weight

Fe(III)EDTA: Iron complex with ethylenediaminetetraacetic acid

Fe: iron

HNO_3 : Nitric acid

ICP-MS: inductively coupled plasma mass spectroscopy

K: Potassium

K_2SO_4: Potassium sulfate

KCl: Potassium chloride

KH_2PO_4: Kalium hydrogen phosphate

$MgSO_4$: Magnesium sulphate

$MnSO_4$: Mangenese sulfate

$(NH_4)_6Mo_7O_{24}$: Ammonium heptamolybdate

RCBD: Randomised complete block design

RG-II: rhamnogalacturonanII

RH: relative humidity

ROS: reactive oxygen species

SEM: Scanning electron microscopy

$ZnSO_4$: Zinc sulfate

ψw : Water potential

Statistics

a: Sorbitol

b: Replicates

c: Calcium

p: Pots

s: Segments

t: Treatments

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 Background

The Sprengel-Liebig Law of the Minimum states that yield is proportional to the amount of the most limiting nutrient. This law has been adapted to science in plant nutrition and soil fertility. Optimal terms of plant production require a good knowledge of the availability of the 16 essential macro- and micronutrients in the soils and nutrient consumption by the planted crops. In many countries low yields and product quality limit plant production, one factor involved to improve the production is a good fertilization management.

The goal of this study was to develop approaches for a better understanding of underlying mechanisms in boron (B) foliar fertilization, effecting foliar B absorption and mobility in the plants.

1.1.1 Boron

About 90 years ago boron was discovered as an essential nutrient in plants (*Warington*, 1923). Even though the demand for B on a molar basis is higher than for any other micronutrient in dicotyledons, knowledge of its physiological role is still limited. Boron deficiency appears worldwide in crop production and is reported in over 80 countries on 132 crops. In the last decades, efforts have been made to understand the role of B in the cell wall formation, membrane integrity and the compound formation with sugar alcohols (*Brown* et al., 2002; *Takano* et al., 2005; *Bielsk*, 2005; *Miwa* et al., 2006; *Kato* et al., 2009; *Wimmer* et al., 2009). In commercial plant production, providing a sufficient B supply is particularly important for yield formation (pollination) (*Wojcik* et al., 1999; *Khayyat* et al., 2007), fruit quality and crop storability (*Wojcik* et al., 1999), and stress tolerance (*Cakmak and Römheld*, 1997). Boron foliar fertilization seems to be a target oriented and optimal solution to cure B shortage in plants. However, scientific experiments on foliar B fertilization showed controversy effects, ranging from positive to no effects at all (*Rerkasem* et al., 1988; *Schon and Blevins*, 1990; *Hanson*, 1991a,b; *Cakmak and Römheld*, 1997; *Wojcik* et al., 1999; *Zhang*, 2000; *Freeborn* et al., 2001; *Ross* et al., 2006; *Khayyat* et al., 2007; *Wojcik* et al., 2008).

Boron characteristics

Boron in plants is mostly bonded to oxygen as borate or boric acid (BA). Boric acid has three valence electron bondings and reacts in solution as electron acceptor (lewis acid).

$$B(OH)_3 + H_2O \leftrightarrow B(OH)_4^- + H_3O^+$$

Boron belongs to the III. main group in the periodic system and represents the only non-metal in this group. Because of the strong binding of the electrons on the atom nucleus the formation of B cations and the formation of metallic bindings do not occur naturally, as in other elements of the III. main group, such as aluminium, gallium, indium, thallium.

In plants, B forms compounds with a variety of polyhydroxy compounds, via ester formation (*Power and Woods*, 1997). Most of B in plants is bonded to rhamnogalacturonanII (RG-II), a complex polysaccharide of the pectic fraction of primary cell walls.

Besides its stabilizing function in cell wall structure, B plays an important role in membrane integrity. Boron deficiency causes an inhibition of membrane uptake of a number of nutrients and leakage of nutrients, e.g K-efflux, out of the cell (*Cakmak* et al., 1995) (Tab.1.1). Apart from the role in cell wall stabilisation and membrane integrity there is also evidence for a variety of enzymes to interact with B (*Power and Woods*, 1997).

Tab. 1.1. Leakage of K^+ (µg K^+ [4 leaf disks]$^{-1}$ [2h]$^{-1}$) from leaves of soybean grown in nutrient solution for 2 weeks with sufficient (15 µM) and deficient (0 µM) B supply. Leakage of K^+ was measured with 1-cm leaf disks floating in H_2O. Each value is the mean of 2 replications. B status was measured in mg kg^{-1} dry weight (source: S. Will).

Treatments	Control (15 µM B)	Deficiency (0 µM B)
B status µM*g^{-1} DW	30	5
K^+ (beginning)	18	54
K^+ (after foliar B supply)	16	23
K^+ (3 days after foliar B supply)	21	28

Another very important function of B in some plant species is the transport of carbohydrates. In many species B is immobile in the phloem, but in plants with sorbitol, mannitol or fructose as a major carbohydrate, B mobility takes place. The formation of stable compounds, such as di-sorbitol borate ester (Fig. 1.2), requires excessive sorbitol concentrations in the fluid (*Brown and Hu*, 1996).

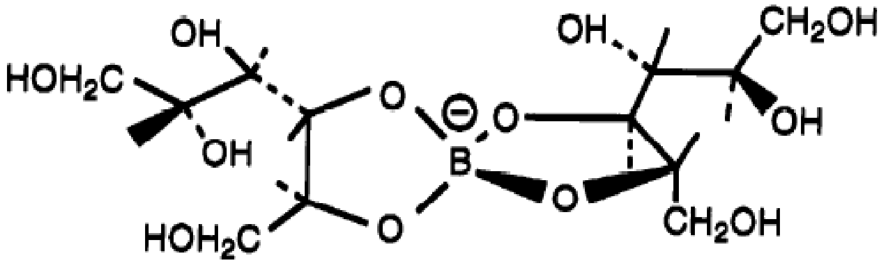

Fig. 1.2. Structural representation of the bis(sorbitol) borate ester, one of several isomers identified in the phloem of sorbitol-rich plant species (*Brown and Hu*, 1996).

Boron deficiency in plants

Most soils have relatively low B contents; only soluble B in soils is available for plants and this is usually about 10% of the total soil B content. The occurrence of B deficiency depends on multiple factors mainly caused by the reduction of the availability of soluble B in the soil, such as e.g. weather conditions (drought, high precipitation), soil conditions (low pH soils: B leaching, calcareous soils: B fixation) and the cultivated crop species (*Shorroks*, 1997). Physiological responses of plants to B deficiency include the loss of membrane integrity and cell wall stability, which result in the development of structural damage in the generative and vegetative organs of crop plants such as blossom wilting and necrosis, reduced pollen production and poor fruit set in the generative organs. Damages in the vegetative organs are for example, cracked stem in celery, stalk rot of cauliflower, heart rot and internal black spot of beets, top rot of tobacco, internal cork of apples, abnormal stomata and distorted guard cells in cauliflower and coffee and decreased stomatal conductance and transpiration rates in navel orange and cotton (*Sharma and Sharma,* 1987; *Blevins and Lukaszewsky,* 1998; *Sheng* et al., 2000; *Oosterhuis and Zhao*, 2001; *Rosolem and Leite,* 2007). Many other effects associated with B imbalances have been described, but the direct role of B in metabolism is still little understood.

Sensitivity to B deficiency is not only species dependent, but may also vary between varieties. In Northern Thailand, seed quality of 19 soybean cultivars was assessed according to their response to B deficiency. The results showed a wide range of responses. Sensitive cultivars showed up to 75% hollow hard symptoms, whereas insensitive cultivars showed only 1% (*Rerkasem*, 1993). The first visual effects of B deficiency can be observed in young leaves and meristematic tissues in case of limited B re-translocation, as well as in roots (*Poss* et al., 1999) (Fig. 1.3, 1.4).

Fig. 1.3. Soybean plants under deficient (left) and sufficient (right) B supply in the nutrient solution (source: S. Will).

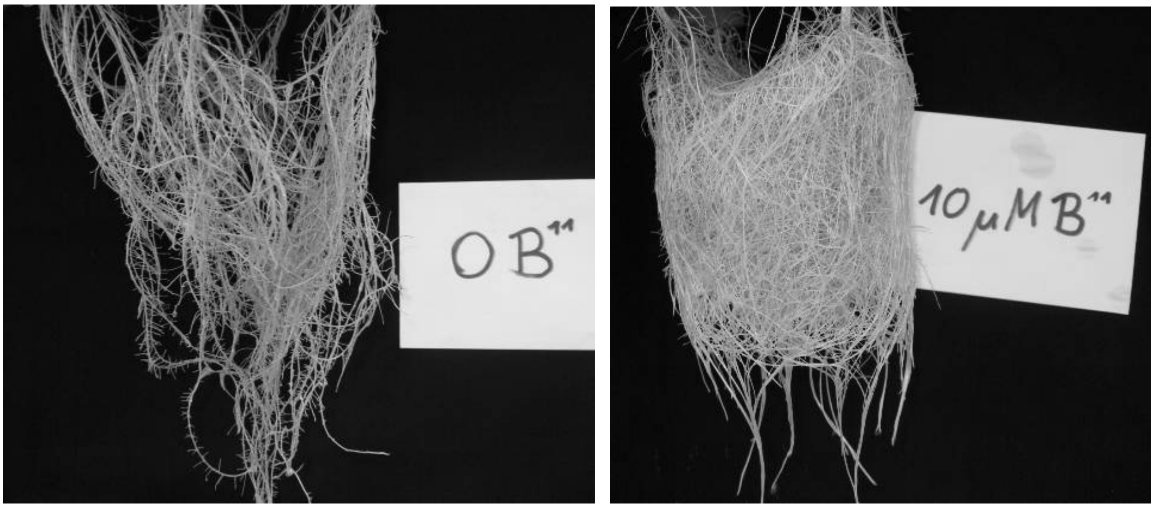

Fig. 1.4. Roots of soybean treated under deficient (left) and sufficient (right) B supply in the nutrient solution (source: S. Will).

Boron toxicity in plants

Natural B toxicity occurs in soils associated with recent volcanism, in arid and semi-arid environments or may derive from mining deposits, fertilizers or irrigation water. B toxicity can considerably limit plant production (*Miwa* et al., 2007), but information on physiological consequences of B toxicity is fragmentary (*Nable* et al., 1997). *Brown and Hu* (1996) described symptoms of toxicity such as the death of cambial tissues and stem die back, fruit disorders (gummy nuts, internal necrosis) and bark necrosis. A loss in membrane integrity in association with B toxicity was reported by *Alpaslan and Gunes* (2001).

In case of limited B-re-translocation, B toxicity symptoms are visible in older leaves especially in the leaf tips where the transpiration stream ends (*Poss* et al., 1999). Visual symptoms of B intoxication in soybean plants included abnormal leaf extension and veinal browning (Fig. 1.5).

Fig. 1.5. Impact of B intoxication in soybean plants, upnormal leaf expansion (left) and veinal browning (right) (source: S. Will).

Boron mobility in plants

In most plant species, B is phloem immobile and distribution of B within a plant mainly follows the transpiration stream.

Within the cell wall and cytoplasm, B quickly forms stable compounds (mainly mono- and diesters) and contributes to the water insoluble fraction. Thus, re-translocation from source to sink organs is not easily accomplished. In a wide range of plants, sugar alcohols (also called polyhydric alcohols or polyols) are present in the phloem sap. Most common are the straight-chained hexiols such as mannitol and sorbitol (*Bielski*, 2005). They contain cis-diol groups which can form stable compounds with B. These compounds facilitate the re-translocation from old leaves to "sink" organs such as young developing leaves, roots, fruits and meristematic tissues (*Shelp* et al., 1988; *Delgado* et al., 1994; *Brown and Hu*, 1996; *Brown* et al., 1999). Boron mobility was evidenced in plants mainly belonging to the Rosaceae family (e.g., apple, cherry, peach) having large quantities of the sugar-alcohol sorbitol in the phloem sap, and also in those rich in mannitol largely corresponding to the families of Apiaceae (carrots and celery), Brassicaceae (broccoli, cauliflower), Fabaceae (pea, common bean) and Oleaceae (olive) (*Bielski*, 1982; *Brown and Shelp*, 1997). *Blevins and Lukaszewski* (1998) found a large quantity of the sugar alcohol pinitol in the phloem sap of soybean, but the possibility of B bonding and re-translocation remains unclear (*Bielski*, 2005). *Lehto* et al. (2004) suggested a possible role of B compound formation with inositol or pinitol in Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) H. Karst), but stable compounds could not directly be demonstrated.

1.1.2 Foliar fertilization

In case of micronutrients, foliar fertilization is considered to be a fast and target oriented tool to cure deficiencies and is commonly applied within the pest control in agricultural practices. Foliar absorption from liquid solutions may take place via the cuticle, cuticular cracks and imperfections, through stomata, trichomes or specialised epidermal cells. Two mechanisms of foliar absorption have been discussed and characterized to a certain extent, namely, the cuticular pathway and the stomatal pathway (*Fernández and Eichert*, 2009). Regarding the mechanisms of cuticular absorption, apolar, lipophilic compounds have been proposed to follow a diffusion-dissolution process (*Riederer and Friedmann*, 2006). In contrast, the mechanisms of absorption of hydrophilic solutes through the cuticle are currently not fully understood (*Fernández and Eichert*, 2009) and it has been hypothesized, it may occur via "aqueous pores" (*Schönherr*, 1976, 2000, 2006). There is only indirect evidence for the existence of polar pores, since it has not been possible so far to detect such structures in plant materials with the microscopical techniques currently available.

While the significance of the stomatal pathway on the uptake of foliar sprays has been a matter of controversy for many years, recent evidence shows that it can largely contribute to the absorption process (*Eichert* et al., 2008). Investigations carried out with fluorescent tracers provided evidence for stomatal absorption along the surface via a diffusion process (*Eichert* et al., 2008). *Eichert and Burkhardt* (2001) found that less than 10% of the total stomata contributed to the total absorption, but the number of penetrated stomata could be increased by a re-wetting/drying cycle.

Foliar absorption requires dissolution of the applied salt. Hence, once the drop of fertilizer solution hits the leaf surface, the time remaining in a liquid phase seems to be crucial. An expanded liquid phase on the leaf surface is influenced by two factors, relative humidity (RH) and deliquescence humidity (DRH) of the salt, which describes the minimal water vapor pressure enabling sugars and salts to absorb water from the surrounding air and to go into solution (*Eichert* et a.l, 1998). The relative humidity at which deliquescence first occurs is a characteristic of the individual substance (*Fernández and Eichert*, 2009). Boric acid has a

DRH of 98% indicating that foliar-applied solutions containing pure boric acid will only stay in solution in almost water saturated air. Mixtures of two substances lower the DRH below the DRH of the individual substance (*Salameh* et. al, 2005).

Since nutrient sprays are applied in the field, there is a limited chance to control the mechanisms associated with the rate of absorption and bioactivity of the nutrients applied to the foliage, but spraying plants when stomata are open and under conditions limiting the rate of solution drying might be a possible control tool. However, the importance of improving the properties of foliar nutrient formulations has long been recognized (*Fernández and Eichert*, 2009), and is currently a matter of scientific interest as key strategy to optimize the performance of foliar sprays (e.g., *Kraemer* et al., 2009; *Val and Fernández*, 2011). Adjuvants, such as surfactants lowering the surface tension of water and facilitating the process of stomatal infiltration or humectants prolonging the process of droplet drying, have been shown to improve the effectiveness of nutrient sprays when applied to horticultural crops (*Blanco* et al., 2010; *Val and Fernández*, 2011; *Eichert and Fernández*, 2012).

1.2. Hypothesis and outline of this study

The main hypotheses addressed in this thesis are:

I. The rates of foliar B absorption and subsequent translocation depend on plant B status. This hypothesis is based on observations with other mineral nutrients such as phosphorous (P). It was observed that the rate of P absorption and subsequent translocation by the leaves of phosphorus-deficient plants was higher then in the control (*Marschner*, 1995).

II. The application of B-sorbitol (sorbitol borate ester) can facilitate the translocation of foliar-applied B in species with B phloem immobility. Natural phloem mobility of B takes place in species with polyols, e.g. sorbitol, as a major carbohydrate, which form stable compounds with B. The foliar application of B together with polyols may thus induce B mobility in plants lacking polyols in the phloem.

III. Foliar B absorption can be enhanced by applying the formulation to the abaxial leaf surface especially in hypostomatal plants (lychee), by taking advantage of the stomatal uptake pathway.

IV. The effectivity of foliar nutrient absorption can be stimulated by the supplementation of adjuvants in the spray formulation. Humectants are considered to increase absorption rates due to the extension of the period were the drop remains in a liquid phase, enabling absorption of the applied nutrient. Foliar B absorption can be enhanced by the addition of humectant adjuvants in the foliar formulation.

V. In contrats to root cells, absorption by green leaf cells is directly stimulated by light. (*Marschner*, 1995). Therefore, foliar B absorption and subsequent translocation will be higher after diurnal vs. nocturnal application.

VI. Rates of nutrient absorption by leaves decline with leaf age due to metabolic activity (sink activity) and increase in the thickness of the cuticle (*Marschner*, 1995). Foliar B absorption will be reduced in mature vs. immature leaves due to metabolic activity and the increase in the thickness of the cuticle.

This study is based on one published paper (Chapter 2), one paper under revision process (Chapter 3) and one submitted paper (Chapter 4).

Hypothesis I and II were tested and discussed in Chapter 2. It includes an article on the impact of different parameters, such as plant B status on foliar B absorption and subsequent translocation and the addition of polyol borate ester to increase B mobility after foliar absorption in soybean plants. Hypotheses II, III and IV were audited and discussed in Chapter 3. The article is based on data collected in lychee and soybean plants after using different foliar formulations either applied to the adaxial or abaxial leaf surface to increase B foliar absorption and subsequent translocation. Hypothesis III, V and VI were tested and discussed in Chapter 4. Chapter 4 includes an article based on data collected from lychee plants. The effect of different parameters on foliar B absorption and distribution, such as leaf side, leaf age and time of application was studied.

The manuscript continues with a general discussion (Chapter 5), a section of references and will be completed with summaries in English and German.

1.3 General Material and methods

1.3.1 Plant species

This study was part of the research project SFB 564 phase III (https://sfb564.uni-hohenheim.de/). Field experiments on foliar B fertilization conducted on lychee trees (*Litchi chinensis* Sonn. cv. `Hong Huay`) grown in Mae Sa Mai, Chiang Mai Province, Northern Thailand, resulted in a complete failure (no increased foliar B concentrations) by spraying mainly the adaxial leaf surface. It was concluded that another focus for future research work should explain the failure of foliar applications of B. This failure was in accordance with various observations in farmers' fields. Aims of the model experiments were to increase B absorption and mobility within the plants. Lychee is a slow growing tree, therefore, another plant species was selected to conduct experiments on different parameters influencing B absorption and subsequent translocation. Parameters causing a strong impact on absorption and subsequent translocation were then studied on lychee. Prerequisite for the second species was economical relevance of B deficiency in the production areas and a fast and equal growth development. Soybean [*Glycine max* (L.) Meer. cv. 'Oak Erin'] fulfilled the requirements and was therefore selected.

For experiments in the climate chamber and greenhouse in Hohenheim, lychee plants were propagated by air layering of stems with equal size and diameter from the cultivar `Hong Huay' cultivated in the research fields of the Chiang Mai University.

Lychee (Litchi chinensis Sonn.)

Lychee fruits are considered as one of the most popular fruits in China. Originated in southern China and in Northern Vietnam, the commercial production has spread to many areas of the world, such as South-East Asia, Taiwan, Japan, Australia, South Africa, Antilles, Brazil and USA (Florida and Hawaii) (*Othmann and Subhadrabandhu*, 1995). The trees require a moist tropical environment with cold periods for flower initiation. Expanding the production is mainly limited by the exact ecological requirements of the trees. The optimal tissue B concentration in mature lychee leaves ranges from 25 to 60 µg g^{-1} dry weight (DW) (*Menzel*, 2002).

Soybean (Glycine max (L.) Meer.)

Soybean is classified into the group of oil crops and takes a leading position on a global scale. Soybean has been grown as a commercial crop primarily in temperate ecologies for thousands of years. The main production areas are USA, Brazil, Argentina and China, but there is also a very important potential role for soybean in many cropping systems of the tropics and subtropics (*Thoenes*, 2004). Soybean plants require well drained highly fertile soils with a good supply of organic matter. The optimal tissue B concentration in mature soybean leaves ranges from 20 to 50 µg g^{-1} DW (*Woodruff*, 1979).

1.3.2 Foliar formulations

All applied foliar formulations consisted of a basic solution containing 50 mM ^{11}B labeled boric acid (BA) and the adjuvant Plantacare (Plantacare, Cognis, Düsseldorf, Germany). In pre-experiments different adjuvants in various concentrations were tested in order to assess the rate of the wetted area and amount of retention on the leaf surface.

Seven adjuvants commonly used in agricultural practice were consecutively evaluated in various concentrations according to the application recommendation of the manufacturer for both species. The aim of this test was to evaluate the optimal concentration and to experience the handling of the adjuvants on the two species used in this study. For the drop application method, which was used for the presented experiments, high retention of the formulation was the prerequisite for evaluation. Highest retention was observed with adjuvants based on oil. As oil based formulations generate severe damage on lychee leaves after treatment, Plantacare as the second best was evaluated as the adjuvant for the basic solution. According to preliminary experiments the adjuvant concentration did not affect the amount of solution retained by leaves of both species. Therefore the concentration of Plantacare was the same in both species.

1.3.3 Nutrient solution

The nutrient solution was a standard solution used at the Plant Nutrition Unit of the Universität Hohenheim. Before the onset of the experiments nutrient analyses were conducted within the timeframe of the experiments to analyse the development of nutrient concentrations (K, Mg, Ca, Fe) during plant development. All nutrients analyzed were in accordance with the recommended sufficient concentration from literature. The fluctuation in the pH values ranged from 5.5 to 6.5 and did not affect the availability of B as below pH 7 more than 99% of total B prevails in the form of BA.

1.3.4 B-polyol compounds

In plant fluids B exists mainly as mononuclear species such as $B(OH)_3$ or $B(OH)_4^-$, polynuclear species are observed with increasing pH (> 10).

In plant species with polyols, e.g. sorbitol, as a major carbohydrate, the compound formation of bis(sorbitol)borate ester enable B mobility within the plant. The terminology “B-sorbitol complexes” is commonly used in literature. However, B bondings to polyols are covalent bondings and therefore the chemical terminology is sorbitol borate ester. In Chapter 2 the correct terminology was modified after publication. Therefore, the published paper includes the term B-sorbitol/mannitol-complexes.

CHAPTER 2

ABSORPTION AND MOBILITY OF FOLIAR-APPLIED BORON IN SOYBEAN AS AFFECTED BY PLANT BORON STATUS AND APPLICATION AS A POLYOL BORATE ESTER[i]

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[i] **This chapter has been reprinted from:**

Will S., Eichert T., Fernández V., Möhring J., Müller T., Römheld V. (2011): Absorption and mobility of foliar-applied boron in soybean as affected by plant boron status and application as polyol complex. Plant Soil (344), 283-293. With permission by Springer Verlag (Copyright 2011). Nomenclature of polyol complex was changed in this chapter into polyol borate ester.

2. Absorption and mobility of foliar-applied boron in soybean as affected by plant boron status and application as a polyol borate ester

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2.1. Abstract

In the present study (i) the impact of plant Boron (B) status on foliar B absorption and (ii) the effect of applying polyol borate ester (sorbitol or mannitol) on B absorption and translocation was investigated. Soybean (*Glycine max* (L.) Meer.) plants grown in nutrient solution containing 0 µM, 10 µM, 30 µM or 100 µM ^{11}B labelled boric acid (BA) were treated with 50 mM ^{10}B labelled BA applied to the basal parts of two leaflets of one leaf, either pure or in combination with 500 mM sorbitol or mannitol. After one week, ^{10}B concentrations in different plant parts were determined. In B deficient leaves (0 µM ^{11}B), ^{10}B absorption was significantly lower than in all other treatments (9.7% of the applied dose vs. 26%-32%). The application of BA in combination with polyols increased absorption by 18-25% as compared to pure BA. The absolute amount of applied ^{10}B moving out of the application zone was lowest in plants with 0 µM ^{11}B supply (1.1% of the applied dose) and highest in those grown in 100 µM^{11}B (2.8%). The presence of sorbitol significantly decreased the share of mobile ^{10}B in relation to the amount absorbed. The results suggest that ^{11}B deficiency reduces the permeability of the leaf surface for BA. The addition of polyols may increase ^{10}B absorption, but did not improve ^{10}B distribution within the plant, which was even hindered when applied as sorbitol borate ester.

2.2. Key words

B deficiency, B toxicity, foliar absorption, mannitol, sorbitol, soybean, water potential

2.3. Introduction

Warrington (1923) proved B to be an essential micronutrient for higher plants. Even though the demand for B on a molar basis is higher than for any other micronutrient in dicotyledons, knowledge of its physiological role is still limited. Boron deficiency appears worldwide in crop production and is reported in over 80 countries on 132 crops. The occurrence of B deficiency depends on multiple factors, such as e.g. weather conditions (drought, high precipitation, etc.), soil conditions (low pH soils B leaching, calcareous soils B fixation) and the cultivated crop species (*Shorroks*, 1997). Physiological responses of plants to B deficiency include the loss of membrane integrity and cell wall stability, which result in the development of structural damage in crop plants like for instance, cracked stem in celery, stalk rot in cauliflower, heart rot and internal black spot in beet, top rot in tobacco and internal cork in apple (*Blevins and Lukaszewski*, 1998). Several studies showed that B deficiency induces leaf structural changes, including abnormal stomata and distorted guard cells in cauliflower (*Sharma and Sharma*, 1987) and coffee (*Rosolem and Leite*, 2007) or decreased stomatal conductance and transpiration rates in navel orange and cotton (*Oosterhuis and Zhao*, 2001; *Sheng* et al., 2009). Many other effects associated with B imbalances have been described, but the direct role of B in metabolism is still little understood.

In commercial plant production, providing a sufficient B supply is particularly important for yield formation (pollination) (*Khayyat* et al., 2007; *Wojcik* et al., 1999), fruit quality and crop storability (*Wojcik* et al., 1999), and stress tolerance (*Cakmak and Römheld*, 1997). In addition to B deficiency, B toxicity can also considerably limit plant production (*Miwa* et al., 2007). Natural B toxicity occurs in soils in arid and semi-arid environments or may derive from mining deposits, fertilizers or irrigation water. Information available on B toxicity is fragmentary (*Nable* et al., 1997). *Brown and Hu* (1996) described symptoms of toxicity such as the death of cambial tissues and stem die back, causing fruit disorders (gummy nuts, internal necrosis) and bark necrosis. A loss in membrane integrity in association with B toxicity was reported by *Alpaslan and Gunes* (2001).

In most plant species, B is phloem immobile and distribution of B within a plant mainly follows the transpiration stream. The first visual effects of B deficiency can be observed in young leaves and meristematic tissues, whereas B toxicity symptoms are mainly visible in older leaves especially in the leaf tips where the transpiration stream ends (*Poss* et al., 1999).

Within the cell wall and cytoplasm, B quickly forms stable compounds (mainly mono- and diesters) and contributes to the water insoluble fraction. Thus, re-translocation from source to sink organs is not easily accomplished in the plant. In a wide range of plants, sugar alcohols (also called polyhydric alcohols or polyols) are present in the phloem sap. Most common are the straight-chained hexiols such as mannitol and sorbitol (*Bielski*, 2005). They contain cis-diol groups which can form stable esters with B. These compounds facilitate the re-translocation from old leaves to “sink” organs such as young developing leaves, roots, fruits and meristematic tissues (*Shelp* et al., 1988; *Delgado* et al., 1994; *Brown and Hu*, 1996; *Brown* et al., 1999). Boron mobility was evidenced in plants mainly belonging to the Rosaceae family (e.g., apple, cherry, peach) having large quantities of the sugar-alcohol sorbitol in the phloem sap, and also in those rich in mannitol largely corresponding to the families of Apiaceae (carrots and celery), Brassicaceae (broccoli, cauliflower), Fabaceae (pea, common bean) and Oleaceae (olive) (*Bielski*, 1982; *Brown and Shelp*, 1997). *Blevins and Lukaszewski* (1998) found a large quantity of the sugar alcohol pinitol in the phloem sap of soybean, but the possibility of compound formation with B and re-translocation remains unclear (*Bielski*, 2005). *Lehto* et al. (2004) suggested a possible role of B compound formation with inositol or pinitol in Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) H. Karst), but stable compounds could not be directly demonstrated.

In the present study the impact of plant B status on B foliar absorption and the effect of polyol borate ester application on improving absorption and translocation was investigated in soybean. Some experiments carried out with soybean cultivar “Oak Erin” suggested that pinitol (i.e. the polyol detected in soybean) does not significantly contribute to B mobility. In contrast to sorbitol and mannitol, pinitol is a cyclic polyol. Since the process of B binding with pinitol remains unclear, it was not investigated as a candidate for foliar B application trials.

Thereby, to assess the effect of polyol borate ester application, sorbitol and mannitol were selected since stable B compounds with these polyols have been previously reported (*Hu and Brown*, 1997). In preliminary trials (data not shown) a 1:10 B:sorbitol ratio was found to increase the rate of foliar B absorption and translocation in soybean plants. Hence, the following two hypotheses were tested, namely: (i) plant B status may affect the absorption and the within-plant mobility of foliar-applied B and (ii) foliar application of sorbitol borate- and mannitol borate ester can increase absorption and the within-plant mobility of B.

2.4. Material and Methodes

Pre-treatment

Soybean seeds (*Glycine max* (L.) Meer., cv. “Oak Erin”) were soaked for 1 hour (h) in 10 mM $CaSO_4$ solution and then transferred to filter paper moistened with 2.5 mM $CaSO_4$ until radicles emerged. Seedlings were planted into 3 liter (L) plastic pots (4 plants per pot) containing continuously aerated nutrient solution (pH 5.5) of the following composition: 0.88 mM K_2SO_4, 0.1 mM KCl, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 10 µM $^{11}B(OH)_3$ (enrichment 99.8%), 0.5 µM $MnSO_4$, 0.2 µM $CuSO_4$, 0.02 µM $(NH_4)_6Mo_7O_{24}$, 1 µM $ZnSO_4$ and 100 µM Fe(III)EDTA. The nutrient solution was prepared with de-ionized water and changed on a weekly basis. Plants were cultivated in a climate chamber (Universität Hohenheim, Germany) with a radiation of approximately 1000 µmol $m^{-2}s^{-1}$. Day (14 h) and night (10 h) temperatures were kept at 24 and 20°C respectively, with a relative humidity (RH) of 60%. After cultivation in full-strength nutrient solution for 2 weeks, the 16 pots were divided into 4 groups which were consequently supplied for 2 weeks with a nutrient solution containing 0 µM, 10 µM, 30 µM or 100 µM ^{11}B -labelled BA,

Foliar Formulations

Foliar treatment solutions were prepared with a basic de-ionized water solution containing 50 mM ^{10}B labelled boric acid (BA) plus 0.3% (v/v) surfactant (Plantacare, Cognis, Düsseldorf, Germany). Sorbitol and mannitol were used at a concentration of 500 mM, because concentrations of B and sorbitol in ratio 1:10 facilitates the formation of 1:2 polyol borate esters (*Hu and Brown*, 1997). The basic solution (BaSol) was used as the control in order to compare whether the polyols contribute to the quantitative absorption and/or affect the within-plant mobility of absorbed B. Treatments of the experiment were as follows:

1. Boron (B): Basic solution (BaSol)
2. Mannitol (BM): BaSol with B:mannitol ratio 1:10 (w/w)
3. Sorbitol (BS): BaSol with B:sorbitol ratio 1:10 (w/w)

All chemicals were of analytical grade (Merck, Darmstadt, Germany).

Data collection and sampling design

Data were collected from 16 pots containing 4 plants per pot, in total 64 plants. The pots were set using a split plot design with 4 replicates. The main plot factor pre-treatment had 4 levels: nutrient solutions with 0, 10, 30, and 100 µM ^{11}B concentration, the sub plot factor foliar formulation has three levels: basic solution, basic solution with sorbitol and basic solution with mannitol. The fourth plant within each pot was used to measure the water potential.

Application

Foliar treatment solutions were applied on leaflets of the last fully-expanded leaves. Treatments were supplied via application of 16* 2.5 µL drops (40 µL in total) of the formulation on two adjacent leaflets per leaf. Soybean leaves consist of three leaflets, two are paired and one is the upper leaflet. Drops were applied to the lower half of the paired leaflets (Fig. 2.1). Leaves were harvested after 1 week and separated into segments. Distribution of the applied ^{10}B was separately determined for the different segments as indicated in Fig.2.1: Segment 1: fraction remaining in the application zone, segment 2: fraction of ^{10}B in the leaf tip indicating acropetal translocation via transpiration stream and segment 3: fraction in non-treated leaflets of the treated leaf indicating short distance basipetal translocation via phloem. All samples from segment 1 were carefully rubbed under de-ionized water between gloved thumb and forefinger for 20 seconds to remove trapped material (*Eichert and Goldbach*, 2008).

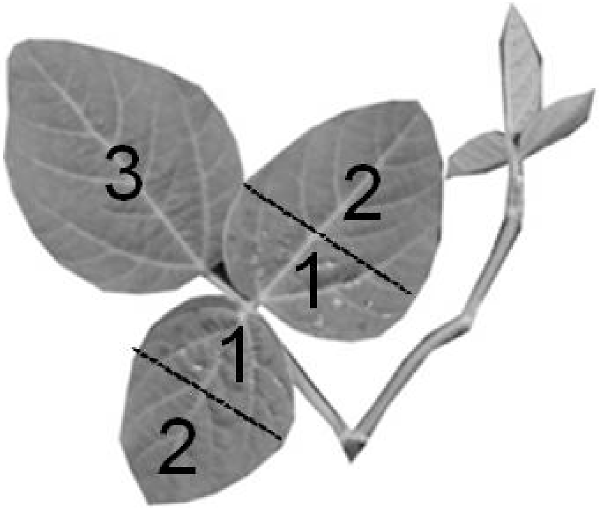

Fig. 2.1. Schematic presentation of soybean leaf segments used for the analysis of ^{10}B absorption and translocation. 1: application zone (lower part of a leaflet), 2: leaf tip, 3: non-treated leaflet of a treated leaf.

Analytical methods

Harvested leaves were dried in the oven at 65°C for 2 days. Ground dry leaf samples (0.05 – 0.1 g) were weighed in quartz crucibles. The samples were ashed in the oven with increasing temperatures (200 °C, 300 °C, 400 °C and 500 °C for 1, 1, 1 and 2 h, respectively). Then samples were cooled down overnight. Next day, samples were rewetted with some drops of 3% H_2O_2-solution and after drying, ashed again in the oven for 3 hours at 500°C. The ash was dissolved in 5 ml mixed acid solution [3.3% v/v HNO_3 + 10 ppb Beryllium (Be)] and centrifuged for 2 minutes at 4000 x g (Hettich Universal 30F). Boron isotopes (^{10}B, ^{11}B) were determined by inductively coupled plasma mass spectroscopy (ICP-MS, ELAN 6000, Perkin-Elmer, Überlingen, Germany), using Be as an internal standard. B concentrations and contents in each segment were calculated.

Water potential

Leaf water potentials were measured using the Scholander pressure chamber method (*Scholander* et al., 1965). From each pot one plant was randomly selected (n=4), the last fully-developed leaves of soybean plants were harvested and immediately fixed into the Scholander pressure chamber. For standardization of the moment when the xylem fluid appeared, tissue paper was held carefully on the top of the leaf stem. As soon as a liquid drop was visually observed, the pressure was recorded. This method was implemented to facilitate the visual detection of the sap appearance, since in former experiences with drought stressed plants, only small and disperse drops similar to foam could be hardly seen to come out of the soybean leaf petiole.

Scanning electron microscopy (SEM) examination

Leaf surfaces were examined under a scanning electron microscope (S- 3400 N, Hitachi, Tokyo, Japan; acceleration potential 15 kV, working distance 10–11 mm). Leaves from the different treatments were dried at room temperature, making sure that the surface remained flat. For observation of either the upper or lower leaf side, approximately 1 cm^2 sections were excised, and sputtered with gold. Different areas of the leaf sections were subsequently directly observed under the microscope. The abaxial and adaxial surface of five leaves was examined for each treatment. The length and width of stomatal pores (n=100) was assessed by the programme Image-Pro Plus 6 (Bethesda, USA).

Statistics

A mixed model approach was used for statistical analysis. For fixed effects general least square means were estimated and presented with their standard error in the results. An univariate analysis was performed for the ^{10}B concentration of each segment, the sum of segment 2 and 3, the propotion of ^{10}B in segment 2 or 3 compared to all segments, for the water potential and the ^{11}B concentration. A multivariate analysis was used for a combined analysis of ^{10}B over all segments. In addition, the water potential und the ^{11}B concentration were used as covariates for ^{10}B, but were dropped from the model as they had no significant influence.

To reach homogeneous residual variation for univariate and multivariate analysis, the data were logarithmically transformed for the traits ^{10}B and ^{11}B. For analysing the proportions of segment 2 or 3 the ^{10}B data were transformed using the logit as link function. In both cases estimated means were back transformated for presentation. The shown standard errors of these means were back transformed using the delta method.

The model for the univariate analysis is given by:

$y_{ijklm} = \mu + r_k + \alpha_i + \beta_j + (\alpha\beta)_{ij} + p_{kl} + e_{ijklm}$, (Equation 1)

where r_k is the effect for the k^{th} replicate, p_{kl} is the main plot error effect of the l^{th} pot in the k^{th} replicate, α_i is the i^{th} pre-treatment effect and β_j is the j^{th} nutrient solution effect. $(\alpha\beta)_{ij}$ denotes the interaction effect of the i^{th} pre-treatment and the j^{th} nutrient solution. e_{ijklm} denotes the subplot error or residual error effect of the i^{th} pre-treatment, j^{th} nutrient solution of the m^{th} plant in the l^{th} pot in the k^{th} replicate. All factors and interactions were taken as fixed. The main and sub plot error were taken as random. The replicate effect was treated as fixed ignoring all inter block information.

For the multivariate analyses the model is given by:

$y_{ijklmn} = \mu + r_{kn} + \alpha_i + \beta_j + (\alpha\beta)_{ij} + (\alpha\gamma)_{in} + (\beta\gamma)_{jn} + (\alpha\beta\gamma)_{ijn} + p_{kln} + e_{ijklmn}$, (Equation 2)

where γ_n denotes the n^{th} segment, and interactions and all other effects are denotes as in equation (1). For the pot effects p_{kln} of the three segments and for the residual error effects e_{ijklmn} of the three segments an unstructered variance-covariance matrix was assumed a priori. Because of small or fixed main plot variance component estimates the variance-covariance structure for the analysis of ^{10}B was simplified by dropping the covariances between main plot effects of one plant. Thus the optimal variance-covariance structure included heterogeneous variances for segments but no covariances. An Akaike Information Criterion (AIC) (*Akaike*, 1974) based model selection approach was used to find this model.

2.5. Results

Plant B status

Growth of soybean plants under various isotopically-labelled BA concentrations in the nutrient solution resulted in plants with different ^{11}B tissue concentrations. Plants grown in full-strength nutrient solution with 0, 10, 30 or 100 µM ^{11}B had average ^{11}B tissue concentrations of 2.1 (± 0.2), 50.9 (± 5.2), 86.7 (± 8.9) and 103.7 (± 10.7) µg ^{11}B g^{-1} DW, respectively.

Visual symptoms

Symptoms were observed in plants with 0 µM ^{11}B and 100 µM ^{11}B supply. Plants grown without ^{11}B showed deficiency symptoms such as diminished root and shoot growth. Root development was significantly decreased in plants without ^{11}B supply in comparison to plants treated with 10, 30 or 100 µM ^{11}B (1.4 ± 0.1, 4.6 ± 0.9, 5.3 ± 0.5, 4.8 ± 0.4 g DW, respectively). Roots were brownish in colour and shoot development was decelerated, due to the dying off of apical meristems. Leaves became very hairy, rigid, dark green, small and interveinal necrosis appeared. The inclination of the leaves was abnormal. They grew vertical and leaf tips pointed downwards. Moreover, alterations in the surface morphology of leaves in plants with 0 µM ^{11}B were observed. Stomata appeared closed, collapsed and sunken underneath the epidermis (Fig. 2.2). Whereas the pore lengths of B deficient leaves did not differ from leaves grown under adequate (10 µM ^{11}B) B supply (-B: 8.4 ± 1.3 µm, +B: 8.5 ± 1.3 µm, n = 100), the pore widths differed significantly. In B deficient leaves average pore widths were 0.1 ± 0.3 µm, while with adequate B supply widths were 3.2 ± 1.3 µm (n = 100).

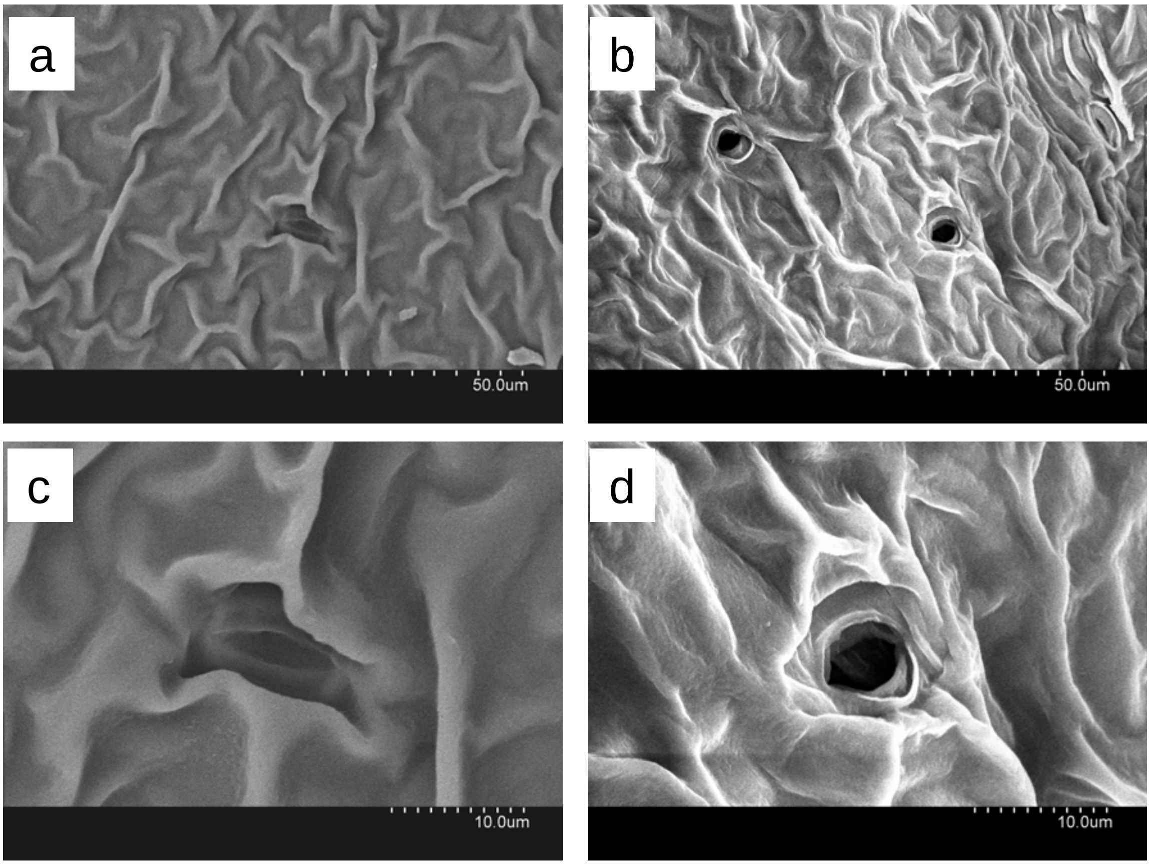

Fig. 2.2. SEM micrographs of the abaxial leaf surface of soybean leaves. Stomata appeared closed, collapsed and sunken underneath the epidermis on plants grown without ^{11}B (a, c) and developed regularly on plants treated with 10 μM^{11}B in the nutrient solution (b, d).

Abnormal leaf inclinations were also observed in association with 100 µM ^{11}B supply. Furthermore, older leaves were also rigid and showed veinal browning on the lower leaf surface, with black spots on the upper leaf surface. In all treatments, the dry matters of the treated leaves varied between 300 to 400 mg and no clear trend could be detected in association with the different B regimes. Development and phenology of roots and shoots of plants with 10 µM ^{11}B and 30 µM ^{11}B supply was in accordance to normal growth of the species.

Necrotic spots appeared beneath the applied droplets in some of the treatments. The degree of damage depended on the composition of the formulations and the plant ^{11}B status. Regardless of the foliar formulations applied to the B-deficient plants (0 µM ^{11}B) necrotic spots never developed on the treated leaf areas. Increased phytotoxicity symptoms were observed in plants cultivated in 30 and 100 µM ^{11}B. The degree of damage was most severe after the application of formulations containing sorbitol.

Water status

Water potential (ψ_w) measurements showed highest values of -0.59 ± 0.05 MPa and -0,61 ± 0.06 MPa in plants with 0 µM ^{11}B and 100 µM ^{11}B supply, respectively. In plants with 10 µM ^{11}B and 30 µM ^{11}B supply, ψ_w was lower with values of -0.78 ± 0.13 MPa and -0.74 ± 0.12 MPa, respectively

Absorption and mobility

Both B absorption and B translocation were significantly affected by plant ^{11}B status and the addition of polyols, whereas interactions between these 2 factors were not significant (Table 2.1). When applied as pure BA 18.2% of the foliar-applied ^{10}B was absorbed by the leaves, while with the addition of sorbitol or mannitol the proportion of absorbed ^{10}B increased to 22.9% and 25.4%, respectively (Fig. 2.3a). Plants with 0 µM ^{11}B supply showed the lowest ^{10}B contents representing only 9.7% of the applied dose, whereas in the other treatments 26.5% to 32.3% of the applied ^{10}B penetrated the leaf surfaces (Fig. 2.3b).

Tab. 2.1. Results of statistical analysis of ^{10}B contents in segments 1 (application zone), 2 (upper part of treated leaf), and 3 (neighbouring leaflet) as affected by the ^{11}B supply during pre-culture ("B status") and the foliar application as pure boric acid or boric acid in combination with polyols ("foliar treatment").

Source of variation	Segment 1	Segment 2	Segment 3
B status	<0.001	<0.001	0.0005
Foliar treatment	0.0038	0.0045	0.0253
Interaction	0.4901	0.3671	0.4829

The share of mobile ^{10}B which moved out of the application zone towards the tips of the treated leaves and the adjacent leaflet ranged from 1.1% to 2.8% of the applied dose (Fig. 3d). It was lowest in plants with 0 µM ^{11}B supply and overall highest in the plants grown under 100 µM ^{11}B supply. The addition of mannitol did not significantly affect the share of applied ^{10}B found in other plant parts, whereas sorbitol overall lead to a significant reduction in mobile foliar-applied ^{10}B (Fig. 2.3c).

While the share of mobile ^{10}B expressed as % of the applied ^{10}B is important in practical terms, the significance of this parameter for the analysis of within-plant ^{10}B mobility is rather limited, because it depends on both, the absorption process and the subsequent translocation in the plant. Therefore, we expressed the amounts of ^{10}B detected in other plant parts as % of the total amount that penetrated the leaves. The share of penetrated ^{10}B that moved to the tips of the treated leaflets (segment 2, Fig. 2.1) was higher in plants with 0 µM ^{11}B supply. In plants grown under 30 µM ^{11}B the share of penetrated ^{10}B was the lowest (Fig. 2.3f). The addition of both polyols significantly decreased ^{10}B movement into the tips of the treated leaflets (Fig. 2.3e).

Similar effects were found in the neighbouring leaflet (segment 3). The highest shares of penetrated ^{10}B were detected in plants grown in 0 µM ^{11}B or 100 µM ^{11}B (Fig. 2.3h), and polyols decreased relative mobility (Fig. 2.3g).

B concentrations in other plant parts were below the detection limit of 1µg g^{-1} DW. This assumption derives from former experiments where ^{10}B measured in other plant parts were below the detection limit.

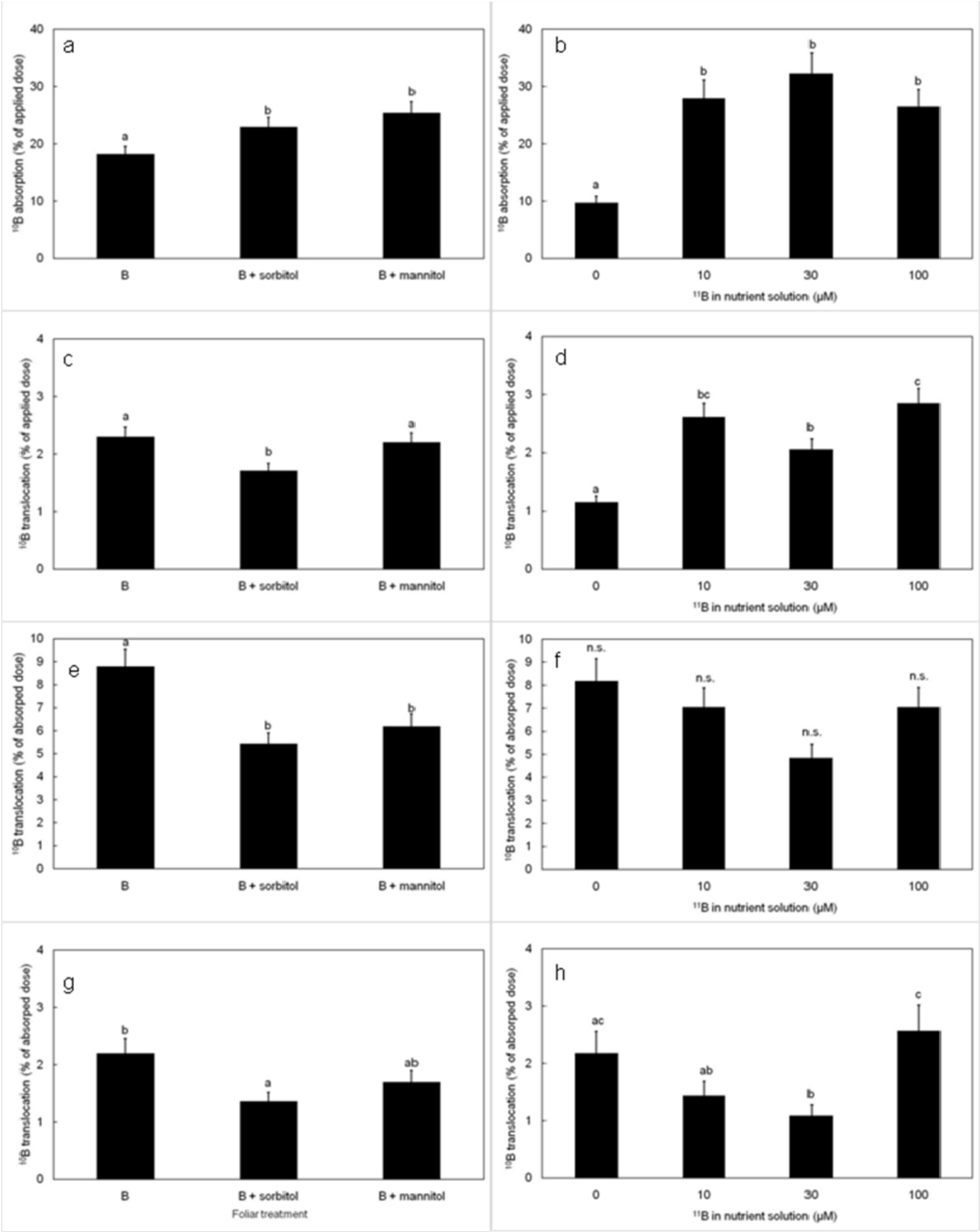

Fig. 2.3. Effect of polyols (a, c, e, g) and different ^{11}B supply during growth (b, d, f, h) on foliar absorption of ^{10}B (a, b), total ^{10}B translocation to the tips of the treated leaflets and neighbouring leaflets as percentage of the applied dose (c, d), and ^{10}B translocation as percentage of absorbed B to tips of the treated leaflets (e, f) and neighbouring leaflets (g, h). Plants were treated either with 50 mM ^{10}B-labelled boric (B), 50 mM labelled boric acid and 500 mM sorbitol (B+sorbitol) or 50 mM labelled boric acid and 500 mM mannitol

(B+mannitol). Error bars represent the standard errors of the means. Values marked by the same letter are not significantly different.

2.6. Discussion

Symptoms of different B root supply

Two weeks after the onset of the ^{11}B treatments, plants showed different visual symptoms. The greatest effect was observed in plants grown without ^{11}B, indicating that they suffered from B deficiency. Shoots and roots showed a significant reduction in growth and development. Moreover, leaf surface morphology alterations were found in plants grown under ^{11}B shortage. Stomata of B-deficient leaves were closed, collapsed and sunken underneath the epidermis (Fig. 2.2). Several studies showed that B deficiency induced leaf structural changes including abnormal stomatal morphology and altered functionality (*Sharma and Sharma*, 1987; *Oosterhuis and Zhao*, 2001; *Rosolem and Leite*, 2007; *Sheng* et al., 2009) but the underlying mechanisms of this physiological response to B deficiency remain speculative.

Boron deficiency and B toxicity affect membrane permeability (*Cakmak* et al., 1995; *Alpaslan and Gunes*, 2001), resulting in membrane leakage and as a consequence K-efflux. Potassium is particularly important for the osmotic regulation of stomatal aperture. Due to the possible K membrane leakage in B-deficient or B -intoxicated plants this regulation could be dysfunctional, which may also explain the higher leaf water potentials at the lowest or highest B supply observed in this study. Another possible explanation for stomatal closure in B deficient plants could be the involvement of B in the structure of the cell walls and microfibrilles of the guard cells enabling stomatal opening.

The cultivation of plants under 100 µM ^{11}B in the nutrient solution induced toxicity symptoms affecting shoot but not root growth as observed visually and by measurement of the root dry mass. The shoots of plants treated with 100 µM ^{11}B supply did not differ in size and development as compared to plants with 10 µM ^{11}B and 30 µM ^{11}B concentrations in the nutrient solution, but the oldest leaves showed toxicity symptoms such as black necrotic spots. *Nable* et al. (1997) reported that under toxic B supply roots had adequate B concentrations in comparison to the toxic B concentrations in the shoots.

Effects on absorption of foliar-applied B

Plants with no ^{11}B supply experienced a significant reduction of foliar ^{10}B absorption as compared to plants grown under 10 µM ^{11}B, 30 µM ^{11}B and 100 µM ^{11}B (Fig. 2.3b). The absorption of ^{10}B was about thrice higher in all treatments in comparison to the 0 µM ^{11}B treatment. This strong decrease in foliar ^{10}B absorption under B-deficiency is rather unexpected and deserves further attention. Foliar absorption is driven by a concentration gradient across the leaf surface and modulated by the permeability of the leaf surface. In theory, a higher B concentration gradient after foliar B application could be expected in B-deficient versus B-sufficient leaves. However, lower ^{10}B penetration rates were determined in B deficient plants. The limited rate of ^{10}B absorption by B-deficient leaves must be most likely caused by a reduced permeability of the leaf surface. In leaves of plants grown without ^{11}B supply, stomata were shrunken and closed, which was earlier reported to reduce absorption of foliar-applied solutes via the stomatal pathway (*Eichert and Burkhardt*, 2001; *Eichert and Goldbach*, 2008). Additionally, with closed stomata, less transpiration water was released which otherwise may have re-condensated on the leaf surface (*Burkhardt* et al., 1999) and kept foliar-applied solutes partly dissolved and mobile even though the surrounding bulk atmosphere was dry (see below). Possibly, B deficiency also induced alterations in cuticular structure, as was recently reported for Fe deficiency in peach and pear trees (*Fernández* et al., 2008). The alteration in leaf structure due to nutrient deficiencies may limit the efficiency of foliar fertilization.

The addition of polyols increased the absorption of foliar-applied B in all treatments. Generally, polyols could enhance B absorption by lowering the deliquescence humidity (DRH) of the deposited substances. This would extend the period of time during which foliar-applied B is mobile and can be absorbed, if RH of the air is above the DRH of the mixture of components (*Fernández and Eichert*, 2009). The RH during the experiment was 60%, which is well below the DRHs of the components, and accordingly this humectant effect should not have affected absorption. However, it has to be taken into account that leaf surfaces are surrounded by a laminar layer in which RH is higher than ambient RH (*Burkhardt and Eiden*, 1994). As already mentioned above, water transpired by the leaves may substantially contribute to an increase in humidity, and therefore the humectant effect of polyols could have increased B absorption despite the low ambient RH. This argument may also explain why polyols did not affect B absorption in plants without B supply because B deficiency induced stomatal closure which probably reduced the amount of water released by the leaves.

Effects on B mobility

The absolute percentage of foliar-applied ^{10}B that moved out of the treated leaf parts ranged from 1.1 to 2.8% of the applied dose, and the effect of ^{11}B pre-culture on absolute ^{10}B mobility was similar to that on ^{10}B absorption, i.e. the lowest amount of ^{10}B moving out of the treated leaf parts was found in plants pre-treated without ^{11}B supply. Polyols also significantly affected ^{10}B mobility, and overall lowest translocation was observed after the addition of sorbitol (Fig. 2.3c), even though the absolute absorption rate in this treatment was significantly higher than with pure BA (Fig. 2.3a). This might be due to the occurrence of many leaf necrotic spots in this treatment, which could have fixed ^{10}B in the dead tissues, thus preventing its translocation.

To gain further mechanistic insight into the effects of the ^{11}B status of plants and added polyols on ^{10}B mobility, we calculated the shares of translocated ^{10}B in relation to the amount absorbed by the leaf. Highest relative translocation rates were observed in plants pre-cultured in 0 µM ^{11}B or 100 µM ^{11}B (Fig. 2.3f, h). While high translocation rates in plants with high ^{11}B contents can be explained by the saturation of possible B binding sites in the cell wall leaving more free B for translocation, the reason for the relatively high shares of translocated ^{10}B in plants without ^{11}B supply is less obvious.

We found evidence that in this treatment stomata were disturbed and, like in plants growing under 100 µM ^{11}B, leaves sustained higher water potentials than plants cultivated under 10 µM ^{11}B or 30 µM ^{11}B indicating that both under B deficiency and high B supply the average transpiration rates were probably lower than under adequate supply, as it was reported by *Eichert* et al. (2010) for Fe deficient peach leaves. According to results obtained with *Ricinus communis* L., low transpiration rates may enhance phloem mobility of foliar-applied B (*Eichert and Goldbach*, 2010), and possibly this was also the case in the present study with soybean.

Both polyols reduced the relative B mobility as compared to the application of BA alone. This may be due to the conversion of small uncharged BA molecules into relatively large, negatively charged polyol borate ester. While BA is moderately plasmalemma-permeable and may thus easily diffuse into the phloem, the large ionic compounds are probably rather excluded from passive trans-membrane transport reducing phloem mobility. This is in contrast to the situation in plants with natural polyol-assisted B mobility, where bonding takes place not until BA has entered the phloem.

2.7. Conclusion

The results of this study indicate that B deficiency symptoms may reduce B absorption through the leaf surface. From an agronomic point of view this negative feedback loop may limit the chance to alleviate B deficiency by foliar fertilization, and it can be concluded that B should therefore be applied before severe deficiency symptoms may occur. The application of B as B-sorbitol compound proved to increase absorption but reduced within-plant B mobility. Therefore, humectants that may have the same positive effect on B absorption as sorbitol, but that may not hinder B mobility should be selected in future research attempts.

CHAPTER 3

BORON FOLIAR FERTILIZATION IN SOYBEAN AND LYCHEE: EFFECTS OF SIDE OF APPLICATION AND FORMULATION ADJUVANTS[ii]

[ii] **This chapter has been reprinted from:**

Will S., Eichert T., Fernández V., Müller T., Römheld V.(2012); Boron foliar fertilization of soybean and lychee: Effects of side of application and formulation adjuvants, JPNSS (175);180-188. With permission by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim (Copyright 2012).

3. Boron foliar fertilization in soybean and lychee: Effects of side of application and formulation adjuvants

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3.1 Abstract

Boron (B) deficiency is a problem of agronomic significance in many species and production areas of the world. Experiments to assess the rate of absorption and translocation of foliar-applied ^{10}B boric acid (BA) were carried out on lychee (*Litchi chinensis* Sonn.) and soybean (*Glycine max* (L.) Merr.) plants grown in nutrient solutions containing ^{11}B. The hypotheses tested were that: (i) absorption rates depend on the leaf side to which B solutions are applied, (ii) that humecting adjuvants ($CaCl_2$ or sorbitol) increase absorption and, (iii) B-sorbitol may increase within-plant mobility of absorbed B. Isotopically-labeled BA solution (50 mM) was applied as individual drops to the basal parts of a pair of leaflets with or without $CaCl_2$ (0.5 mM) and/or sorbitol (50 mM or 500 mM) either to the adaxial or to the abaxial leaf side. Generally, B absorption in lychee was much lower than in soybean. The adaxial leaf surfaces of lychee leaves, which are lacking stomata, were nearly impermeable, while the stomatous abaxial surfaces were permeable to BA solutions. In this species, no translocation of ^{10}B to other leaf parts and no effect of adjuvants on ^{10}B absorption were recorded. In contrast, ^{10}B was absorbed both by adaxial and abaxial leaf surfaces of amphistomatous soybean leaves, but absorption rates were 4-fold higher after treatment of the abaxial as compared to the adaxial side. Treatments containing 500 mM sorbitol led to increased ^{10}B absorption and enhanced acropetal ^{10}B movement, whereas adding only 50 mM sorbitol had no significant effect. Application of 0.5 mM $CaCl_2$ in combination with 500 mM sorbitol decreased the rate of ^{10}B absorption, compared to the performance of 500 mM sorbitol alone. None of the adjuvants improved ^{10}B basipetal translocation. It is concluded that in both species treatment of the lower leaf sides promotes foliar B absorption, probably due to stomatal uptake, and that humectants may enhance the foliar absorption process.

3.2 Key words

Boron, foliar absorption, Boron translocation, sorbitol, deliquescence relative humidity, adjuvants

3.3 Introduction

Boron application either as soil or foliar fertilizers is a widely used strategy to improve yield formation (pollination), fruit quality, fruit storage capability and tolerance towards abiotic stress (reduction of reactive oxygen species (ROS) development). Boron shortage is often a result of low B availability in the soil and foliar B fertilization is commonly used. However, according to the existing reports the efficacy of B foliar fertilization to improve such factors may range from positive results *(Schon and Blevins,* 1990*; Hanson,* 1991a,b*; Cakmak and Römheld,* 1997*; Wojcik et al.,* 1999*; Ross et al.*, 2006*; Khayyat* et al., 2007*; Wojcik* et al., 2008) to no effects (*Rerkasem* et al., 1988*; Zhang,* 2000; *Freeborn* et al., 2001; *Liebisch* et al., 2009, *Sotiropoulos*, 2010). The inconsistent results associated with foliar B application indicate the need to carry out more research efforts to better understand the mechanisms of B foliar absorption. The absorption of solutes by the foliage may take place via the cuticle, cuticular cracks and imperfections, through stomata, trichomes or specialised epidermal cells. The major mechanisms of foliar absorption described are the cuticular pathway and stomatal pathway (*Fernández and Eichert*, 2009). Regarding the mechanisms of cuticular absorption, apolar, lipophilic compounds have been proposed to follow a dissolution-diffusion process (*Riederer and Friedmann*, 2006). In contrast, the mechanisms of absorption of hydrophilic solutes through the cuticle are currently not fully understood (*Fernández and Eichert*, 2009), and it has been hypothesized that it may occur via “aqueous pores” (*Schönherr*, 2006). There is only indirect evidence for the existence of “aqueous pores” since it has not been possible so far to detect such structures in plant materials with the microscopical techniques currently available.

While the significance of the stomatal pathway on the uptake of foliar sprays has been a matter of controversy for many years, recent evidence shows that it can largely contribute to the absorption process (*Eichert* et al., 2008). Investigations carried out with fluorescent tracers provided evidence for stomatal absorption along the surface via a diffusion process (*Eichert* et al., 2008).

Foliar absorption requires dissolution of the applied substance. Hence, once the drop of the fertilizer hits the leaf surface, the time remaining in a liquid phase seems to be crucial. An extended liquid phase on the leaf surface is influenced by two factors relative humidity (RH) and deliquescence relative humidity (DRH) of the foliar applied solute, which describes the minimal water vapor pressure enabling sugars and salts to absorb water from the surrounding air and to go into solution (*Burkhardt and Eiden*, 1994). The relative humidity at which deliquescence first occurs is a characteristic of the individual substance (*Fernández and Eichert*, 2009). The DRH of boric acid is 98%, indicating that foliar-applied solutions containing pure boric acid will only stay in solution in almost water-saturated air. Mixtures of two substances lower the DRH below that of the individual substances (*Salameh* et al., 2005).

Since nutrient sprays are applied in the field, there is a limited chance to control the mechanisms associated with the rate of absorption and bioactivity of the nutrients applied to the foliage, but spraying plants when stomata are open and under conditions limiting the rate of solution drying. However, the importance of improving the properties of foliar nutrient formulations has long been recognized (*Fernández and Eichert*, 2009), and is currently a matter of scientific interest as key strategy to optimize the performance of foliar sprays (e.g., *Kraemer* et al., 2009; *Val and Fernández*, 2011). Adjuvants such as surfactants that will lower the surface tension of water and may facilitate the process of stomatal infiltration and humectants that will prolong the process of droplet drying, have been shown to improve the effectiveness of nutrient sprays when applied to horticultural crops (*Blanco* et al., 2010; *Val and Fernández*, 2011; *Eichert and Fernández*, 2012).

In this study different mixtures of BA with solutes having a lower DRH and hence providing a humectant effect (i.e., $CaCl_2$ and sorbitol with DRHs of 32% and 69%, respectively; *Salameh* et al., 2005), were investigated with respect to foliar B absorption and translocation in two crop species, namely, lychee (*Litchi chinensis* Sonn., hypostomatal) and soybean (*Glycine max* (L.) Merr., amphistomatal). Lychee represents a species with B immobility (*Konsaeng* et al., 2005), probably due to a lack of sugar alcohols for B binding. In soybean plants B accumulates in mature leaves resulting in a B downward gradient from mature to young leaves. However, under B deficient conditions *Brown* and *Shelp* (1997) found this downward gradient to disappear indicating a redistribution of B. In the present study, the variety *Glycine max* (L.) Meer. cv. ‘Oak Erin’ was utilized. In earlier experiments with this variety (data not shown) foliar applied ^{10}B could never be detected in other plant parts, neither in sufficient, nor in deficient pre-treated plants. Thereby, for this variety B-mobility seems to be very restricted regardless of the plant B status.

The following hypotheses were tested: a) Foliar B absorption can be enhanced by applying the treatments to the abaxial leaf surface, especially in hypostomatal leaves, b) the rate of B absorption can be enhanced by adding sorbitol and $CaCl_2$ as adjuvants, c) B-sorbitol (sorbitol borate ester) may facilitate the translocation of foliar-applied B in species with B phloem immobility.

3.4 Materials and methods

Plant material

Experiment 1

Soybean seeds (Glycine max (L.) Meer., cv. ‘Oak Erin’) were soaked for 1 hour (h) in 10 mM $CaSO_4$ and germinated on filter paper moistened with 2.5 mM $CaSO_4$ until radicles emerged. Seedlings were transferred into 3 L plastic pots (4 plants per pot) containing continuously aerated and weekly exchanged nutrient solutions (pH 5.5) of the following composition: 0.88 mM K_2SO_4, 0.1 mM KCl, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 10 µM H_3BO_3 ^{11}B labeled (99.8%), 0.5 µM $MnSO_4$, 0.2 µM $CuSO_4$, 0.02 µM $(NH_4)_6Mo_7O_{24}$, 1 µM $ZnSO_4$, 100 µM Fe(III)EDTA (*Will* et al., 2011). Plants were cultivated for 4 weeks in a climate chamber with a radiation of approximately 1000 µmol m-2s-1. The RH in the chamber was around 60% and day (14 h) and night (10 h) temperatures were kept at 24 and 20°C respectively.

Experiment 2

Lychee (*Litchi chinensis* Sonn.) trees were propagated by air layering of stems with equal size and diameter from the cultivar `Hong Huay’ cultivated in the research fields of the Chiang Mai University in Thailand. Rooted plants of about 50 cm height were pre-cultured in a continuously aerated nutrient solution, containing ^{11}B as the only B isotope which was supplied during the previous year to achieve a homogenous leaf and root growth. The nutrient solution composition and cultivation procedures were the same as described above for soybean.

Formulations

Foliar treatment solutions were prepared in de-ionized water, with a basic solution containing 50 mM ^{10}B labeled boric acid and 0.3% (v/v) surfactant (Plantacare, Cognis, Düsseldorf, Germany). Different combinations of $CaCl_2$ and sorbitol were added as formulation adjuvants. Sorbitol was applied at 2 different ^{10}B:sorbitol ratios, i.e., 1:1 (w/w) and 1:10 (w/w), hence favoring the formation of two different compounds (i.e., mono- and bi-sorbitol borate ester). The employed ratios derive from the findings of *Hu and Brown* (1997) who showed in-vitro that the formation of bi-sorbitol borate ester requires an excess of sorbitol. In Experiment 1, the basic solution was used as control to compare whether the adjuvants contributed to increase the absorption rate quantitatively. Treatments of the two experiments were as follows:

Experiment 1 (soybean):
Boron (B): Basic solution (BaSol)
CaCl2 (BCa): BaSol with 0.5 mM $CaCl_2$
Sorbitol (BS1): BaSol with 50 mM sorbitol
Sorbitol (BS2): BaSol with 500 mM sorbitol
Sorbitol (BCaS1): BaSol with 50 mM sorbitol and 0.5 mM $CaCl_2$
Sorbitol (BCaS2): BaSol with 500 mM sorbitol and 0.5 mM $CaCl_2$

Experiment 2 (lychee):
Control (Ctr.): de-ionized water with 0.3% (v/v) surfactant
Boron (B): Basic solution (BaSol)
Sorbitol (BS2): BaSol with 500 mM sorbitol

All chemicals were of analytical grade (Merck, Darmstadt, Germany).

Application and sampling design

Foliar treatments were separately applied on leaflets of the last fully-developed leaves. Treatments were supplied by the application of 16 droplets with a volume of 2.5 µL each (i.e., 40 µL in total) of the formulation on 2 adjacent leaflets per leaf. Soybean leaves consist of three leaflets, 2 are paired and 1 is the upper leaflet. Drops were applied as individual drops to the basal parts of a pair of leaflets (Fig. 3.1). Lychee leaves consist of 6 adjacent leaflets. The middle pair of the adjacent leaflets was used for drop application (Fig. 3.1). Leaves were harvested after 1 week and separated in 4 segments as indicated in Fig. 3.1. Distribution of the applied ^{10}B was separately determined for the different segments: Segment 1: fraction of ^{10}B remaining in the application zone, segment 2: fraction of ^{10}B in the leaf tip indicating acropetal translocation via transpiration stream, segment 3: fraction in non-treated leaflets of the treated leaf indicating short distance basipetal translocation via phloem and segment 4: growing tissue of a new flush indicating long distance basipetal translocation via phloem. All samples from segment 1 were carefully rubbed under de-ionized water between gloved thumb and forefinger for 20 seconds to remove trapped material (*Eichert and Goldbach*, 2008).

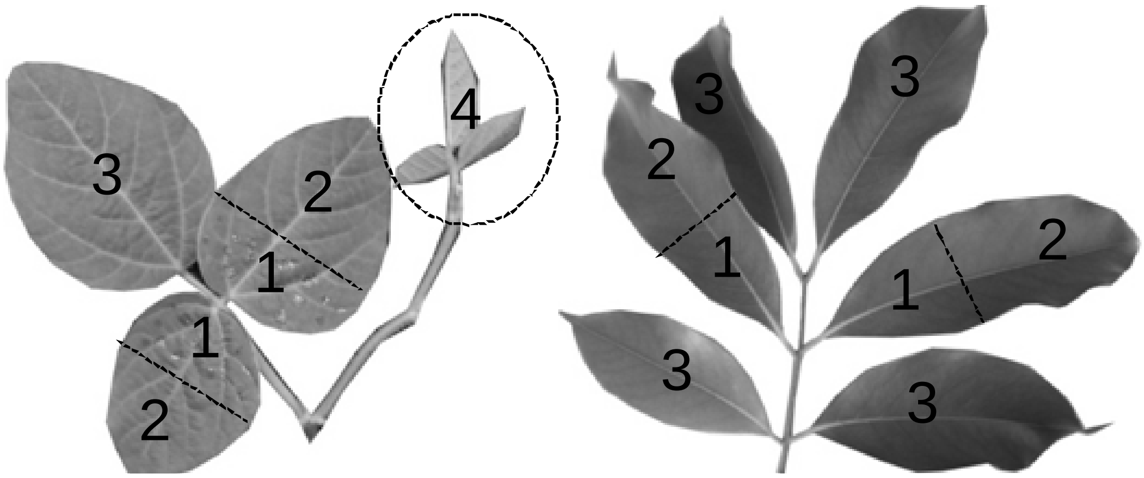

Fig. 3.1. Schematic presentation of leaf segments of soybean (left) and lychee (right) leaves for sampling and analysis after treatment by drop application (right, Soybean) of different formulations. 1: application zone (lower part of a leaflet), 2: leaf tip (acropetal translocated ^{10}B fraction), 3: non-treated leaflet of a treated leaf (short distance basipetal translocated ^{10}B fraction), 4: growing young flush above the treated leaf (long distance basipetal translocated ^{10}B fraction).

Analytical methods

Harvested leaves were dried at 65°C for 2 days. Ground dry leaf samples (0.05 – 0.1 g) were weighed in quartz crucibles. The samples were ashed in the oven with increasing temperatures (200 °C, 300 °C, 400 °C and 500 °C for 1, 1, 1 and 2 h respectively). Samples were then cooled down overnight. The next day, samples were rewetted with some drops of 3% H_2O_2-solution and after drying, ashed again in the oven for 3 h at 500°C. The ash was dissolved in 5 mL mixed acid solution (3.3% v/v HNO_3 + 10 ppb Beryllium (Be)) and centrifuged for 2 minutes at 4000 x g (Hettich Universal 30F). Boron isotopes (^{10}B, ^{11}B) were determined by inductively coupled plasma mass spectroscopy (ICP-MS, ELAN 6000, Perkin-Elmer, Überlingen, Germany), using Be as an internal standard. B concentrations in each segment were calculated.

Scanning electron microscopy (SEM) examination

Leaf surfaces were examined under a variable pressure scanning electron microscope (S-3400 N, Hitachi, Tokyo, Japan; acceleration potential 15 kV, working distance 10–11 mm). Leaves from the different treatments were dried at room temperature, making sure that the surface remained flat. For observation of either the adaxial or abaxial leaf side, approximately 1 cm2 sections were excised, and sputtered with gold. Different areas of the leaf sections were subsequently directly observed under the microscope. The abaxial and adaxial surfaces of five leaves were examined for each species.

Data collection and statistics

In Experiment 1, data were collected from 22 pots (*p*) with at least 2 plants per pot randomized as block design with four replicates (*b*). In replicate 1 to 3, 6 pots with 12 to 14 plants were used to test each of the 12 treatments (*t*) once. In replicate 4, only 4 pots with in total 9 plants existed. The remaining 3 treatments were replicated on plants grown in pots of other replicates. Therefore each treatment is replicated four times, but not always once per pot. This resulted in an unbalanced dataset.

Data were logarithmically transformed prior to the analysis and a multivariate analysis treating different segments (*s*) as different traits was used.

The model to analyse these data is given by equation 1:

$$y_{ijkl} = \mu + (bs)_{ij} + (ps)_{ijk} + t_l + s_j + (st)_{kj} + e_{ijkl} \quad , \tag{1}$$

i, *j*, *k* and *l* were subscripts for replicate, segment, pot, and treatment. Interactions were denoted by parentheses including the main effects. Effects for pot and error were assumed as random, all other effects were assumed as fixed. For both random effects an unstructured 4 × 4 matrix was assumed. A model selection approach had shown no heterogeneity within the pot effect, thus only a variance component for pot was assumed. For the error structure positive correlations between segments 1, 2, and 4 and a heterogeneous variance were found. An alternative analysis split the treatment effect in three factors: the addition of calcium (*c*), the concentration of sorbitol (*a*) and the leaf surface side of application (*l*). The model is then given by equation 2:

$$\begin{aligned} y_{ijkl} &= \mu + (bs)_{ij} + (bps)_{ijk} + c_m + a_n + l_o + s_k + (ca)_{mn} + (cl)_{mo} + (al)_{no} + (cal)_{mno} \\ &+ (sc)_{km} + (sa)_{kn} + (sl)_{ko} + (sca)_{kmn} + (scl)_{kmo} + (sal)_{kno} + (scal)_{kmno} + e_{ijkmno} \quad , \end{aligned} \tag{2}$$

where the subscripts m, n, and o denoted for the stage of calcium addition, concentration of sorbitol, and the side of application. All other effects and interaction effects are denoted as in equation 1.

In Experiment 2, data were collected from 24 plants randomized to 6 pots (*p*) with four plants per pot using a randomised complete block design (RCBD) with six replicates. From each plant the ^{10}B concentration within the leaf segments (*s*) was measured (Fig. 3.5). Within each block plants were treated with ^{10}B on the abaxial or adaxial leaf surface (*l*) with and without the addition of sorbitol (*a*). The analysis was performed using a multivariate analysis of a two factorial RCBD treating observations of different segments as different traits. Thus the model is given by

$$y_{ijkl} = \mu + p_i + (ps)_{ij} + l_k + a_l + s_j + (la)_{kl} + (ls)_{kj} + (as)_{lj} + (las)_{klj} + e_{ijkl} \tag{3}$$

where *yijkl* is the mass of boron in segment *j* of the plant from pot *i* with application on side *k* and the addition *l*. Parentheses are used for interactions. Data were logarithmically transformed prior to the analysis to reach homogeneous error variances. The presented estimates were back transformed for presentation only. Additionally, estimated standard errors were back transformed using the delta method. All effects except the error were treated as fixed effects, so inter-block information was ignored. It was assumed that the error effects of different segments on one plant were correlated, thus an unstructured 3 × 3 matrix for the error effects of one plant was assumed. An Akaike Information Criterion (AIC) (*Akaike*, 1974) based model selection approach was used to find the best model. While the variances in different segments varied, no covariance was found, thus in the final model a heterogeneous error variance was assumed.

3.5. Results

Experiment 1

In all treatments, growth and development of the plants were very homogenous throughout the experiment. One day after foliar application, leaves showed necrotic spots beneath the applied droplets. The degree of damage depended on the composition of the formulation and the leaf side of application (Fig. 3.2). Leaves treated on the adaxial leaf surface showed only slight damage in the area where drops of treatments were applied (i.e., relating to the application of B, BCa, BS1 and BS1Ca). Severe damage appeared in the treatments BS2 and BS2Ca. Application of the different formulations to the abaxial leaf surface consistently resulted in necrotic spots, the highest degree of damage being associated with the S2 treatment.

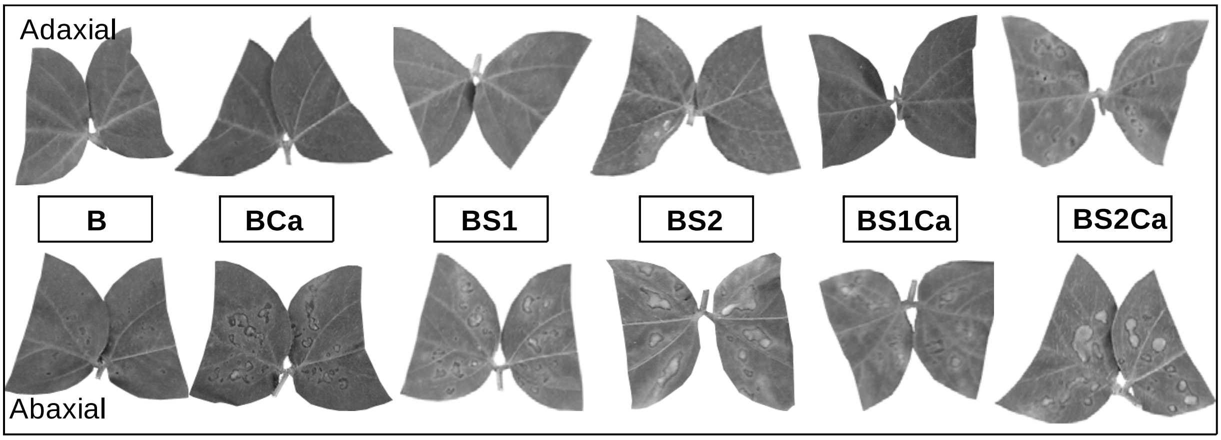

Fig. 3.2. Application zone (segment 1) of treated soybean leaves after foliar drop application of $^{10}B\ (OH)_3$ in combination with different adjuvants. B: 50 mM $^{10}B(OH)_3$ with 0.5% surfactant as Basic solution (BaSol), BCa: BaSol with 0.5 mM $CaCl_2$, BS1: BaSol with 50 mM sorbitol, BS2: BaSol with 500 mM sorbitol, BCaS1: BaSol with 50 mM sorbitol and 0.5 mM $CaCl_2$, BCaS2: BaSol with 500 mM sorbitol and 0.5 mM $CaCl_2$.

In segment 1, the results differed greatly depending on the leaf side of application. Treatment of the adaxial leaf surface resulted in foliar ^{10}B concentrations of 77 µg g^{-1} ^{10}B dry weight (DW). The ^{10}B concentrations increased significantly up to 312 µg g^{-1} ^{10}B DW when applied to the abaxial leaf surface (Fig. 3.3b). The supplementation of sorbitol (S1 and S2) significantly enhanced ^{10}B concentrations in segment 1 in comparison to pure BA application. Treatment S2 showed the highest absorption rates. Concentrations in the treated leaves were 100, 154 and 245 µg g^{-1} ^{10}B DW after applying pure BA, S1 or S2, respectively (Fig. 3.3a).

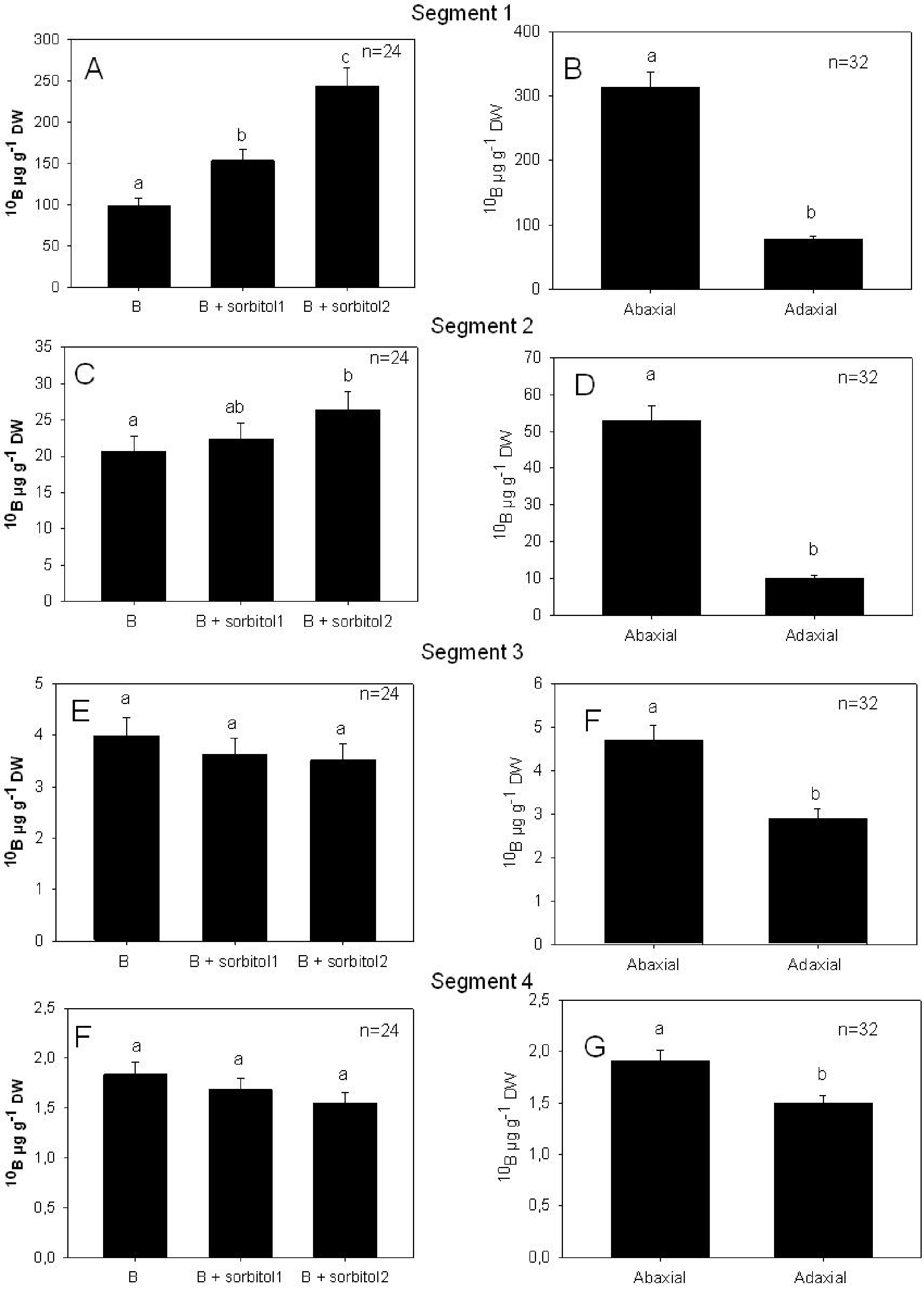

Fig. 3.3. Effect of polyol supplementation (A,C,E,F) and application to the abaxial or adaxial leaf surface (B,D,F,G) on foliar absorption and within-plant mobility of ^{10}B in soybean. A, B: segment 1, application zone, C, D: segment 2, leaf tip, E, F: segment 3, not treated leaflet, G,

H: new leaf. Boron was applied either as pure boric acid (50 mM B) or in combination with 50 mM sorbitol (“sorbito1”) or 500 mM sorbitol (“sorbitol 2”). Error bars represent the standard errors of the means.

The addition of $CaCl_2$ in the BA solution and S1 treatments increased ^{10}B absorption in comparison to the absence of $CaCl_2$ (Fig. 3.4a,b).

When $CaCl_2$ was added to S2, lower ^{10}B absorption rates were recorded as compared to the performance of S2 alone (Fig. 3.4c).

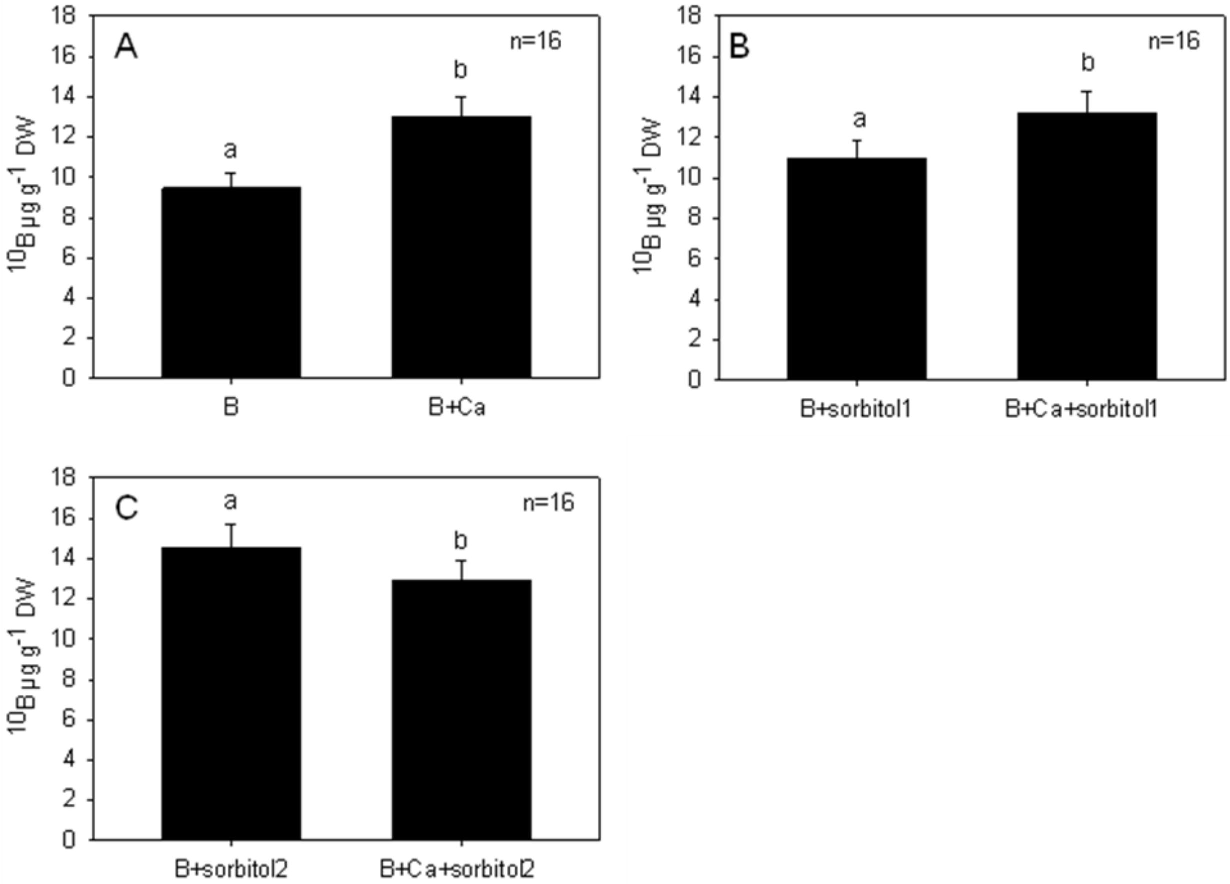

Fig. 3.4. Effect of $CaCl_2$ supplementation 0.5 mM on foliar absorption of ^{10}B by soybean leaves. A: 50 mM ^{10}B without sorbitol, B: 50 mM ^{10}B and 50 mM sorbitol C: 50 mM ^{10}B and 500 mM sorbitol. Dataset combined from application to the different leaf sides. Error bars represent the standard errors of the means.

On average, the ^{10}B concentrations in segment 2 (leaf tip) were lower than in segment 1 by a factor of six (Fig. 3.3d). As in segment 1, application to the abaxial leaf surface led to significantly higher ^{10}B concentrations as compared to the application to the adaxial leaf surface. In contrast to segment 1, differences in ^{10}B concentrations between application of pure BA and application with adjuvants were only significant in treatment S2 (Fig. 3.3c).
In segment 3, foliar ^{10}B concentrations were lower than in segment 2 by a factor of 10 (Fig. 3.3f). After foliar application to the abaxial leaf surface, ^{10}B concentrations in segment 3 were significantly higher than after application to the adaxial leaf surface. No significant differences in ^{10}B concentrations could be observed between application of pure boric acid and application with adjuvants (Fig. 3.3e).
In segment 4, small differences in leaf ^{10}B concentrations after application to the different leaf sides were measured (Fig. 3.3g), and no differences between the different treatments were found (Fig. 3.3f).
The ^{10}B concentrations measured in other plant parts were below the detection limit of 1µg ^{10}B g^{-1} DW (data not shown).

Experiment 2

No damage symptoms were observed on lychee leaves after foliar application of the different formulations.

In segment 1 the results differed greatly between the leaf sides of application. Treatment of the abaxial leaf surface resulted in significantly higher ^{10}B concentrations as compared to the application to the adaxial leaf surface by a factor of 7 (Fig. 3.5a). No significant differences in ^{10}B concentrations could be observed after the application of pure BA in comparison to its application with adjuvants.
In segment 2 and 3 the ^{10}B concentrations were below the detection limit of 1µg g^{-1} DW for any of the treatments applied to the leaves (Fig. 3.5b,c).

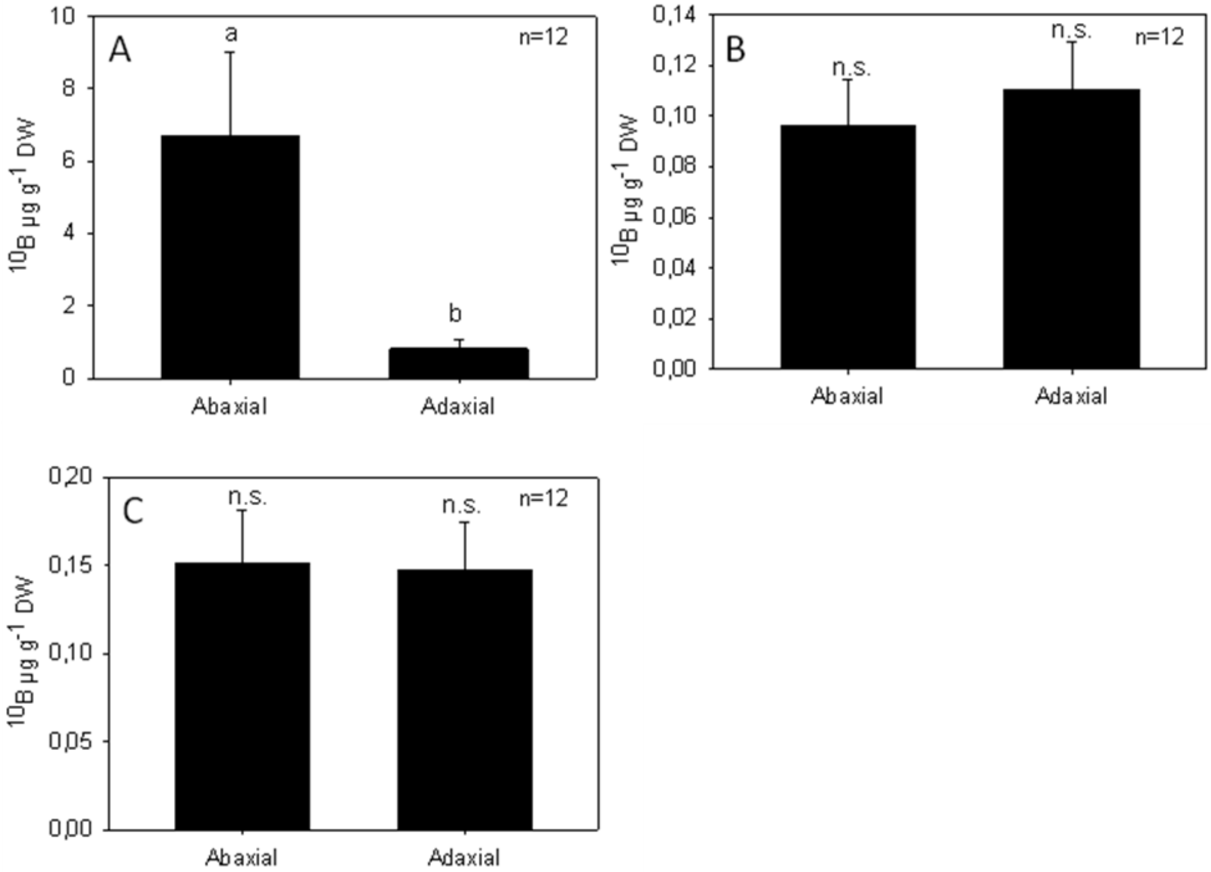

Fig. 3.5. Average ^{10}B concentrations in different segments of lychee leaves after drop application of BaSol either to the abaxial or adaxial leaf surface. A: segment 1, B: segment 2 and C: segment 3 (Fig.3.1). Error bars represent the standard errors of the means.

3.6. Discussion

In this study, experiments were carried out to assess the rate of absorption and mobility of foliar applied B. Both species were selected because B deficiency in these two crops has economic relevance in some production areas of the world. A hypostomatal and an amphistomatal species were used to assess the permeability to B solutions of adaxial versus abaxial leaf sides.

In both species the absorption of foliar-applied B was strongly leaf side dependent. Boron absorption through the abaxial side was more than four-fold (soybean) or seven-fold (lychee) higher than through the adaxial side. A higher permeability of abaxial versus adaxial leaf sides has been reported in several studies (e.g., as reviewed by *Fernández and Ebert* (2005) and *Fernández and Eichert* (2009) and references therein). This has been attributed to cuticular permeability differences between both leaf sides, and to the fact that stomata may not always occur on the adaxial side (e.g., as in lychee) or could be present at lower densities in contrast to the abaxial side (e.g., as in soybean) (Fig. 3.6).

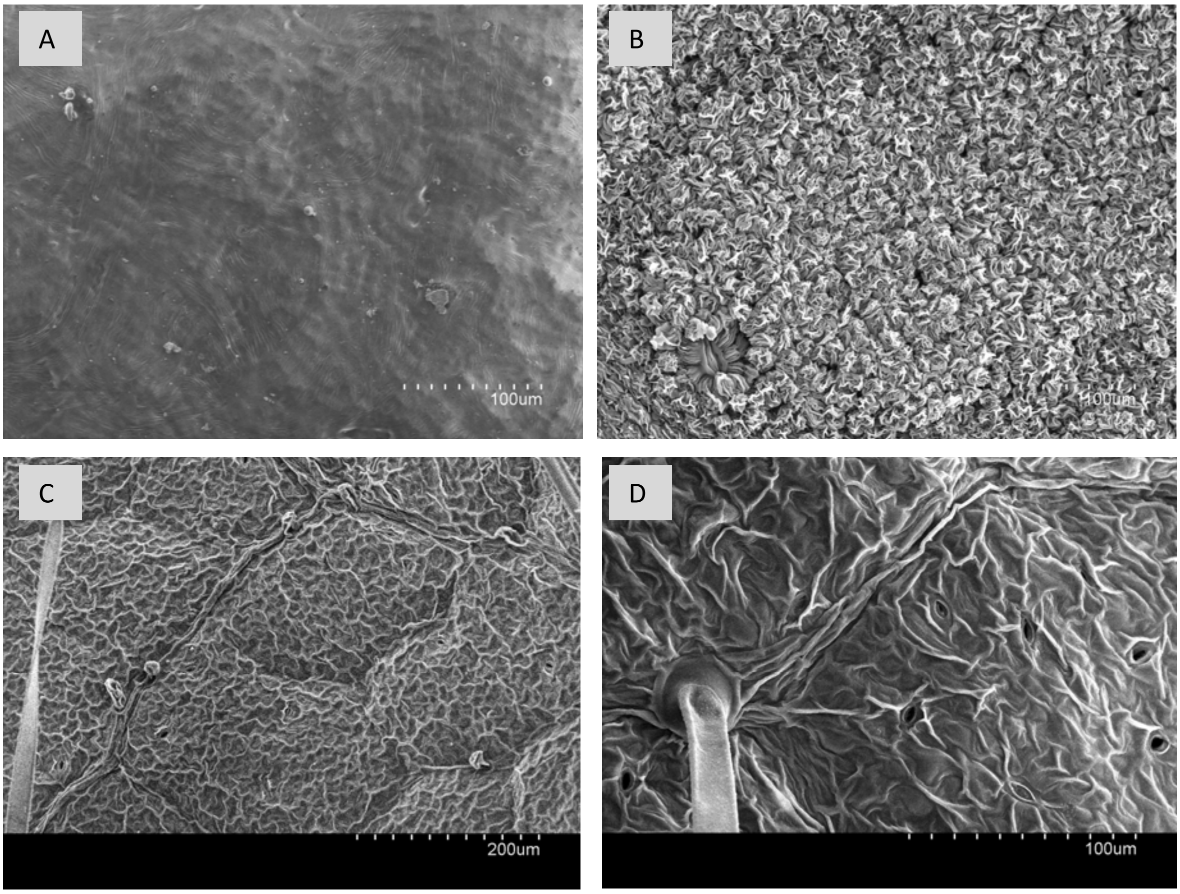

Fig. 3.6. SEM micrographs of: (A) adaxial lychee leaf surface (x300), (B) abaxial lychee leaf surface (x500), (C) adaxial soybean leaf surface (x200) and (D) abaxial soybean leaf surface (x500).

Permeability and transport characteristics of the cuticular are determined by wax thickness and to a greater extent wax microstructure. The physiochemical characteristics of the waxes are strongly influenced by radiation intensity, water status of the plant, temperature, nutrient status of the plants and by mechanical damage, such as e.g. wind (*Shepherd and Griffiths*, 2006; *Eichert* et al., 2010). Under controlled conditions in the climate chamber radiation mainly hits the adaxial leaf side keeping the abaxial leaf side shaded, a phenomenon that is also likely to occur under field conditions but to a lower extent. The prevailing growing conditions may have an effect on different physicochemical characteristics of leaf epicuticular waxes as shown e.g., by *Baker* (1974) and *Koch* et al. (2006), but their direct effect on the permeability of foliar-applied nutrient solutions is currently unclear and requires further research efforts.

Nowadays, it is still not possible to quantify the absorption of solutions via stomata as compared to the cuticle, but there is concluding evidence that the contribution of stomata to overall foliar absorption can be significant (*Eichert and Goldbach*, 2008; *Eichert* et al., 2008). Furthermore, on leaf sides having stomata transpiration water may re-condensate on the leaf surface (*Burkhardt* et al., 1999) and keeps foliar-applied solutes partly dissolved and mobile even though the surrounding bulk atmosphere may be dry.

The addition of adjuvants significantly affected foliar B absorption in soybean. In most cases sorbitol enhanced foliar absorption and this effect was more remarkable with increasing sorbitol concentrations in the treatment solution. The addition of $CaCl_2$, that will provide a humectant effect to the formulation, also improved the rate of B absorption. This can be explained by the DRH decrease of the mixture of solutes as compared to pure BA, which prolonged the length of time during which the applied solutions remained liquid onto the leaf surface. The higher concentration of sorbitol (S2) probably had a stronger humectant effect (i.e., it decreased more the DRH of the mixture) than the lower concentration (S1). The higher humectancy associated with increasing sorbitol concentrations resulted into significantly higher B absorption rates, which is in agreement with other studies showing higher nutrient uptake rates associated with the addition of humectants (e.g. *Fernández* et al., 2009). The addition of $CaCl_2$ to high sorbitol concentrations decreased B absorption as compared to high sorbitol concentrations alone, which may be the result of the interaction between negatively charged sorbitol borate ester and Ca^{2+} (*Wimmer and Goldbach*, 1999). A reduction of cuticular penetration rates of commercial fungizide formulations in relation to $CaCl_2$ was also reported by *Schlegel* et al. (2005).

In lychee, no effect of sorbitol in improving the rates of B absorption could be identified, which might be associated with the lower penetration rates and therefore, the low detection limits to measure any substantial B increases as compared to the more permeable soybean leaves.

In soybean, acropetal translocation of B increased with increasing B absorption and no additional sign of sorbitol effect was gained. Foliar ^{10}B application slightly increased ^{10}B concentrations in neighboring leaflets and newly growing leaves, indicating that a small part of B was transported in the phloem. Sorbitol supplementation had no effect on basipetal translocation, which is in line with earlier reports, where sorbitol supplementation increased absorption but did not have an effect on basipetal translocation (*Will* et al., 2011). In lychee, there was no translocation of ^{10}B after foliar application in neither of the treatments, suggesting that ^{10}B in the application zone was rapidly fixed in the apoplast.

3.7. Conclusions

In both crop species, treatment of the abaxial leaf surface led to significantly higher ^{10}B absorption rates as compared to ^{10}B absorption by adaxial leaf sides. Whereas the underlying physiological processes (differences in cuticular composition or effect of stomata) remain unclear, these results are of practical relevance for lychee growers. According to our field observations in Thailand, farmers mainly apply B foliar fertilizers by methods favoring the moistening of the adaxial leaf surface. Applying foliar fertilizers by alternative techniques like “fogging” will help to target the abaxial leaf surface and to improve the performance of foliar sprays. Fogging heads and e.g., electrostatic spraying techniques produce very small droplets which may improve wetting of the entire plant. However, the lower droplet sizes may limit the amount of liquid that is deposited onto the plant surface and solution drying may occur more rapidly. In soybean production, the application of B foliar fertilizers to the abaxial leaf surface might not be essential as B was also absorbed through the adaxial leaf side. Nevertheless, more research is required to improve the efficiency of foliar sprays by the development of effective formulations and spraying methods that also target the abaxial leaf side.

The supplementation of BA with sorbitol did not affect B basipetal translocation. However, both sorbitol and $CaCl_2$ improved foliar absorption, most likely by lowering the DRH. The addition of humectants is especially relevant for foliar applied fertilizers with a high DRH, such as BA. Future research needs to be implemented to determine suitable adjuvant-active ingredient combinations and concentrations that may improve the rate of absorption, translocation and bioactivity of foliar nutrient sprays.

CHAPTER 4

BOROMN FOLIAR ABSORPTION AND DISTRIBUTION IN LYCHEE (*LITCHI CHINENSIS* SONN.) LEAVES AS AFFECTED BY THE LEAF SIDE, LEAF AGE AND TIME OF APPLICATION[iii]

[iii] Submission: Scientia Horticulturae

4 Boron foliar absorption and distribution in lychee leaves (*Litchi chinensis* Sonn.) as affected by the leaf side, leaf age and time of application

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4.1. Abstract

Boron (B) deficiency in lychee (*Litchi chinensis* Sonn.) is a problem of agronomic importance in some production areas of the world. Foliar fertilization is a widespread strategy to cure B deficiency but the results are very variable. Experiments to assess the rate of absorption and translocation of foliar-applied, isotopically-labelled boric acid (BA) were carried out on lychee plants. The effect of applying BA to mature or immature leaves and diurnal or nocturnal application were investigated in terms of B absorption and subsequent translocation within the plant, one week after treatment. The contribution of stomata to the absorption process was assessed by applying the solutions either to the adaxial or to the abaxial leaf side, of which only the abaxial side contains relevant amount of stomata. The mobility of ^{10}B was evaluated by analysing acropetal (xylem transport) and basipetal (phloem transport) translocation within different leaf segments.

The adaxial leaf surface (lacking stomata) of lychee leaves was nearly impermeable to BA solutions, while the rate of absorption of stomatous abaxial surface was 4 times higher as compared to the adaxial side. The leaf side factor did not affect ^{10}B mobility in the plant.

The factors leaf age and time of application did not affect absorption. Acropetal and basipetal translocation of foliar applied ^{10}B increased significantly after foliar application of BA to mature in comparison to immature leaves. Nocturnal application of BA resulted in significantly enhanced basipetal translocation of ^{10}B, but did not effect acropetal

translocation. In conclusion, nocturnal application of B foliar fertilizer mainly to the abaxial leaf surface (e.g. by fogging instead of spraying) might be recommended.

4.2. Key words

Boron; foliar absorption; leaf side; leaf age; diurnal; nocturnal; re-translocation

4.3. Introduction

Boron (B) deficiency causes many anatomical, physiological and biochemical changes in plants. Several reviews provided an overview of the role of B in plant metabolism (*Shelp* et al., 1995, *Goldbach and Wimmer*, 2007, see references therein).

Boron application either as soil or foliar fertilizers is a widely used strategy in plant production to improve yield formation (pollination), fruit quality, storage capability and stress tolerance. Especially in plant species, where B re-mobilization is very limited, continous B availability is essential throughout the year. The occurrence of B deficiency depends on multiple factors, such as weather conditions (drought, high precipitation, etc.), soil conditions (low pH: B leaching, calcareous soils: B fixation), low soil temperatures or low transpiration where root growth and activity are inhibited and the tolerance of the cultivated crop species towards B deficiency (*Shorroks*, 1997). Under conditions, such as drought or high precipitation, the effectiveness of soil fertilization can be significantly reduced. Foliar fertilization could be a target-oriented alternative with high economic benefit (*Sakar* et al., 2007) to correct deficiencies. However, the efficacy of B fertilization in scientific experiments may range from positive (*Schon and Blevins*, 1990; *Hanson*, 1991a,b; *Cakmak and Römheld*, 1997; *Wojcik* et al., 1999; *Ross* et al., 2006; *Khayyat* et al., 2007; *Wojcik* et al., 2008) to no effects at all (*Rerkasem* et al., 1988; *Zhang*, 2000; *Freeborn* et al., 2001). The mechanisms of foliar absorption of solutes by the foliage were controversially discussed in the last decades, but are still not fully understood. Absorption may take place via the cuticle, cuticular cracks and imperfections, through stomata, trichomes or specialised epidermal cells. Two mechanisms of foliar absorption have been discussed and characterized to a certain extent, namely, the cuticular pathway and the stomatal pathway (*Fernández and Eichert*, 2009). Regarding the mechanisms of cuticular absorption, apolar, lipophilic compounds have been proposed to follow a dissolution-diffusion process (*Riederer and Friedmann*, 2006). In contrast, the mechanisms of absorption of hydrophilic solutes through the cuticle are still not fully understood (*Fernández and Eichert*, 2009) and it has been hypothesized, that it may occur via "aqueous pores" (*Schönherr*, 2006).

The stomatal pathway can largely contribute to the absorption process (*Eichert* et al., 2008). Investigations carried out with fluorescent tracers provided evidence for stomatal absorption along the surface via a diffusion process (*Eichert* et al., 2008). *Eichert and Burkhardt* (2001) found that less than 10% of the total stomata contributed to the total absorption, but the number of penetrated stomata could be increased by a re-wetting/drying cycle.

Besides the different pathways, the qualitative and quantitative nature of the epicuticular waxes is important for foliar absorption (*Baker and Hunt*, 1981). The distribution of nutrients within the plant following foliar absorption contributes to the efficiency of the overall fertilization strategy.

Several studies reported nutrient disorders mainly due to B and Zn deficiency in field crops and fruit trees in South East Asia (*Sharrocks* 1997, *Dong* 1997, *Bahadur* 1998) and Northern Thailand (*Sruamsiri* et al., 2005; *Roygrong*, 2009). *Roygrong* (2009) concluded that B and Zn deficiencies in lychee orchards in Northern Thailand was casually linked with low flowering, fruit set and fruit quality. First field trials in Northern Thailand including B foliar fertilization mainly on the adaxial leaf surface resulted in non-significant increase of B concentrations in the leaves (*Roygrong*, 2009).

In this study, different factors affecting B foliar absorption and mobility were investigated in lychee (*Litchi chinensis* Sonn.). This fruit crop represents a hypostomatal species with B immobility (*Konsaeng* et al., 2005).

The following hypothesis were tested (i) foliar B absorption is influenced by leaf age, (ii) the application of B to the abaxial leaf surface may increase B foliar absorption, owing to the presence of stomata, and (iii) the application of diurnal or nocturnal foliar B treatments may affect the rate of foliar absorption and mobility in the plant.

4.4. Materials and methods

Pre-treatment

Lychee plants derived from vegetative reproduction of the cultivar 'Hong Huay' by air-layers were pre-cultured in continuously-aerated nutrient solution containing ^{11}B as the only B isotope supplied for one year to achieve a homogenous leaf and root growth and nearly 100 atom % ^{11}B in the newly developed plant tissue (data not shown). The nutrient solution was of following composition: 0.88 mM K_2SO_4, 0.1 mM KCl, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.25 mM KH_2PO_4, 10 µM H_3BO_3 ^{11}B labeled (99.8%), 0.5 µM $MnSO_4$, 0.2 µM $CuSO_4$, 0.02 µM $(NH_4)_6Mo_7O_{24}$, 1 µM $ZnSO_4$, 100 µM Fe(III)EDTA . It was prepared with de-ionized water (adjusted to 6.5 pH) and was changed on a weekly basis. Plants were cultivated in a climate chamber, with a relative humidity (RH) of 60% and day (14 h) and night (10 h) temperatures of 24 and 20°C, respectively.

Application

Foliar treatment solution (pH 6.3) contained 50 mM ^{10}B as isotopically labelled boric acid in de-ionized water and 0.3% (v/v) surfactant (Plantacare, Cognis, Düsseldorf). Lychee leaves mainly consist of six adjacent leaflets. Treatments were supplied to the middle pair as indicated in Fig. 1 either to the adaxial or abaxial leaf surface (factor 1) of leaves of a young developing flush (immature) or on leaves of the second fully developed flush (mature) (factor 2). Diurnal or nocturnal application (factor 3) was done via drop application of 16 * 2.25 µL drops (8 drops on each leaflet) of the formulation. The light source at night application in the climate chamber was a standard lamp (25 Watt) covered with dark green foliage and turned on only during the treatment. For each of the resulting eight treatments (2x2x2), a corresponding control treatment (formulation without boron application) was conducted (factor 4). The complete design, thus, resulted in a total number of 16 (8x2) treatments including the controls.

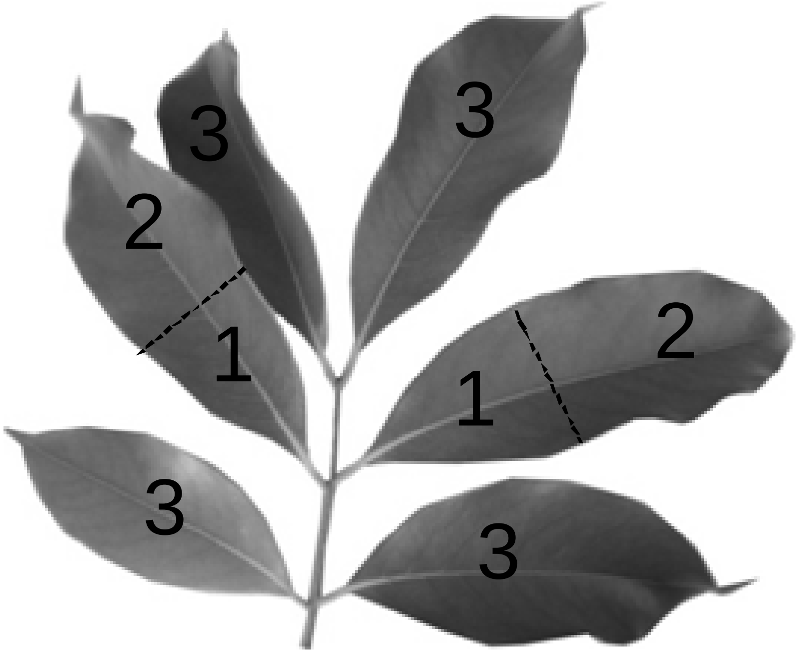


Fig. 4.1. Schematic presentation of lychee leaf segments used for the analysis of B absorption and translocation. 1: application zone (lower part of a leaflet), 2: leaf tip, 3: non-treated leaflets of a treated leaf.

Sampling design and analytical methods

Leaves were harvested after 1 week and separated into 3 segments as indicated in Fig.4.1 Distribution of the applied ^{10}B was separately determined for the different segments: Segment 1: fraction remaining in the application zone, segment 2: fraction of ^{10}B in the leaf tip indicating acropetal translocation via transpiration stream and segment 3: fraction in non-treated leaflets of the treated leaf indicating short distance basipetal translocation via phloem. All samples from segment 1 were carefully rubbed under de-ionized water between glove wearing thumb and forefinger for 20 seconds to remove trapped material (*Eichert and Goldbach*, 2008).

Harvested leaves were dried in the oven at 65 °C for 2 days. Ground dry leaf samples (0.05 – 0.1 g) were weighted in quartz crucibles. The samples were ashed in the oven with increasing temperature at 200 °C, 300 °C, 400 °C and 500 °C for 1, 1, 1 and 2 h, respectively (*Pfeffer* et al., 2001). Afterwards, samples were cooled down overnight. Next day, samples were rewetted with some drops of 3% H_2O_2-solution and after drying, ashed again in the oven for 3 hours at 500°C. The ash was dissolved in 5 ml mixed acid solution (1:30 HNO_3 + 10 ppb Beryllium as internal standard) and centrifuged for 2 minutes at 4000 x g. Boron isotopes

(^{10}B, ^{11}B) were measured using an inductively coupled plasma mass spectroscopy (ICP-MS). Boron concentrations and contents in each segment were calculated.

Statistics

For the eight control treatments two replicates and for the eight treatments with boron application three replicates were conducted resulting in a total of 40 applications and leaf samplings conducted at 40 tree limbs (t) of 20 plants (p) using a randomized complete design (RCD). In the statistical model, the 16 treatments were divided in boron application (α), leaf side of B application (β), time of application (γ) and age of treated leaves (δ) corresponding to the four factors described above. From each plant, the ^{10}B and the ^{11}B concentration within the different leaf segments (s) were measured (Fig. 1). The analysis was performed using a multivariate analysis of a four factorial RCD treating observations of different segments as different traits. To simplify the formulation of the model, we only give the model for the univariate case. The model was then expanded to a multivariate model using the method described in *Piepho and Möhring* (2011). The univariate model is given by

$$y_{ijlmno} = \mu + p_i + t_{ij} + \alpha_l + \beta_m + \gamma_n + \delta_o + (\alpha\beta)_{lm} + (\alpha\gamma)_{\mathrm{ln}} + (\alpha\delta)_{lo} + (\beta\gamma)_{mn} + (\beta\delta)_{mo}$$
$$+ (\gamma\delta)_{no} + (\alpha\beta\gamma)_{lmn} + (\alpha\beta\delta)_{lmo} + (\alpha\gamma\delta)_{lon} + (\beta\gamma\delta)_{mno} + (\alpha\beta\gamma\delta)_{lmno} + \varphi_{ij} + e_{ijlmno}$$

where y_{ijlmno} and φ_{ij} are the concentration of ^{11}B and ^{10}B, respectively, in a certain leaf segment of the j^{th} limb of tree i. The indices l, m, n and o are used for levels of the four treatment factors boron application, leaf side of application, time of application and age of treated leaves. Interactions were denoted by parentheses of the corresponding main effects. φ_{ij} was used as a covariate. While the four treatment effects were taken as fixed, the effects of plant, limb of tree and error were taken as random. Data were logarithmically transformed prior to the analysis to reach homogeneous error variances within segments. The presented estimates were back transformed for presentation only. Additionally, estimated standard errors were back transformed using the delta method. Finally, the unstructured variance-covariance structure for random effects was simplified using an Akaike Information Criterion (AIC) (*Akaike*, 1974) based model selection approach. The compound symmetry structure fits best implying the same variance for all segments and a homogeneous covariance between segments. F-tests were used to find significant effects. Multiple comparisons using a t-test were only used after significant F-tests were found.

4.5. Results

All significant effects are shown in Tab.4.1. the factors leaf age and leaf segment as well as their interactions showed significant effects on ^{10}B concentrations. Interactions between the leaf segment and leaf side and time of application were also significant. This indicates that in different leaf segments the factors leaf age, leaf side and time had different effects on ^{10}B concentrations. Therefore the results are examined for each segment.

Statistical analyses showed a significant effect of the pre-treatment with ^{11}B, when calculated as covariate. Leaves with higher tissue ^{11}B concentrations were found to have higher concentrations of ^{10}B (data not shown).

Tab.4.1. Results on statistical analysis assessing the effects on ^{10}B concentrations in the leaf tissue. Only significant effects are shown. Data was analyzed as described in the section Materials and Methods.

Effect	Parameter	P
Covariate	^{11}B	0.0048
Main effects	Leaf age	0.0072
Interactions	Segment x leaf side of application	0.0029
	Segment x leaf age	0.0146
	Segment x time of application	0.0424

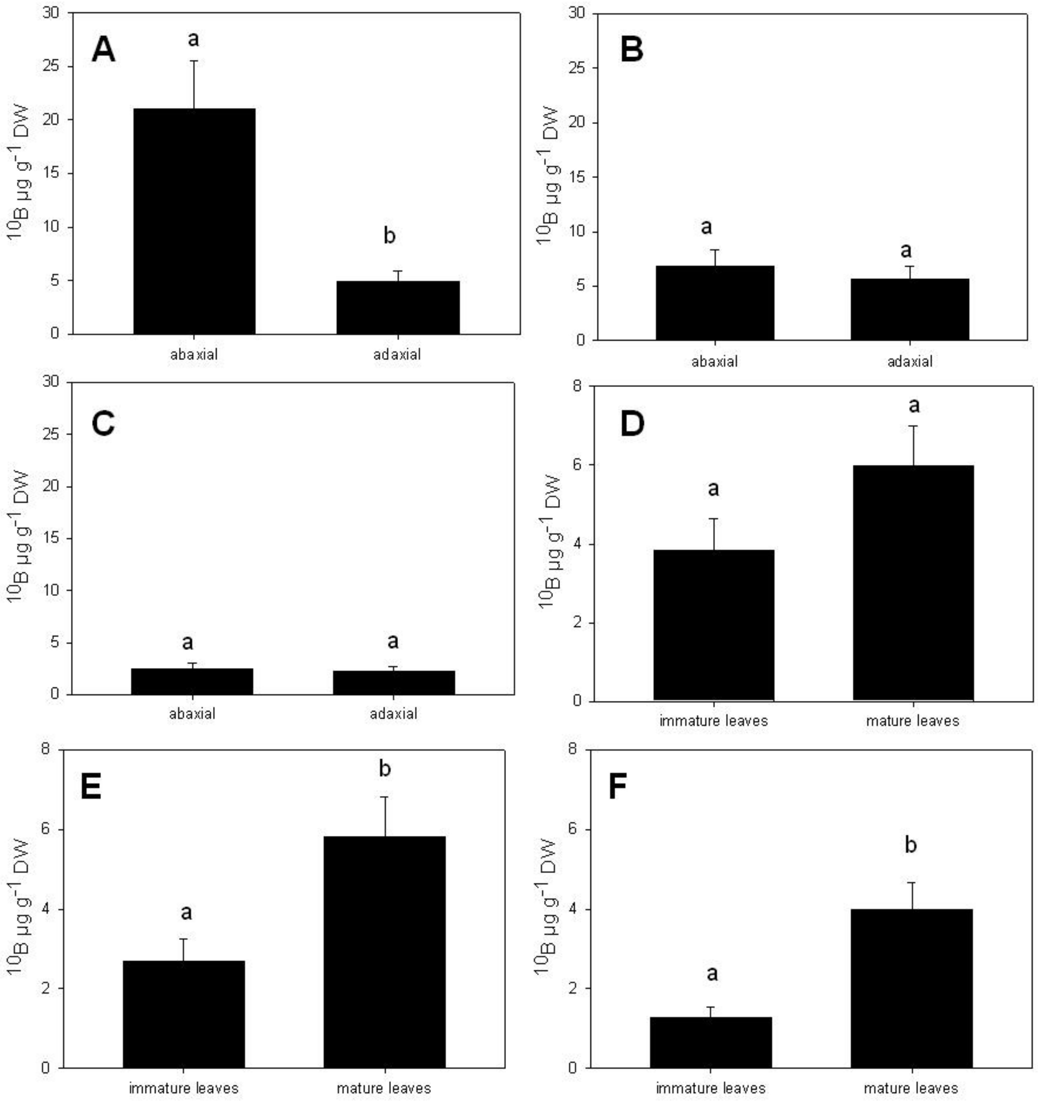
In segment 1 the average ^{10}B concentrations of about 20 µg*g^{-1} DW were significantly higher after application of ^{10}B to the abaxial leaf surface in comparison to the adaxial leaf surface with concentrations of about 5 µg*g^{-1} DW ^{10}B (Fig 4.2.A). Treatment of mature leaves tend to higher ^{10}B concentrations in comparison to young growing leaves, however, the difference here was not significant (Fig 4.2.D). The diurnal versus nocturnal application showed no differences in ^{10}B concentrations (Fig 4.2.G).

In segment 2, similar tissue ^{10}B concentrations were measured irrespective of the treated leaf side (Fig 4.2.B). ^{10}B concentrations after B application to mature leaves were significantly higher in comparison to immature leaves (Fig 4.2.E).

The foliar application of ^{10}B after diurnal versus nocturnal application also showed no differences in ^{10}B concentrations as in segment 1 (Fig 4.2.H).

In segment 3, significant differences in ^{10}B concentrations were observed in treatments of mature leaves as compared to immature leaves (Fig 4.2.F). Nocturnal BA application led to significantly higher ^{10}B concentrations in segment 3 in contrast to the diurnal BA treatments (Fig 4.2.I).

Overall, the nocturnal application to the abaxial leaf surface of mature leaves showed the same tendencies within the different segments. Although not always significant, in all segments the concentrations of ^{10}B were higher, when applied nocturnal to the abaxial leaf surface of mature leaves in comparison to diurnal application to the adaxial leaf surface of immature leaves, respectively.


A
B
C
D
E
F
^{10}B µg g^{-1} DW
0
5
10
15
20
25
30
0
2
4
6
8
a
b
abaxial
adaxial
immature leaves
mature leaves

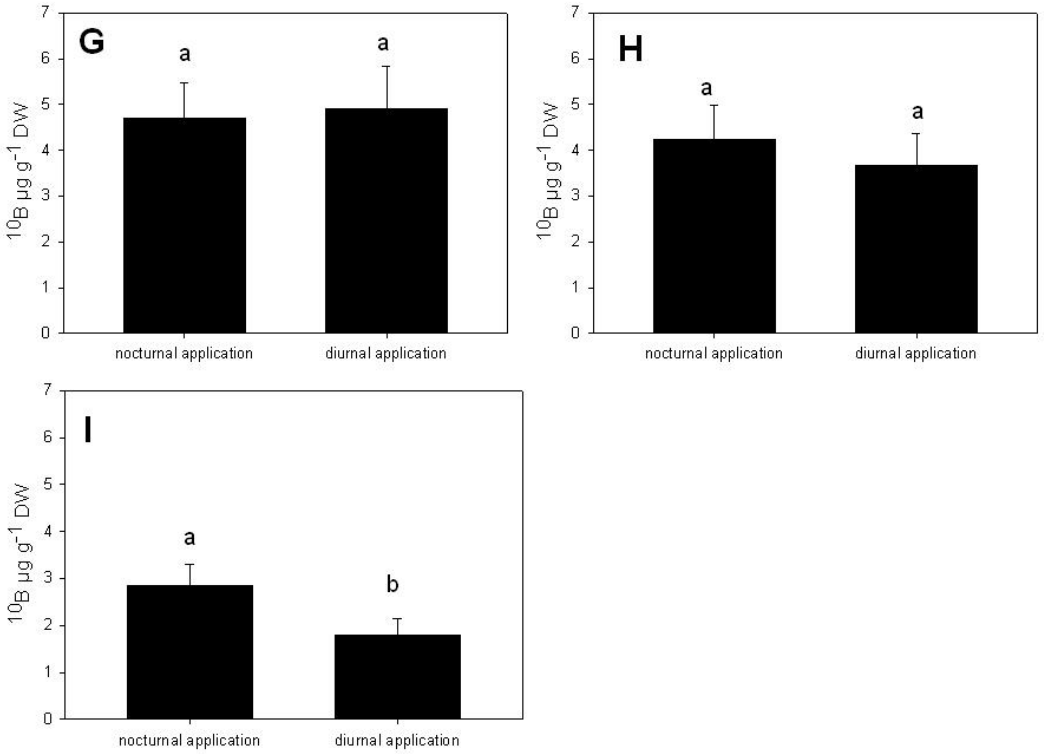


Fig. 4.2. Average ^{10}B concentrations in different segments of lychee leaves referring to Fig. 1 after foliar application of BA solution to the abaxial or adaxial leaf surface to mature and immature leaves and after diurnal or nocturnal application. Segment 1 (A,D,G): application zone, segment 2 (B,E,H): leaf tip and segment 3 (C,F,I): not treated leaflets. Error bars represent standard errors of the means. Different small letters indicate significant differences (t-test, $p<0.05$).

4.6 Discussion

Leaves with higher tissue ^{11}B concentrations were found to have a higher rate of absorption of foliar applied ^{10}B. This is consistent with the findings in previous experiments on soybean, where the B status of the plant influenced foliar absorption positively too (*Will* et al., 2011).

A higher permeability of abaxial vs. adaxial leaf sides has been reported in several studies for different plant species (see reviews by *Fernández and Ebert*, 2005 or *Fernández and Eichert*, 2009 and references therein). This was attributed to differences in cuticular permeabilities between both leaf sides or to the fact that in many species, as in lychee (Fig. 4.3), adaxial leaf sides are lacking stomata. It is still not possible to quantify the absorption of solutions via the stomata pathway as compared to the cuticle, but it was shown that the contribution of stomata to overall foliar absorption can be significant (*Eichert and Goldbach*, 2008; *Eichert* et al., 2008). On leaf sides containing stomata, transpiration water may re-condensate on the leaf surface (*Burkhardt* et al., 1999) and keeps foliar-applied solutes partly dissolved and mobile even though the surrounding bulk atmosphere is dry.

Very restricted absorption across the adaxial plant cuticle was also shown for pear, apple and grapevine leaves (*Schlegel* et al., 2005). In addition, lychee leaves have a thick layer of epicuticular waxes on both leaf sides. *Baker and Hunt* (1981) studied the penetration of foliar applied 1-naphthylacetic acid during leaf growth on different plant species. Absorption decreased during leaf growth in species, such as *Vitis vinifera* and *Eucalyptus globulus.* In contrast, foliar absorption of 1-naphthylacetic increased during leaf extension in *Malus hypehensis* leaves. In the case of lychee, however, we recorded increased rates of foliar B absorption and mobility in association with mature, fully-expanded leaves which appeared to be more permeable than young, developing leaves.

Recent evidence showed that after foliar application, the rate of B distribution via phloem was enhanced in relation to the interruption of the transpiration stream (*Eichert and Goldbach*, 2010; *Will* et al., 2011). In this study the translocation of foliar applied ^{10}B out of the treated leaflet was significantly higher after nocturnal versus diurnal application. During night time, stomatal closure may limit the transpiration flow and improve the rate of B distribution from the point of application.

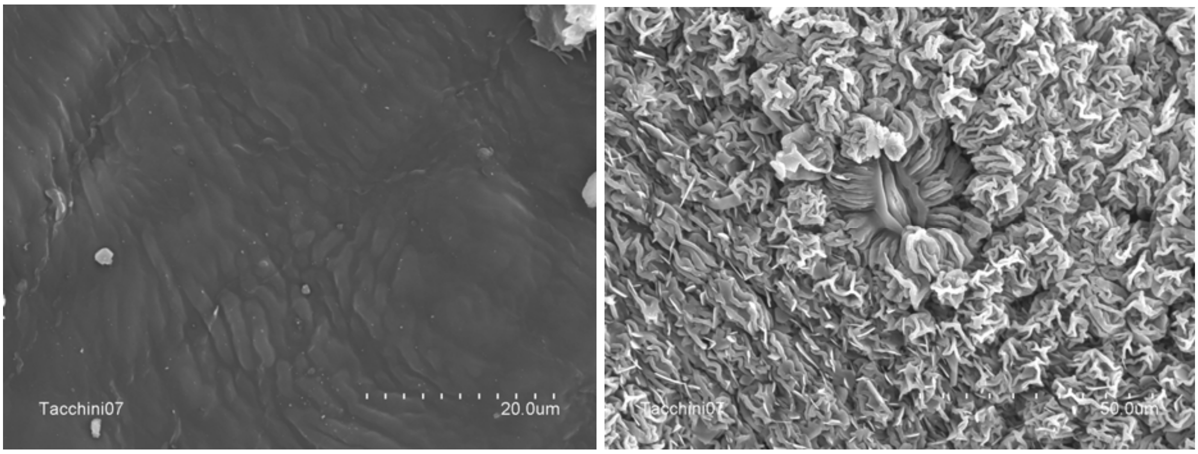


Fig 4.3. SEM micrographs of (left) adaxial lychee leaf surface and (right) abaxial lychee leaf surface.

4.7. Conclusions

The efficacy of foliar B fertilization can be limited in lychee, especially if only the adaxial leaf surface is targeted as it commonly occurs with the spraying techniques available in many lychee orchards in Northern Thailand. The practical implications of our results clearly show that B foliar sprays should be applied nocturnally to the abaxial leaf surface of lychee leaves to improve the efficacy of the treatments, e.g. by fogging instead of spraying. As reported for soybean (*Will* et al., 2011), it was observed that leaf B nutrient status had an impact on B absorption. If foliar B absorption is impaired by B deficiency, B must be applied as early as possible. Identifying B deficiency in lychee is however very limited by visual indicators alone. Based on our findings, future research is needed to further develop an adapted management practice for lychee orchards by optimizing spray time, leaf side of application and consideration of the developmental stage of the leaf flushes to increase the efficiency of foliar B sprays.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5. GENERAL DISCUSSION AND CONCLUSION

5.1. General discussion

In many production areas of the world B deficiency limits plant production. Boron shortage is often a result of low B availability in the soil and foliar B fertilization is commonly used. The lack of consistent results associated with foliar B application indicates the need to carry out more research efforts to better understand the mechanisms of B foliar absorption.

Nevertheless, with precise knowledge of the underlying mechanism of foliar absorption and subsequent translocation of the specific nutrient, foliar fertilization can become a practice with high efficiency in plant production.

In the last decades many efforts have been made to understand the physiological as well as mechanistic processes of foliar absorption.

The hypotheses addressed in this thesis concerning foliar B fertilization will be discussed and evaluated within this section.

I. *The rates of foliar B absorption and subsequent translocation depend on plant B status. This hypothesis is based on observations with other mineral nutrients such as phosphorous (P). It was observed that the rate of P absorption and subsequent translocation by the leaves of phosphorus-deficient plants was higher then in the control (Marschner, 1995).*

The plant B status had a very strong impact on foliar B absorption and subsequent translocation in soybean and foliar B absorption in lychee.

In contrast to the findings described in *Marschner* (1995), a significant reduction of foliar B absorption in soybean plants with no root B supply as compared to plants grown under 10 µM B, 30 µM B and 100 µM B in the nutrient solution were observed. Foliar B absorption in soybean was about thrice higher in all treatments in comparison to the treatment with no B root supply (strong deficiency).

In lychee, absorption was correlated with the B status of the plants. High B contents in the leaf tissue resulted in increased absorption rates.

This high decrease in foliar B absorption under B-deficiency was rather unexpected. Foliar absorption is driven by a concentration gradient across the leaf surface and is modulated by the permeability of the leaf surface. In theory, a higher B concentration gradient after foliar B application could be expected in B-deficient versus B-sufficient leaves.

The limited rate of B absorption in B-deficient leaves must be most likely caused by a reduced permeability of the leaf surface, due to induced alteration in the cuticular structure, or to the fact that stomata were shrunken and closed, as it was observed with SEM on soybean. Disfunctioning and closure of stomata was earlier reported to reduce absorption of foliar-applied solutes via the stomatal pathway (*Eichert and Burkhardt*, 2001; *Eichert and Goldbach*, 2008). Additionally, with closed stomata, less transpiration water was released which otherwise may have re-condensated on the leaf surface (*Burkhardt* et al., 1999) and kept foliar-applied solutes partly dissolved and mobile.

To gain further mechanistic insights into the effect of plant B status on subsequent translocation of foliar absorbed B, the shares of translocated B in relation to the amount absorbed were calculated. Highest relative translocation rates were observed in soybean plants pre-cultured in 0 µM B or 100 µM B. While high translocation rates in plants with high B contents can be explained by the saturation of possible B binding sites in the cell wall leaving more free B for translocation, the reason for the relatively high shares of translocated B in plants under B deficiency is less obvious.

It was evidenced that in soybean plants with no or 100 µM B root supply, leaves sustained higher water potentials than plants cultivated under 10 µM B or 30 µM B indicating that both under B deficiency and high B supply the average transpiration rates were probably lower, as it was reported by *Eichert* et al. (2010) for Fe deficient peach leaves. According to results obtained with *Ricinus communis* L., low transpiration rates may enhance phloem mobility of foliar-applied B (*Eichert and Goldbach*, 2010).

II. The application of B-sorbitol (sorbitol borate ester) can facilitate the translocation of foliar-applied B in species with B phloem immobility. Natural phloem mobility of B takes place in species with polyols, e.g. sorbitol, as a major carbohydrate, which form stable compounds with B. The foliar application of B together with polyols may thus induce B mobility in plants lacking polyols in the phloem.

The addition of polyols increased the absorption rates of foliar-applied B compared to application of BA alone. Generally, polyols could enhance B absorption by lowering the deliquescence humidity (DRH) of the deposited substances. The RH during the experiment was 60%, which is well below the DRHs of the components, and accordingly this humectant effect should not have affected absorption. However, it has to be taken into account that leaf surfaces are surrounded by a laminar layer in which RH is higher than ambient RH (*Burkhardt and Eiden*, 1994).

Both polyols (mannitol, sorbitol) added to the foliar formulations reduced the relative B mobility as compared to the application of BA alone. This may be due to the conversion of small uncharged BA molecules into relatively large, negatively charged polyol borate ester. While BA is moderately plasmalemma-permeable and may thus easily diffuse into the phloem, the large ionic compounds are probably rather excluded from passive trans-membrane transport reducing phloem mobility. This is in contrast to the situation in plants with natural polyol-assisted B mobility, where bonding takes place not until BA has entered the phloem.

III. Foliar B absorption can be enhanced by applying the formulation to the abaxial leaf surface especially in hypostomatal plants (lychee), by taking advantage of the stomatal uptake pathway.

Absorption of foliar-applied B was strongly affected by the leaf side of application in soybean and lychee. Boron absorption through the abaxial leaf side was more than three-fold (soybean) or seven-fold (lychee) higher than through the adaxial side.

The positive effect of foliar absorption via abaxial vs. adaxial leaf sides has been reported in several studies (see reviews by *Fernández and Ebert*, 2005; *Fernández and Eichert*, 2009; and references therein). This was attributed to differences in cuticular permeabilities between both leaf sides or to the fact that on adaxial leaf sides stomata are lacking (as in lychee) or less frequent than on the abaxial side (as in soybean).

Permeability and transport characteristics of the cuticle are determined by wax thickness and to a greater extent wax microstructure. The physiochemical characteristics of the waxes are strongly influenced by radiation intensity, water status of the plant, temperature, nutrient status of the plants and by mechanical damage (*Shepherd and Griffiths*, 2006; *Eichert* et al., 2010). Under controlled conditions in the climate chamber radiation mainly hits the adaxial leaf side keeping the abaxial leaf side shaded, a phenomenon that is also likely to occur under field conditions. The prevailing growing conditions may have an effect on different physicochemical characteristics of leaf epicuticular waxes as shown e.g., by *Baker* (1974) and

Koch et al. (2006), but their direct effect on the permeability of foliar-applied nutrient solutions is currently unclear and requires further research efforts.

Nowadays, it is still not possible to quantify the absorption of solutions via stomata as compared to the cuticle, but there is concluding evidence that the contribution of stomata to overall foliar absorption can be significant (*Eichert and Goldbach*, 2008; *Eichert* et al., 2008). Furthermore, on leaf sides having stomata transpiration water may re-condensate on the leaf surface (*Burkhardt* et al., 1999) keeping foliar-applied solutes partly dissolved and mobile even though the surrounding bulk atmosphere may be dry

IV. The effectivity of foliar nutrient absorption can be stimulated by the supplementation of adjuvants in the spray formulation. Humectants are considered to increase absorption rates due to the extension of the period were the drop remains in a liquid phase, enabling absorption of the applied nutrient. Foliar B absorption can be enhanced by the addition of humectant adjuvants in the foliar formulation.

In this study different mixtures of BA with solutes having a lower DRH and hence providing a humectant effect (i.e., $CaCl_2$ and sorbitol with DRHs of 32% and 69%, respectively; *Salameh* et al., 2005), were investigated with respect to foliar B absorption and subsequent translocation.

The addition of humectants significantly enhanced foliar B absorption in soybean, which is in agreement with other studies showing higher nutrient absorption rates associated with the addition of humectants (e.g. *Fernández* et al., 2009). This effect was more remarkable with increasing sorbitol concentrations in the treatment solution. The higher concentration of sorbitol probably had a stronger humectant effect (i.e., it decreased more the DRH of the mixture) than the lower concentration.

The addition of $CaCl_2$ to high sorbitol concentrations decreased B penetration as compared to high sorbitol concentrations alone, which may be the result of the interaction between negatively charged sorbitol borate ester and Ca^{2+} (*Wimmer and Goldbach*, 1999). A reduction of cuticular penetration rates of commercial fungizide formulations in relation to $CaCl_2$ was also reported by *Schlegel and Schönherr* (2004).

In lychee, the addition of humecting adjuvants in the foliar formulation did not affect the rates of B absorption, which might be associated with the lower penetration rates and therefore, the low detection limits to measure any substantial B increases as compared to the more permeable soybean leaves.

V. *In contrast to root cells, absorption by green leaf cells is directly stimulated by light. (Marschner, 1995). Therefore, foliar B absorption and subsequent translocation will be higher after diurnal vs. nocturnal application.*

The application of foliar formulations nocturnal or diurnal showed no significant differences in foliar B absorption on lychee leaves. But, subsequent translocation of foliar applied B out of the treated leaflet was significantly higher after nocturnal versus diurnal application. During night time stomatal closure may limit the transpiration flow and improve the rate of B distribution from the point of application. Recent publications showed increased B distribution via phloem, after foliar application, in relation to the interruption of the transpiration stream (Chapter 2; *Eichert*, 2010).

VI. *Rates of nutrient absorption by leaves decline with leaf age due to metabolic activity (sink activity) and increase in the thickness of the cuticle (Marschner, 1995). Foliar B absorption will be reduced in mature vs. immature leaves due to sink activity and the increase in the thickness of the cuticle.*

In case of lychee, increased rates of foliar B absorption in association with mature, fully-expanded leaves were recorded, which appeared to be more permeable than young, developing leaves. The higher absorption rates in mature lychee leaves might have been an effect of favorable wax compositions of mature leaves in comparison to immature leaves. *Baker and Hunt* (1981) studied the penetration of foliar applied 1-naphthylacetic acid during leaf growth on different plant species. Absorption decreased during leaf growth in species, such as *Vitis vinifera* and *Eucalyptus globulus*. In contrast, foliar absorption of 1-naphthylacetic increased during leaf extension in *Malus hypehensis* leaves.

After foliar B absorption, subsequent translocation was significantly higher in mature lychee leaves compared to immature leaves. Nevertheless, the total amount of B that moved out of the application zone was hardly above the detection limit. The finding can be linked to metabolic activity (source activity), but re-translocation to sink tissues did not occure.

5.2. Concluding remarks and final recommendations

The efficiency of foliar B absorption and subsequent translocation is determined by numerous factors requiring interdisciplinary approaches. The results presented in this thesis suggest an important role of stomata for the performance of foliar B sprays. There are three possible functions of stomata regarding foliar B fertilization. First, the direct absorption through the stomatal pores, second, the transpiration water increasing air humidity in the laminar layer keeping B in solution and mobile, and third, closed stomata increasing phloem mobility of B, due to the interruption of the transpiration stream.

Increased phloem mobility with closed stomata could be demonstrated in Chapter 2 and, to some extent, in Chapter 4 (nocturnal application). In both crop species, treatment of the abaxial leaf surface led to significantly higher B absorption rates as compared to B absorption by adaxial leaf sides. The underlying physiological processes (differences in cuticular composition or effect of stomata), however, could not be elucidated in this work. According to our field observations in Thailand, farmers mainly apply B foliar fertilizers by methods favoring the moistening of the adaxial leaf surface. Applying foliar fertilizers by alternative techniques like “fogging” will help to target the abaxial leaf surface and to improve the performance of foliar sprays. Fogging heads and e.g., electrostatic spraying techniques produce very small droplets which may improve wetting of the entire plant. However, the lower droplet sizes may limit the amount of liquid that is deposited onto the plant surface and solution drying may occur more rapidly. In soybean production, the application of B foliar fertilizers to the abaxial leaf surface might not be essential as B was also absorbed through the adaxial leaf side. Nevertheless, more research is required to improve the efficiency of foliar sprays by the development of effective formulations and spraying methods that also target the abaxial leaf side.

In future management practices of foliar B fertilization the influence of B deficiency on foliar B absorption need to be taken into account. Foliar B sprays have to be applied before severe deficiency is observed.

Foliar fertilization of mature lychee leaves was more effective as compared to immature leaves. Due to the very limited phloem mobility of B in lychee, B absorption in immature leaves should be improved in further research attempts. It was also suggested that young flushes of lychee trees are important locations for hormonal signal for flower initiation.

Strong necroses on these flushes under B deficieny are assumed to limit flower initiation and total yield (*Roygrong*, 2009). The cuticular morphology (wax composition, stomata) of immature leaves should be studied in more detail to understand the limitation of absorption. It could help to create an optimal spray formulation for practical implications.

Experiments on the effect of different foliar formulations on subsequent translocation showed only very restricted mobility and foliar applied B was only detectable within the treated leaves. The relevance of innovative spray formulations (polyol supplementation) to increase B phloem mobility seems to be very limited. The statistical results were significant but the small rates of B moving out of the application zone might not have relevance for practical implications. The supplementation of polyols to improve phloem mobility did not show sufficient effects for both species.

The effect of nocturnal application on increasing B mobility after foliar B application in lychee leaves might be due to closed stomata, as it was suggested before.

In conclusion, absorption of foliar applied B is highly dependent on plant B status and can be enhanced by humectant adjuvants supplementation (polyols, $CaCl_2$) in soybean and application of foliar formulations to the abaxial leaf surface in soybean and lychee.
All other parameters studied, such as time of application, leaf age and polyol supplementation to improve subsequent translocation need to be further investigated to gain a better understanding and to identify the relevance for foliar fertilization.

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SUMMARY

Foliar fertilization is an agricultural practice to supply plants with a specific nutrient in times of low soil availability of the nutrient or low root activity, e.g. during generative growth. The focus of this study was placed on Boron (B). Boron is an essential micronutrient for higher plants and deficiency appears in many countries on numerous crops throughout the world. B fertilization is often applied as foliar fertilizer, but the efficiency is inconsistent.

The possible physiological function of B in plants is described in Chapter 1 within the general introduction. The experiments were conducted on two disparate crop species (soybean and lychee) and the impact of different parameters on foliar B absorption and subsequent translocation was studied.

The first study (Chapter 2) shows the impact of plant B status on foliar B absorption and subsequent translocation in soybean. The limited absorption of foliar applied B in B deficient plants was observed in soybean and in lychee. The physiological study was developed for soybean plants pre-treated with different B root supply, ranging from deficient to B intoxicated plants. In addition, different formulations were tested in order to increase the subsequent B translocation after foliar B absorption. For this reason polyols (mannitol, sorbitol) were added to the foliar formulations, as they form stable polyol borate ester with B and these compounds enable phloem mobility in some species.

Lowest absorption was observed in plants with B deficiency and B intoxication, whereas the share of subsequent B basi translocation was highest. Results correlated with measurements on stomata opening and water potential. The interruption of the transpiration stream, indicated by high water potentials in B deficient and intoxicated plants, might facilitate B phloem translocation, as it was shown in recent publications.

Absorption rates were increased in treatments with polyol supplementation, probably due to a humectants effect (lowering the DRH). Subsequent translocation could not be improved by the addition of polyols.

In Chapter 3 the impact of leaf side of application and adjuvant supplementation on foliar B absorption and subsequent translocation was studied on lychee and soybean.

The effect of the adjuvants $CaCl_2$ and sorbitol as humectant adjuvants and mannitol and sorbitol as B-binding adjuvants were investigated.

Both plant species differed greatly in total absorption rates. Boron absorption through the abaxial leaf side was more than three-fold (soybean) or seven-fold (lychee) higher than through the adaxial side
The addition of adjuvants significantly enhanced the rate of B absorption in soybean, but had no effect on B absorption in lychee. The positive results of adjuvant supplementation in soybean might be attributed to the humectants effect. Subsequent translocation could not be increased in neither of the treatments.
The results on foliar B absorption via the abaxial leaf surface in both species suggest a high demand for future research, e.g. techniques to spray the abaxial leaf surface.

In Chapter 4 the focus was to assess the impact of different parameters on foliar B absorption and subsequent translocation in lychee. B solutions were applied on the adaxial versus the abaxial leaf surface of mature or immature leaves. In addition, nocturnal versus diurnal application was studied.
Absorption was significantly increased after application to the abaxial leaf surface. The parameters leaf age and time of application did not affect absorption.
Subsequent translocation of foliar absorbed B increased significantly after foliar application of B to mature in comparison to immature leaves.
Nocturnal application of B resulted in significantly enhanced basipetal B translocation.
The efficacy of foliar B fertilization can be limited in lychee, especially if only the adaxial leaf surface is targeted as it commonly occurs with the spraying techniques available in many lychee orchards. The practical implications of our results clearly show that B foliar sprays should be applied nocturnally to the abaxial leaf surface.

In conclusion, absorption and subsequent translocation of foliar applied B can be increased by different parameters in lychee and soybean. Plant B status and leafside of application showed a strong impact on foliar B absorption. All results are discussed in Chapter 5.

ZUSAMMENFASSUNG

Die Blattdüngung spielt eine wichtige Rolle im Pflanzenbau und findet Anwendung, wenn aufgrund von verminderter Verfügbarkeit im Boden oder während geringer Wurzelaktivität (generatives Wachstum) Versorgungsengpässe entstehen.

In dieser Arbeit wurde der Mikronährstoff Bor (B) als Grundlage für die Forschungsarbeiten gewählt. Bor ist ein lebensnotwendiger Mikronährstoff für höhere Pflanzen, und Mangelerscheinungen wurden bereits in vielen Ländern an unterschiedlichsten Pflanzen beschrieben. Bor wird oft in Kombination mit Pflanzenschutzmaßnahmen über das Blatt gedüngt. Die Effektivität der B-Blattdüngung ist aber sehr variabel.

Die physiologische Funktion von Bor in der Pflanze und deren Auswirkung für den Pflanzenbau wird in Chapter 1 beschrieben. Die Experimente wurden an zwei unterschiedlichen Pflanzenarten (Sojabohne, Litschi) durchgeführt und Einflussfaktoren auf die B-Blattaufnahme und nachfolgende Verlagerung getestet.

Im zweiten Kapitel (Chapter 2) wurde das Thema ‚Einfluss des B Versorungsgrades der Pflanzen auf die B Aufnahme und nachfolgende Verlagerung an Sojapflanzen‘ und der Einfluss von Additiven auf die B Verlagerung untersucht.

Die Pflanzen wurden mit unterschiedlichen Borangeboten angezogen. Der Versorgungsgrad reichte von B Mangel bis B Toxizität. Zusätzlich wurden verschiedene Zusätze der Formulierung für die Blattapplikation zugesetzt, um die Verlagerung nach der Blattaufnahme zu verbessern. In Sojapflanzen ist B immobil. Die Zusätze in der Applikationslösung waren Polyole (Mannitol, Sorbitol), die stabile Verbindungen mit Bor bilden. In einigen Pflanzenarten kann B in dieser gebundenen Form im Phloem verlagert werden.

Die B Blattaufnahme war stark beeinflusst vom B Versorgungsgrad der Pflanzen. B Mangel und Toxizität führten zu geringerer B Absorption über das Blatt, als bei Pflanzen mit guter bis sehr guter Versorgung. Die anschließende basipetale Verlagerung des aufgenommenen B war prozentual zur Gesamtaufnahme in den mangel- und toxischen Pflanzen am höchsten. Die Ergebnisse zeigten eine gute Korrelation mit den Wasserpotential Messungen und der Auswertungen der elektronenmikroskopischen Aufnahmen der Stomata. Eine Drosselung des Transpirationsstroms, erkennbar an den hohen Wasserpotential Werten der Pflanzen mit B Mangel und Toxizität, könnte die B Phloem Verlagerung begünstigt haben, wie kürzlich veröffentlichte Untersuchungen zeigen.

Die B Blattaufnahme wurde durch die Zusätze in der Düngerlösung erhöht, mit größter Wahrscheinlichkeit ist dies auf einen ‚humectant' Effekt zurück zu führen, der den flüssigen Zustand der Düngerlösung verlängert und somit das Zeitfenster zur B Aufnahme vergrößert hat. Die B Verlagerung konnte durch die Zusätze nicht verbessert werden.

Im dritten Kapitel (Chapter 3) wurde der Einfluss verschiedener Zusätze in der Blattdünger Lösung auf die Aufnahme und anschließende Verlagerung an Soja- und Litschi Pflanzen untersucht. Der Beitrag der Stomata an der B Blattaufnahme wurde durch die Applikation auf die Blattober- und Blattunterseite bewertet.

Beide Pflanzenarten zeigten sehr starke quantitative Unterschiede in der B Blattaufnahme. Litschi Pflanzen nahmen kaum Bor über die Blattoberseite auf (keine Stomata), wogegen über die Unterseite das 7-fache der B Menge aufgenommen wurde. Die Blätter der Soja Pflanzen absorbieten B über beide Blattseiten, wohingegen die B Aufnahme über die Blattunterseite 3-mal größer war. Soja Blätter haben mehr Stomata an der Blattunterseite.

Die B Blattaufnahme wurde durch die Zusätze in der Blattdüngerlösung in Soja Pflanzen erhöht, die nachfolgende B Verlagerung wurde aber nicht beeinflusst. Litschi Pflanzen zeigten keine veränderte Aufnahme und Verlagerung mit Zusätzen in der Düngerlösung.

Die Ergebnisse zeigen, dass es einen großen Forschungsbedarf bezüglich der Applikationstechniken und Zusatzstoffe gibt, um zukünftig die Blattunterseite verstärkt zu benetzen und die Blattdüngerlösungen möglichst lange in einer flüssigen Phase auf dem Blatt zu erhalten.

Im dritten Abschnitt (Chapter 4) wurde der Schwerpunkt der Untersuchungen auf den Einfluss verschiedener Parameter auf die B Blattaufnahme und anschließende Verlagerung an Litschi Pflanzen gelegt. Die B Blattdünger Lösungen wurden entweder auf die Blattober- oder Blattunterseite von vollständig entwickelten oder noch wachsenden Blättern appliziert. Die Applikation erfolgte am Tag oder in der Nacht. Die B Aufnahme war signifikant erhöht nach der Applikation auf die Blattunterseite. Die Parameter Blattalter und Applikationszeitpunkt (Tag/Nacht) hatten keinen Einfluss auf die Menge an absorbieten B. Die Mobilität des absorbierten B's wurde dagegen in vollständig entwickelten Blättern und nach Nachtapplikation erhöht.

Die Applikation von Blattdüngern auf die Blattunterseite an Litschi Pflanzen scheint aus pflanzenbaulicher Sicht eine hohe Relevanz zu haben. Die Applikation bei Nacht könnte nach erfolgreicher B Aufnahme die anschließende Verteilung im Blatt begünstigen.

Die Ergebnisse der drei Studien zeigen, dass die B Blattaufnahme und anschließende Verlagerung durch unterschiedliche Parameter bei beiden Pflanzenarten verbessert werden konnte. Der B-Versorgungsgrad der Pflanzen, sowie die Blattseite auf die appliziert wúrde, zeigten die größten Einflüsse auf die Blattaufnahme. Die Ergebnisse werden ausführlich in Chapter 5 diskutiert.