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BORON: NOTHING BORING ABOUT THIS INTRIGUING ELEMENT

Greice Leal Pereira
Doctor Scientiae

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José Lavres Júnior
(Member)

Rebecca P. G. Omena
(Member)

Dimas Mendes Ribeiro
(Member)

Wagner Campos Otoni
(Member)

Wagner L. Araújo
(Adviser)

To Benedito, my grandfather (in memoriam), for all that means to me

I dedicate

The most important people in my life: my parents (Erany and Leonel) and my brothers (Karina, Roberto, Wagner, Richard e Emerson) for all the values, teachings, dedication, encouragement and never measuring efforts and sacrifices to fulfill my dreams

I offer

"Each dream you leave
Behind is a part of
Your future that
Will no longer exist"

Steve Jobs

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ABSTRACT

PEREIRA, Greice Leal, D.Sc., Universidade Federal de Viçosa, September, 2019. **Boron: nothing boring about this intriguing element**. Adviser: Wagner L. Araújo.

Although Boron (B) is an essential micronutrient for plant growth and development, both deficiency and toxicity of B are important problems that severely affect agricultural production. This fact aside, the impact of these stresses in plants are still poorly understood. Thus, plants may respond differently to B availability through local and systemic signaling, whose mechanisms are yet poorly understood. Previous studies further suggest that ethylene plays a key role in the responses induced by B deficiency in the root system. The main goal of this work was to better understand the physiological and metabolic mechanisms underlying stress caused by B deficiency and excess, as well as to better understand the connections between ethylene and B in modulating plant growth. For this purpose, the responses of different conditions of B availability (deficiency, adequate and toxicity) were investigated in *Arabidopsis* and ethylene mutant tomato (*Solanum lycopersicum*) plants. The results obtained in this work demonstrate physiological and metabolic alterations in response to the contrasting conditions of B and that these responses are likely able to generate energy and maintain normal growth in B deficiency. It was also observed an association between B and ethylene levels mediating physiological and metabolic changes. Finally, our study sheds light on the complex relationship between B and ethylene and their overall effects on plant growth and development. The results described here helps to understand the plant's response mechanism to B deficiency and excess, and paves way for identifying the signaling pathways and genes involved in homeostasis and B accumulation in tissues. Although the absence of alterations in plant growth coupled with changes in fruit yield and seed production observed in response to change in the levels of ethylene is somewhat surprising it is tempting to speculate that pathways of energy metabolism and hormone metabolism are most likely highly interconnected at the whole plant level in a manner that allows the plant to prioritize reproductive organs during senescence under B stressfully conditions. It will be important to establish the functional significance of this observation in future studies in order to fully understand

the molecular regulatory hierarchy regulating ethylene balance at the whole-plant level, particularly in response to fluctuations in B levels.

Key-words: B deficiency, B toxicity, Ethylene, Central Metabolism.

RESUMO

PEREIRA, Greice Leal, D.Sc., Universidade Federal de Viçosa, setembro de 2019. **Boro: nada entediante acerca deste intrigante elemento**. Wagner L. Araújo.

Embora o Boro (B) seja um micronutriente essencial para o crescimento e desenvolvimento das plantas, juntamente com o fato de que tanto a deficiência quanto a toxicidade são problemas importantes que afetam severamente a produção agrícola, o impacto dessas tensões nas plantas ainda é pouco conhecido. Assim, as plantas podem responder diferentemente à disponibilidade de B por meio de sinalização local e sistêmica, cujos mecanismos ainda são pouco compreendidos. Estudos anteriores sugerem também que o etileno desempenha um papel fundamental nas respostas induzidas pela deficiência de B no sistema radicular. O principal objetivo deste trabalho foi entender melhor os mecanismos fisiológicos e metabólicos em resposta a deficiência e excesso de B, bem como entender melhor as conexões entre etileno e B na modulação do crescimento das plantas. Para tanto, foram investigadas as respostas de diferentes condições de disponibilidade de B (deficiência, adequada e toxidez) em plantas de *Arabidopsis* e tomateiro (*Solanum lycopersicum*) mutantes em etileno. Os resultados obtidos neste trabalho demonstram alterações no crescimento, ocorrem alterações fisiológicas e metabólicas em resposta às condições contrastantes de B e que essas respostas provavelmente são capazes de gerar energia e manter o crescimento normal na deficiência de B. Também foi observada associação entre os níveis de B e etileno, mediando alterações fisiológicas e metabólicas. Finalmente, nosso estudo lança luz sobre a complexa relação entre B e etileno e seus efeitos gerais no crescimento e desenvolvimento das plantas. Os resultados deste estudo ajudam a entender o mecanismo de resposta da planta à deficiência e ao excesso de B e abre caminho para identificar as vias de sinalização e os genes envolvidos na homeostase e no acúmulo de B nos tecidos. Embora a ausência de alterações no crescimento das plantas juntamente com as mudanças na produção de frutos e na produção de sementes observadas em resposta à mudança nos níveis de etileno seja algo surpreendente, é tentador especular que as vias do metabolismo energético e do metabolismo hormonal estão provavelmente altamente interconectadas ao nível de planta inteira de uma maneira que permita à planta priorizar os órgãos reprodutivos

durante a senescência sob condições estressantes B. Será importante estabelecer o significado funcional dessa observação em estudos futuros para entender completamente a hierarquia molecular que regula o balanço de etileno em todo o nível da planta, particularmente em resposta as flutuações nos níveis de B.

Palavras-chave: Deficiência de B, Toxidez de B, Etileno, Metabolismo Central

SUMMARY

Overview

Boron (B) is an essential micronutrient for both plant growth and development. Its essentiality was first described in 1923 by Warington by showing that the growth of *Vicia faba* (field bean) and other plants was reduced in the absence of B, but it was rescued following the resupply of B. Following this discovery, it has been widely accepted that B is a necessary and beneficial element for different organisms.

B availability in the soil and its use by plants are mainly regulated by pH. Thus, with the increase of the soil solution pH, lower is the availability/uptake of B. This occurs due to the fact that at pH 7 B is mainly in the form of boric acid (H_3BO_3), that has little affinity to clay minerals, being readily available to plants. As the pH increases, the concentration of B in the form of borate anions ($B(OH)_4^-$) increases, a form having high affinity for the soil colloids, resulting in an increased B adsorption (Goldberg, 1997). Soil composition also interferes with B availability, especially the content of amorphous and crystalline oxides of iron (Fe) and clay silicate capable of adsorbing B, and also the presence of B containing minerals (such as tourmaline). The B available in the soil is mainly associated with soil organic matter (SOM). It is important to mention that these factors related with B availability are directly affected by the climatic conditions (Goldberg, 1997; Goldberg et al., 2005).

B predominates in the soil solution in the non-dissociated form of boric acid (Hu and Brown, 1997), which greatly influences its availability. Thus, usually two extremes, deficiency and toxicity, are observed in the arable areas that drastically affect agricultural production. B deficiency is commonly related to highly leached sandy soils with low pH (acidic conditions) and low organic matter content (Yan et al., 2006), problem that mainly affects regions of Japan, Southeast China, USA and Brazil. On the other hand, toxicity occurs predominantly in regions characterized by alkaline and saline soils, along with low precipitation and scarce leaching (arid and semi-arid regions). In these regions, high B concentrations may either occur naturally in soil and groundwater or be added to soil by fertilizers and irrigation water, making B levels highly toxic to plant growth (Tanaka and Fujiwara, 2008; Camacho-Cristóbal et al., 2008). The regions most affected by

this problem are South Australia, Western Asia and North Africa, North America (California) and South America (Chile) (Gupta et al., 1995; Nable et al., 1997; Roessner et al., 2006; Herrera- Rodríguez et al., 2010). It should be noticed that both B deficiency and B toxicity have been extensively studied for decades in many plants (Camacho-Cristóbal et al., 2008; Camacho-Cristóbal et al., 2011; Landi et al., 2019), yet the underlying mechanisms by which B play its functions in land plants remain rather unclear.

Due to this amplitude in the levels of B present in the soil solution, plants use distinct mechanisms to maintain homeostasis in their tissues (Ozhuner et al., 2013). Usually, this process occurs at the root absorption stage. B uptake by plants in the form of H_3BO_3 occurs by three mechanisms: (i) passive transport through the plasma membrane by the diffusion process; (ii) facilitated transport by NIPs (Nodulin 26-like intrinsic proteins), and (iii) energy-dependent transport (against a concentration gradient) (Marschner, 2012). Although significant advances have been made in understanding the regulation of B transport and the mechanisms related to the maintenance of B levels in plants, their physiological and metabolic impacts remains incipient.

The management for toxicity conditions is more complex than for the deficiency, since the application of fertilizers containing this element (the most common: boric acid and borax) greatly aid in the correction of B deficiencies (Macho-Rivero et al., 2017). Overall, techniques based on the cultivation of tolerant varieties in soils with toxicity have been proposed (Siddiqui et al., 2013). B toxicity affects various aspects of cellular metabolism, and the symptoms exhibited by plants under such conditions are associated with the reduction of vigor and development particularly of the shoot, formation of chlorotic to necrotic spots in the leaves that initially occurs in the margin and in the tips of old leaves, extending the whole plant, reflecting the distribution and the accumulation of B via transpiratory flow (Reid and Fitzpatrick, 2009; Marschner, 2012). In addition, a decreased root growth is observed as a consequence of changes in the cell division of the apical meristem (Aquea et al., 2012). Moreover, increased oxidative stress, as evidenced by lipid peroxidation, coupled with increased activity of antioxidant compounds are also usually observed in response to B excess (Tewari et al., 2010; Schnurbusch et al., 2010; Kayaa and Ashraf, 2015; Pallotta et al., 2014). On the other hand, B limitation usually affects young parts

of plants, especially at the apical meristem. In this context, the main symptoms usually observed under B deficiency include reduction of root elongation (root and lateral root), reduction of leaf expansion, and impacts on reproductive function, including lower flowering, fruit set, seed production and final yield (Goldbach et al., 2001; Abreu et al., 2014). It should be also highilighted that B responses largely depend on the species and their stage of development. It is equally important to mention that B deficiency and toxicity not only reduce yield but also impact crop quality (Brown et al., 2002).

B plays key roles in plants, including cell wall formation and stability (Cakmak, 1995), maintenance of the structural and functional integrity of the plasma membrane (González-Fontes et al., 2013), transport and metabolism of sugars, respiration and metabolism of indoleacetic acid (IAA) (Graham and Webb, 1991), metabolism of RNA (pyrimidine biosynthesis), phenolic compounds, nitrogen and ascorbate (Camacho-Cristóbal et al., 2008). Despite this variety of physiological processes apparently altered by B levels, little or nothing is currently known concerning the impacts of B levels on primary metabolism. Therefore, to study the metabolic impacts of fluctuations in the levels of B is necessary to enhance our understanding on its role in these processes.

The search for a deeper understanding of the mechanisms associated with the regulation of B uptake has been greatly facilitated by the identification and characterization of a series of transporters involved in this process. In *Arabidopsis thaliana*, several B transporters were recently described showing that plants are able not only to perceive the internal and external conditions of B but also to regulate its transport according to its availability, by the expression of transporters, maintaining B homeostasis in tissues (Miwa and Fujiwara, 2011; Miwa et al., 2013). Although these findings were obtained in a model plant, it seems reasonable to assume that this may be applicable to other species, and that this knowledge may be useful in the design of B tolerant plants with high or low B requirement (Miwa and Fujiwara, 2010).

The first B transporter identified in *Arabidopsis thaliana* was BOR1 (Takano et al., 2002). It is an efflux type B transporter, which plays a key role in xylem loading against a concentration gradient. It is generally located in the plasma membrane of the root cells, predominantly in the pericarp cells surrounding the xylem, and it harbors great importance in the transport of B from

the roots to the shoots of the plants, specifically under low B availability (Takano et al., 2002, 2010). In this context, under limiting B conditions, BOR1 is required for the efficient loading of B into xylem. Interestingly, BOR1 accumulation is regulated by the conditions of B at the post-transcriptional level. Briefly, under high levels of B in the soil solution, BOR1 is transported to the vacuole for degradation (Takano et al., 2005, 2010; Kasai et al., 2011). This response is seemingly important to avoid B accumulation in toxic levels in the plant tissues plants when B levels in the soil are relatively high. Although the physiological significance of this transporter has not been elucidated, it seems reasonable to assume its significance under B excess and that further research is clearly required to decipher its physiological function.

NIP5;1, another membrane protein, was characterized as a major aquaporin associated with B uptake as boric acid under B limiting conditions (Takano et al., 2006). It is important to also note that NIP5;1, the major member of the intrinsic protein family, is an important facilitator of the passive flow of water and small uncharged molecules (glycerol, urea, and boric acid). This fact aside, NIP6;1 which is the most similar to NIP5;1, corresponds to a channel that facilitates the permeability of boric acid through the plasma membrane and has recently been found to be completely impermeable to water (Takana et al., 2008). NIP6;1 transcript accumulation occurs in response to the B deficiency in the shoot. This transporter is expressed predominantly in the regions of the shoot nodes, especially in the phloem region (Takana et al., 2008). Although NIP6;1 mutants showed reduced expansion of young leaves only under low B availability, B concentrations were not reduced in old leaves (Tanaka et al., 2008; Miwa and Fujiwara, 2010).

In response to B excess, many plant species are able reduce B accumulation by excluding it mainly in the roots, in order to maintain plant homeostasis (Hamurcu et al., 2016). Under B excess, there is an induction of the B-efflux transporter BOR4 located at the plasma membrane (mainly in the endodermis), which was reported as a homologous BOR1 gene (Miwa et al., 2007, 2014). Plants that increase expression and activity of this transporter exhibit higher tolerance, thus enhancing plant growth when exposed to high levels of B (Miwa et al., 2007, 2013; Kajikawa et al., 2011; Miwa and Fujiwara, 2011). The functional lack of this transporter led to reductions in shoot growth

and symptoms of toxicity under high levels of B. By contrast, over expression of this transporter culminates with higher tolerance to conditions of B excess (Miwa et al. 2014; Lv et al., 2017).

A growing body of evidence indicates a possible interaction between B availability and signaling via ethylene, auxin and reactive oxygen species (ROS), culminating in root growth inhibition (Martín-Rejano et al., 2011; Oiwa et al., 2013; Camacho-Cristóbal et al., 2016; González-Fontes et al., 2016). Notably, ethylene plays a significant role in plant responses to different abiotic stresses, such as hypoxia, heavy metals, salinity, heat, drought, ozone, aluminum toxicity as well as phosphorus and B deficiency (Sun et al., 2007, 2010; Lei et al., 2011; Khan et al., 2013; Habben et al., 2014; Ludwikow et al., 2014; Steffens, 2014; Li et al., 2015a; Yang et al., 2016). Accordingly, B deficiency may induce rapid ethylene biosynthesis, which leads to inhibition of root elongation (Sun et al. 2007; Martín-Rejano et al., 2011; Tian et al., 2014). Ethylene inhibits root growth by mainly stimulating auxin biosynthesis and modulating the basipetal auxin transport towards the elongation zone (Swarup et al., 2007). Ethylene biosynthesis from methionine is regulated by the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and conversion of ACC into ethylene by the enzyme ACC oxidase (ACO) (Kende, 1993; Lyzenga et al., 2012). Both ACS and ACO enzymes are fundamental components for the biosynthesis of ethylene, which are encoded by multigenic families and are strongly regulated by different environmental stimulus (Sun et al., 2010; Schellingen et al., 2015; Li et al., 2015a; Li et al., 2015b, Khan et al., 2015). However, our knowledge on how exactly B levels in conjunction with ethylene impacts leaf primary metabolism and plant growth in general remains rather fragmented.

Preliminary results from our research group indicate a potential role of B in growth regulation mediated by extensive reprogramming of primary metabolism and that this modification resides primarily in changes in carbohydrate and starch metabolism. Further evidence suggests that ethylene is probably involved in root responses to B levels (Camacho-Cristóbal et al., 2016; González-Fontes et al., 2016). As aforementioned, not only phytohormones and in special ethylene but also B, are required for several functions within plant development and it seems also to connect plant growth and carbon metabolism

yet our understanding of the precise molecular and metabolic basis of this phenomena remains poorly elucidated. It is therefore of paramount importance to elucidate what and how the components regulating this hormonal adaptative mechanism are associated with B homeostasis. To investigate how and to which extent B and ethylene impact metabolism in general, here we used both *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) ethylene mutants as a model system to elucidate this intriguingly connections.

Thus, this thesis is largely focused on the investigation of the functions of B on the context of plant growth and leaf carbon metabolism as well as to investigate the functional role of the ethylene in mediating this response. To reach this goal, a range of complementary approaches were used and thus this thesis is organized as a compilation of three independent chapters which discuss the impact of different levels of B on plant growth and central metabolism in the two last chapters. While in the first one on the functions of B on plant development are revisited, and the classical classification of B essentiality is further discussed. In each chapter an introduction and discussion as well as details of the methods used are included. At the end, one chapter entitled “Concluding Remarks” synthesizes the main findings of this work and the main challenges and perspectives in understanding B function during plant development are briefly discussed.

CHAPTER 1. “A necessary evil”: Boron, more than only an essential element for land plants?

Since the role of B during plant growth and development is a recurring theme in plant biology, and given that a growing body of evidence of the function of this micronutrient here we revisited this and further hypothesize the possible role of B in a classic scenario of studies. B is a chemical element that has long been suggested as an essential micronutrient for plants. The main players associated with B uptake and translocation by plant roots have been already identified, showing a sophisticated set of proteins able to cope with B levels in the soil solution. Accordingly, the vast majority of reports suggest B as an essential element for plants. This fact aside, such evidence has been recently questioned (REFERENCE). Here, we provide compelling evidences supporting the essential role for B in mediating developmental program of plants. At the end,

we further posit that the development of new tools to precisely determine B levels *in vivo* is likely necessary to unequivocally demonstrate the essentiality of B.

CHAPTER 2. Physiological and metabolic changes in response to different levels of Boron: extending Boron functions in *Arabidopsis thaliana*

Although B is an essential micronutrient in land plants, the impacts of B deficiency and toxicity, which is generally narrow, remains poorly understood. Both stress (B deficiency and toxicity) are rather common and severely impact the agricultural production. To further investigate the physiological and metabolic mechanisms underlying B stress, *A. thaliana* were fertilized with different B concentrations (0; 0.03; 30; 100; 1000 and 3000 μM H_3BO_3) in the vegetative phase. Here by performing a detailed morphological, physiological, and biochemical characterization of *A. thaliana* plants novel aspects of the influence of B on plant growth and primary metabolism in leaves is provided. Our results demonstrated that adequate levels of B are important role in mediating growth responses by impacting leaf metabolism as highlighted by the changes in carbon assimilation, carbohydrates turnover, and an interesting reprogramming of metabolites levels.

CHAPTER 3. Physiological and metabolic changes in response to Boron levels are mediated by ethylene in tomato plants

To gain further insights into the well-established function of ethylene as growth regulation we investigated the physiological, molecular, and biochemical responses of tomato (*Solanum lycopersicum*) leaves following changes in the concentrations of B. The results show that plants treated with B excess were characterized by maintenance of growth, not being associated with decreases in carbon assimilation due to both reduced photosynthesis rates and stomatal conductance, coupled with impairments in carbohydrates turnover. Taken together, our results suggests that B affects ethylene biosynthesis, most likely by acting in enzymes related with its biosynthesis and reducing carbon assimilation and leading to altered central metabolism. The results obtained are discussed in the context of current models of ethylene and B effects on the regulation of both growth and metabolism.

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Article type: Review

Boron, "A necessary evil": more than only an essential element for land plants?

Authors: Greice Leal Pereira[1], João Antonio Siqueira[1], Samuel Vasconcelos Valadares[2], Adriano Nunes-Nesi[1], Wagner L. Araújo[1*]

[1]*Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-900 Viçosa, MG, Brazil*

[2]*Departamento de Solos, Universidade Federal de Viçosa, 36570-900 Viçosa, MG, Brazil*

[*] Corresponding author:

wlaraujo@ufv.br

Tel: +55 31 3612-5358

Abstract

Cumulative evidences demonstrated the agents associated with Boron (B) uptake and translocation by plant roots, showing a sophisticated set of proteins used to cope with B levels in the soil solution. Although B is a chemical element that has long been assumed as an essential plant micronutrient, this assumption was recently questioned. Here, we gather compelling evidence supporting the essential role for B in mediating plant developmental programs. Overall, the vast majority of plant species studied exhibited B transporters with a tight genetic coordination in response to B levels in the soil that are able to uptake B from soil which is uncommon responses for toxic elements. Moreover, the current tools available to determine B levels cannot exempt B translocation dynamics. We posit here that plants need B for their metabolic activities and in the regulation of root and shoot meristems, promoting plant developmental phases transitions, which is critical for complete of their life cycle. Therefore, plants need to acquire sufficient amounts of B while protecting themselves from its toxic effects. Thus, the development of *in vitro* and *in vivo* approaches is clearly required to accurately determine B levels and therefore to define unambiguously B functions in terrestrial plants.

Key-words: B deficiency, phases transitions, developmental programs

The complexity behind Boron

Boron (B) essentially was first described in the 1920s by the demonstration that root growth of *Vicia faba* L. (field bean) and other plants was reduced in the absence of B, but it was rescued following the resupply of B (Warington, 1923). Later, it was suggested that B played a pivotal role during transition from aquatic to terrestrial environment, driving this evolutionary transition of plants (Lewis et al., 1980). In consonance with this fact, the first vascular plant *Zosterophyllum shengfengense*, shows that B is primordial to originate root system in a terrestrial environment (Lewis et al., 1980).

In land plants, B is considered to play important functions including structural role in cell walls and the maintenance of plasma membrane functions, stimulation of reproductive tissues, improvement of seed quality and its influence on the biosynthesis of some metabolic compounds, i.g. antioxidants and polyphenols. Additionally, involvement in nucleic acid synthesis, phenolic metabolism, carbohydrate biosynthesis and translocation, indole-3-acetic acid oxidase, as well as pollen-tube growth and root elongation (Brown et al., 2002; Shireen et al., 2018; Landi et al., 2019) yet, the molecular mechanisms behind these functions remains largely unknown. Nevertheless, compelling evidence has demonstrated an elaborate system involved in both the uptake and transport of B in different plant (Miwa and Fujiwara, 2010). From a totally different perspective, B was recently assumed to be a rather toxic element, causing drastic damage in plant cells even under low levels (Reid et al., 2004; Landi et al., 2019). Moreover, Lewis (2019) argued against B essentiality considering that the responses of its deficiency are largely due to the toxicity of phenylpropanoids. It is also important to mention that alleles mediating tolerance to high levels of B in the soil also apparently remains in the wheat elite cultivars genome following landrace breeding selection (Pallotta et al., 2014). Remarkably, these alleles of tolerance are widespread in wheat elite cultivars developed on countries with extremely low levels of B in soil, evidencing alternative potential roles in contrasting environments, implying that combining B tolerance alleles through the level of B in soil is important for mediating the developmental program of plants.

Accordingly, B is able to induce molecular pathways regulating developmental phase transitions, which are involved in the coordination of the entrance of plants in adult phase (Xu et al., 2016; Sakamoto et al., 2018).

Over the last century, several independent and complementary studies have provided there of compelling evidence for the involvement of B and its significance for plant development and growth. Remarkably, this aspect was recently contested based in metabolic responses that could be confused with B deficiency responses. Here, we summarize the complex B relationships in the soil as well as how these relationships influence an elaborate system of transporters governing B uptake from rhizosphere. We further discuss the preferential B distribution and remobilization among tissues and its functional implications. Furthermore, we revisited the functions of B in mediating single genetic mechanisms in meristem cells, which might contribute to precisely explain the essentiality of element. Finally, we provide compelling evidence supporting the essentialty of B in mediating plant developmental program. We further posit that the development of new tools to precisely determine B levels *in vivo* is likely necessary unequivocally demonstrate the essentiality of B.

B transport inside of plant and B dynamics

The patterns of B distribution around the rhizosphere and the B behavior in the soil directly affect its availability for plants. Soil solution contains predominantly boric acid (H_3BO_3) and borate anion ($B(OH)_4^-$), in which the chemical equilibrium strongly depends on the soil pH (Klochko et al., 2006). Another variable that strongly influences the physicochemical property of H_3BO_3 is the ionization constant (pK_a). In general, pK_a can be defined as the pH of a solution where the concentration of the undissociated species is equal to that of the ionized species, that means, [HA] = [A]. Thus, in solutions with pH lower than pK_a there is an increase in H_3BO_3 concentration. Unlike, at higher pH, the form ($B(OH)_4^-$), predominates. That being said, due to its small ionization constant (pK_a 9.25), H_3BO_3 is the predominant form under conditions of pH below than the pK_a (Tanaka and Fujiwara, 2008).

Following the mass flow phenomenon, B is directed for roots, where this nutrient might be uptake from the soil solution. The uptake mechanism is regulated predominantly by both non metabolic and metabolic processes. The mechanism of passive diffusion through the plasma membrane was considered an exclusive process for B uptake by roots (Nable et al., 1990; Brown and Hu, 1994; Hu and Brown, 1997). However, using genetic and biochemical approaches the presence of B-specific transporters was demonstrated not only in roots but also in leaves and reproductive organs (Takano et al., 2008; Chatzissavvidis and Therios, 2011).

The activity of the B transporters is tightly regulated in response to the levels of B in the soil solution, optimizing B uptake and use, as well as maintaining nutrient homeostasis in different plant tissues (Takano et al., 2008; Yoshinari and Takano, 2017). Nodulin 26-like intrinsic protein (NIP) family contain the major B transporters, including the root transporters NIP5;1 (boric acid channel) and BOR1(borate exporter), which are assumed as the proteins responsible to maintain B homeostasis (Takano et al., 2008). NIP5;1 is located preferably on the plasma membrane, with its polarity facing the soil side, while BOR1 also is located on the plasma membrane but with its polarity towards stela (Wakuta et al., 2016). According to these transporters activity, the radial B transport from the soil solution into xylem is mediated under low soil levels of B (Sakamoto et al., 2011). By contrast, under B excess, the transporter BOR4 is involved with B exclusion from cells and tissues, enhancing tolerance to B toxicity (Miwa et al., 2007). Thus, a sophisticated system for B uptake and translocation based on families NIP and BOR is present in land plants, which the expression of NIP5 isoforms is highly induced in both roots and shoots (Diehn et al., 2019). Accordingly, transcripts encoding NIP members were also detected in floral tissues and showing expression patterns depending of developmental stage which seems to contribute for B distribution in B deficient flowers, (Diehn et al., 2019). In maize (*Zea mays*), double mutants for functional homologs of BOR1 (*RTE* and *RTE2*) were characterized by slow developmental time of shoot apical meristem (SAM) displaying reduced fertility and small ears (Chatterjee et al., 2014;

Chatterjee et al., 2017). It seems reasonable to assume that an elaborate genetic network allows land plants to cope with B levels via a tightly regulated response to this nutrient.

Once it is accumulated in the plasma membrane, BOR1 is rapidly ubiquitinated and transported to multivesicular bodies (endocytosis), being subsequently targeted into the vacuole for BOR1 degradation (Takano et al., 2010; Wakuta et al., 2016; Yoshinari et al, 2018). Yoshinari et al. (2019) observed that AP2-dependent endocytosis maintains the polar localization of BOR1 to support plant growth under low-B conditions, whereas the B-induced vacuolar sorting of BOR1 is mediated through an AP2-independent endocytic pathway. This response is assumed to be important for plant acclimation to high B conditions (Tanaka and Fujiwara, 2018). Curiously, wheat (*Triticum turgidum* L. var. durum) genes modulating adaptation for B in the soil exhibit alleles origin and dispersion on whole earth, important in tolerance to distinct B levels (Pallotta et al., 2014). Thus, these alleles display a large natural variability mediating B responses, which seems to have been remodeled following selective breeding promoted in elite cultivars owing to contrasting environments of selection (Pallotta et al., 2014). Collectively, compelling evidence obtained during the last decades indicate intrinsic relationships between B transporters and plant development in general, which exhibit evolution patterns to drive plant breeding, highlighting the importance of this element and consequently contrasting with toxic element characteristics. Remarkably, there is virtually no current evidence of any plant that evolved other morphoanatomical or genetic mechanisms to deal with toxic levels of B rather than only using this exquisite regulation of B transporter

Boron translocation and distribution

Following B uptake by the roots, the transpiration actually drives B transported through xylem cells (Marschner, 2012). By analyzing B tolerance, beyond intrinsic regulation mediated by transporters in distinct tolerant species, it was possible to verify that differential B tolerance is also attributed to ability of restricting nutrient translocation of roots into shoots, allowing a high B accumulation

161 in roots these plants are indicated for phytoremediation in areas with high levels of
162 B (Xin and Huang, 2017).
163 Moreover, B can also be translocated allowing its distribution between
164 vegetative and reproductive tissues (Camacho-Cristóbal et al., 2008), although this
165 strongly varies among species. Accordingly, for most plant species B is relatively
166 immobile and thus the most common B deficiency symptom, particularly in restricted
167 mobility plants, is related to the death of the apical meristem. This fact apart, in
168 certain plant species, including apple, nectarine, arabidopsis and citrus (Brown and
169 Hu, 1996; Takano et al., 2001; Wu et al., 2019), B can be translocated in the phloem,
170 apparently in quantities that are sufficient to meet plant requirements. It is important
171 to mention that the translocation occurs via the formation of B-diol complexes that
172 are important for the nutrient remobilization among tissues (Brown and Hu, 1996;
173 Hu et al., 1997). In fact, B can easily bind to cis-hydroxyl groups of sugar alcohols
174 (mannitol and sorbitol), allowing B transport through the phloem (Reid et al., 2004).
175 It seems reasonable to suggest that the molecular aspects involved in both B translocation
176 and remobilization are likely a critical knowledge barrier to B nutrition that must be proper
177 investigated to fully elucidate the essential nature of B for land plants.
178 The precise determinations of B at the cellular level are not currently achieved,
179 given that the actual B measurements do not consider dynamics of element and the
180 differential affinity for B of each B transporter. Therefore, it seems clear that more detailed
181 and high resolution techniques are required and that those must consider the dynamics
182 of this element in both space/time as well as it should consider its dynamics in living cells.
183 We posit that it may be achieved by the development of genetically encoded sensors or
184 chemical sensors for H_3BO_3, similar to nanobiotechnology which allows the development
185 of intelligent plant sensors that communicate chemical signals with the physiological and
186 nutritional state of plants (Giraldo et al., 2019). To this end, the conventional approached
187 suggested is the measurement of B stable isotopes, namely ^{10}B and ^{11}B, allowing the
188 analysis of B pools in plant tissues (Dannel et al., 2000). The recent application of an
189 ablation laser-ICP-MS (Laser Ablation Incuctively Coupled Plasma Mass Spectrometry)
190 allowed the development of a mathematical model that enable high-resolution analyzes

of B distribution along roots of *A. thaliana* in an extremely higher resolution (Shimotohno et al., 2015). Consequently, works using this tool are important in studies of element distribution throughout the plant and behavior of the same specific tissue level.

The revolution obtained recently by the development and characterization of genetically encoded biosensors for cytosolic H_3BO_3, based on the fluorescent proteins of the transporters NIP5; 1 and BOR1 (Fukuda et al., 2018), has clearly contribute for the first visualization of B in living plant cells. The fluorescence intensity in roots and shoots of the transgenic plants were high under B limiting conditions, and gradually decreased with increasing concentration of B (Fukuda et al., 2018), that provide compelling confirmation of the sensor functionality. Such biosensors allow the visualization not only the distribution but also the dynamics of B at physiologically relevant levels in various tissue and cell types. As a result, these sensors can be used by a wider range of purposes, to determine whether the phenotype studied is influenced by differences in concentration of B, demonstrating the importance of B for plant growth and development. With that in mind, it is clear that the revolution afforded by next-generation sequencing means that appropriate tools and resources are becoming available to fully unravel the function of B in different crops. When taken alongside enhanced spatial resolution from techniques such as *in situ* hybridizations (Thisse and Thisse, 2008), promoter-gene fusions (Van Dyk, 2001), metabolite sensors (Wang and Lei , 2018), single-cell sequencing (Wang et al., 2018), and laser microdissection (Nelson et al., 2006), we anticipate that this will open new avenues for understanding B dynamics will ultimately allow increased yield by tailoring approaches to tap into more specific attributes of desired crop species.

Boron mediating developmental transitions in shoot and root

The indirect concept of nutrient essentiality posits that in the absence of determined nutrient the plant does not complete its life cycle (Arnon and Stout, 1939). In this context, developmental transitions are required for land plants to complete their life cycle, and these transitions are regulated through proliferation and differentiation of cells. Notably, a highly specific induction of B transporters has been described for root apical meristem (RAM) and SAM (Durbak et al., 2014; Sakamoto et al., 2019). Thus, it seems reasonable

to suggest that B is most likely a direct regulator of the cell division and differentiation, particularly on these specific sites, which is in good agreement with the assumption that B is indeed an essential micronutrient.

The preferential distribution of B in developing tissues of rice (*Oryza sativa*) is seemingly regulated in consonance with the expression of *member of nodulin 26-like intrinsic protein* (*NIP*), *OsNIP3;1* (Shao et al., 2018). Accordingly, knockout plants for this gene exhibited reductions in B content in new leaves coupled with an improved B translocation to old leaves, suggesting that OsNIP3;1 plays a key role in the distribution of B for developing tissues (Sho et al., 2018). Furthermore, it has been recently demonstrated that B contributes for the maintenance of the identity of root Quiescent Center (QC) and that B deficiency drastically inhibits cell proliferation (Poza-Viejo et al. 2018). In summary, there is a growing body of evidence showing that both meristem activity and fate are regulated in response to B levels, suggesting that this micronutrient play, a major role mediating developmental phases transitions of plants.

Mechanisms associated with root organogenesis are extremely sensitive to B deficiency and it seems that they most likely regulated through of B signalling (Abreu et al., 2014). By using mathematical modeling and further experimental validation, it was established that B flux does not display a continuous increase from root tips towards the mature zone (Shimotohno et al., 2015). Accordingly, B absorbed at the root tip is likely used only at this root site, while mature root zones display a higher importance to drive B transport for the shoot (Shimotohno et al., 2015). It is also important to mention that B acts blocking the accumulation of aluminum (Al) in apical root zone (Li et al., 2018), where Al might induce the QC differentiation reducing cell divisions and root elongation. This is good agreement with the hypothesis that B play a protective role on RAM. Moreover, a reduced cell differentiation observed in *Medicago sativa* root nodules under B deprivation indicates that B plays specific functions in the initial phases of root organogenesis (Reguera et al., 2009). Accordingly, genomic factors inducing cell differentiation that are involved in damages on DNA and DNA double-strand breaks (DSB) are triggered under B high levels in plants (Hu et al., 2016). DSB induced under toxic B levels might be mitigated through the degradation of BRAHMA (BRM) protein given that BRM binds to

acetylated histone residues opening chromatin and turning DNA more exposed for B-related damages (Sakamoto et al., 2018). By the way BRM temporally regulates the expression of miR156, the master regulator the transition of juvenile to adult phase, and as such mutations on BRM accelerated the exit of the vegetative phase (Xu et al., 2016). In fact, B transport is critical for vegetative and reproductive maize development, where Tassel-Less1 (TLS1) protein facilitates meristematic B transport, that seems to be fundamental for meristem fate and inflorescence development (Durbak et al., 2014).

Flowers are singular organs representing the last developmental phases and it is therefore not surprising that efforts should be placed in understanding flower development, an exciting, yet understudied field of plant biology. It has been demonstrated that the type II nodulin intrinsic protein 7;1 (NIP7;1), a facilitator of boric acid transport, is predominately expressed in young flower anthers in a narrow developmental window (Routray et al., 2018). Accordingly, *nip7;1* mutants show several defects in reproductive structures (Routray et al., 2018), indicating that B homeostasis control in both meristem and reproductive organs is critical for biological success of vascular plants. Thus, compelling evidence suggest that obtaining and maintaining an optimal B level in flower related tissues is often imperative for successful plant reproduction.

Since B has been described as an essential micronutrient (Warington, 1923), substantial advances to our understanding of B-related pathways has been achieved illustrating the essential role of B in plants. Nevertheless, Lewis (2019) argued against B essentiality for land plants by proposing an alternative discussion over the direct metabolic effect of B. Briefly, Lewis (2019) suggested that B is, and always has been, potentially toxic for plants and, perhaps more importantly, that this attribute must be fully avoided for normal growth, development and reproduction. This arguably assumption relies on the fact that not only B but also phenolics (compounds considered toxic for cellular metabolism) are interconnected. Thus, plants have evolved the ability to mitigate adverse effects of both B and phenolics by chemical (as organic complexes: *cis*-diols for B and lignin for phenolics) and physical (into vacuoles/apoplast) sequestration (Lewis 2019). Hence, B complexes formed in the cell wall are most likely a mechanism allowing

detoxification of such compound and cannot be presented as an evidence of B essentiality. Here we add further complication to this assumption given that our current knowledge concerning micronutrient gradient and distribution among distinct organs and tissues is rather limited requiring, therefore, significant advances in nutritional microscopic techniques (e.g. development of micronutrient specific sensors and nutritional living-microscopy). We posit here that the temporal control of micronutrient levels in different development phases could help us to unequivocally describe novel and specific roles for B. One can assume that even minimal changes in the levels of B in specific organs (e.g. meristem, anthers and flowers) has probably remained unnoticed in several studies and thus has led to the proposition of B as a not essential micronutrient. Although Lewis (2019) suggested that B is rather a toxic element with which plants have evolved to cope with the metabolic reprogramming suggested still must be unequivocally confirmed. One possible way would be to use mutant plants in the biosynthetic pathways of the free neutralizing agents (e.g. polyphenols) suggested and investigate their response to different B levels, in conjunction with precise quantification methods. In that case one must expect that such plants will either display toxicity effects at much higher B concentrations, without changing the levels of these compounds.

By contrast to the motion of Lewis (2019) we strongly believe that the gradually evolving function of B in the regulation of meristem fate discussed above highlight and put forward the potential role of this micronutrient to act at very low doses and during a very short development window. Therefore, we here postulate that B essentiality should not be discussed only in the context of both biomolecules constitution and from an unnoticed metabolic viewpoint, but also regarding their importance for specific cell-types around RAM and SAM. One can assume that B is likely able to induced cell proliferation and differentiation, triggering a proper development of vascular plants. Although our theoretical growth proposal is rather distinct from the theoretical metabolic mechanism postulated by Lewis (2019), we believe this inference should be considered when designing strategies to test B essentiality.

Concluding remarks

During recent year, the importance of B in plant growth and development has attracted much attention, most likely due to the fact that its specific and complementary functions are not fully yet understood. Yet, significant advances have underlined the essentiality of this element for land plants. Nevertheless, Lewis (2019) purposely not only challenged the essentiality of B in the conventional sense, but also suggests that it is toxic and as such cannot have a primary role. Although the biological significance of such statement remained elusive several exciting and testable research avenues were provided by Lewis (2019).

We provide here another alternative to unequivocally demonstrate the essentiality of B. Once B is absorbed by the roots it is preferably distributed to developing tissues such as meristems and reproductive organs. Although we cannot at the moment rule out the metabolic mechanism suggested before (Lewis, 2019), this differential B distribution provide, at least circumstantial evidence that highlighting the potential role of B in mediating plant development programs, by promoting the transition from vegetative to the reproductive phase, as well as enabling land plants to complete their life cycle. In order to accurately understand the role of B and thus convincingly prove its essentiality, we assume the importance of developing new diagnostic tools that allow the detection of minimal changes in B levels in different tissues and specific cells. The discussion highlighted both in Lewis (2019) and here should attract research from different but complementary field to investigate their hypotheses and add the analytical techniques into the “parts list” of B functions to test the application and relevance of their skills. Although the application of these techniques requires substantial financial investment, it is very likely to bring returns in the form of an improved mechanistic functions of B.

Neither Lewis (2019) nor us here have provided experimental evidence to unequivocally demonstrate B essentiality (or not). However, it clearly open up several research avenues that must be persuaded in the following years. Lewis (2019) expertly present a theoretical proposal for the metabolic mechanism by which B toxicity is not only overcome but also put to reasonably good purpose in particular circumstances. Future directions of research are also accurately presented in Lewis viewpoint with several and

341 independent ideas. We add complexity to this discussion by urging attention to the role
342 played by B in the regulation and control of axillary meristem fate. Understanding the
343 mechanisms behind accepted (and challenged) function of B may help to elucidate how
344 and which extent B is in fact important, and ultimately contribute to understanding its
345 biological function.
346
347 **References and recommended reading**
348 Papers of particular interest, published within the period of review, have been highlighted
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Physiological and metabolic changes in response to different levels of B: extending B functions in *Arabidopsis thaliana*

Greice Leal Pereira[1], *Jorge Alberto Condori Apfata*[1], *Adriano Nunes Nesi*[1] *and Wagner L. Araújo*[1*]

Departamento de Biologia Vegetal
Universidade Federal de Viçosa
36570-900, Viçosa, Minas Gerais, Brazil

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*To whom correspondence should be addressed.
Wagner L. Araújo
Email: wlaraujo@ufv.br
Tel: +55 31 3612-5358

Abstract

Although Boron (B) is an essential micronutrient in land plants, the impacts of B deficiency and toxicity, which is generally narrow, remains poorly understood. Both stresses (B deficiency and toxicity) are rather common and severely impact the agricultural production. To further investigate the physiological and metabolic mechanisms underlying B stress, *Arabidopsis thaliana* were fertilized with different B concentrations (0; 0.03; 30; 100; 1000 and 3000 µM H_3BO_3) in the vegetative phase. High levels of B caused a delay in the initial development as observed by reduced germination rates and root growth despite relatively minor differences in leaf area. Both B deficiency and toxicity severely impacted photosynthesis and respiration without affecting chlorophyll *a* fluorescence. High levels of B were associated with high levels of MDA, reduced chlorophyll and carotenoids content. Nitrate levels were highly influenced by the different B concentrations whereas protein and amino acids were not affected. Both deficiency and toxicity of B culminated with higher leaf levels of fumarate, malate and starch. Our results demonstrate that, despite the absence of growth modifications, physiological and metabolic changes in response to B fluctuations occur most likely to generate energy and sustain normal growth simultaneously reducing oxidative stress impacts.

Keywords: Boron, metabolic changes, plant growth, oxidative stress

Introduction

Boron (B) is an essential trace element with intermediate characteristics between metal and non-metal (Marschner, 2012), whose essentiality for plant growth and development was first described in 1923 by Warington. The functions of B in plants have been extensively studied (Kabata-Pendias, 2011), and include xylem development and lignin biosynthesis (Cakmak, 1995), the transportation and metabolism of sugars (Shelp, 1993), respiration and metabolism of Indole 3-acetic acid (IAA) (Graham and Webb, 1991), metabolism of RNA, phenolic compounds, nitrogen, ascorbate and plasma membrane integrity (González-Fontes et al., 2013). Thus, although a variety of physiological processes appear to be responsive to fluctuation in the levels of B levels, little or nothing is currently known about the impacts of manipulation of these levels on primary energy metabolism.

Suboptimal nutrient supply is a key factor regulating plant growth and development, and in the case of B, two conditions (deficiency and toxicity) are usually observed in arable areas that dramatically affect crop production. B deficiency is most commonly related to sandy soils, highly leached, with acidic pH and low organic matter content (Havlin et al., 2004; Yan et al., 2006). It is important to mention that deficiency symptoms, which depending on the age and species, include stunted root growth, restricted apical meristem growth, reduced chlorophyll content and photosynthetic activity, disruption in ion transport, increased phenolic and lignin contents, and ultimately reduced crop yield (Brown et al., 2002; Goldbach et al., 2001; Wang et al., 2015). B deficiency is more commonly observed than toxicity. Notably, B limitation usually causes effects on young parts of plants, especially in the apical meristem. In this context, the main symptoms usually observed are the reduction of root elongation (main and lateral root), reducing of leaf expansion and reproductive function including impacts on flowering, frui-set, seed production and final yield (Gupta, 1979; Dell and Huang, 1997; Goldbach et al., 2001; Voxeur et al., 2011; Abreu et al., 2014).

B toxicity also impacts crop yield and quality in different agricultural areas worldwide, and it frequently occurs in arid and semi-arid regions, coupled with a low rainfall and very scarce leaching, favoring the accumulation of this element in the

superficial layer of the soil, due to water evaporation, making B levels highly toxic to the plants (Tanaka and Fujiwara, 2007; Camacho-Cristóbal et al., 2008). The symptoms commonly observed in plants under B toxicity are associated with reduced growth, particularly of shoot, onset of chlorosis at the tips and margins of older leaves (Nable et al., 1997; Reid et al., 2004; Reid and Fitzpatrick, 2009). Such symptoms are, presumably, result of the accumulation of the transported B through the mass flow. It is also worth mentioning that, due to minimal redistribution of B in many species of plants, B tends to accumulate in old leaves (Marschner, 2012). Altogether, it seems highly plausible that the commonly observed symptoms in response to B deficiency or toxicity are likely related to biochemical, physiological, and anatomical changes.

As a consequence of the amplitude in the levels of B present in the soil solution, plants use different mechanisms to maintain B homeostasis in tissues (Ozhuner et al., 2013). Usually, this occurs at the root uptake stage. Thus, significant advances have been made in understanding the regulation of B transport in plants (Chatterjee et al., 2014; Hanaoka et al., 2014). Our understanding of the mechanisms associated with the regulation of B uptake has been greatly facilitated by the identification and characterization of distinct transporters involved in this process. In *Arabidopsis thaliana*, several B transporters have recently been identified and their function has been associated with an efficient uptake of B by the root, transporting it to the shoot, and the exclusion of B to the soil solution (Miwa and Fujiwara, 2011; Miwa et al., 2013). Notwithstanding, the effects promoted by either excess or deficiency of B remains poorly understood and particularly the metabolic impacts associated with these effects are virtually unknown. Although compelling evidence on the importance of B in plant growth and development is current available, a more detailed understanding of the mechanisms associated with B transport and usage by plants coupled with the responses of plants submitted to B toxicity or deficiency may provide practical information in plant breeding programs, thus assisting in the search for plants capable of tolerating low or high concentration of B.

It has been suggested that B plays a potential role on growth regulation mediated by extensive reprogramming of primary metabolism and that this modification resides

primarily in changes in carbohydrate and starch metabolism (Han et al., 2008, 2009). Therefore, the present proposal seeks to analyze the physiological and metabolic impacts caused by different concentrations of B in *A. thaliana*. Thus, we hypothesized that plants respond to fluctuations in B levels (deficiency or toxicity) in a relatively similar way, modifying their physiology and their central metabolism to cope with this stress. To investigate the physiological and metabolic mechanisms underlying response to B, *A. thaliana* were fertilized with different B concentrations (0; 0.03; 30; 100; 1000 and 3000 µM H_3BO_3) during the vegetative phase. By performing a detailed morphological, physiological, and biochemical characterization, novel aspects of the influence of B on plant growth and primary metabolism in leaves of *A. thaliana* are provided.

Material and Methods

Growth conditions and plant material

All experiments were carried out with *Arabidopsis thaliana* plants of the Columbia ecotype (Col-0) background. Seeds were surface-sterilized by incubation in 70 % (v/v) ethanol for 2 min. After, the ethanol was replaced by 2.5 % (v/v) sodium hypochlorite and shacked manually for 2 min and kept on room temperature 15 min. Subsequently, the seeds were washed five times by adding 1 ml sterilized water, inverting the tubes for 2 min and spinning the seeds down. After sterilization, seeds were distributed in sterile petri dishes containing modified MS culture medium (Murashige and Skoog, 1962) with different levels of B (0, 0.03, 30, 100, 1000 and 3000 µM B, applied as boric acid (H_3BO_3) and, supplemented with 1 % sucrose (m/v). Plates were further maintained at 4 °C for four days in the dark to promote and synchronize the germination. After 10 days seedlings were transferred to 0.08 dm^3 vessels containing sand (in order to isolate the factor of interest), since it is considered as an inert material. Prior to the installation of the experiment, sand was washed with 0.5 mol L^{-1} HCl solution and held in the solution for 24 h to remove residues of organic matter. Subsequently, it was washed three times with distilled water to remove excess of HCl, before the seedlings were transplanted.

Plants were cultivated in a short (8h/16h) day/night photoperiods with with 65 ± 10% relative humidity at 22 ºC and 150 µmol photons m^{-2} s^{-1}, corresponding to the

photosynthetically active irradiance. MiliQ water was applied daily to the pots to maintain the water supply. Once a week, each vase received a 5 mL of nutrient solution (Hoagland and Arnon, 1950), together with the solution of B in the respective treatments.

Root development and germination index

Seeds were surface sterilized as described above. Following, seeds were distributed in semi-solid medium, in sterile square plates in a vertical position (120 x 120 mm) containing different levels of B (0, 0.03, 30, 100, 1000 and 3000 µM B). The plates were placed in a growth chamber under 22 ºC, 8/16 h day-night photoperiod, 150 µmol photons m^{-2} s^{-1}, and 60-70 % relative humidity. Four days later the first evaluation was carried out, where the apex of the root was marked and the measurements occurred every 2 days for 14 days after germination. The root length was calculated using the ImageJ program (Kimura et al., 1999).

The same process was used to evaluate the germination index by using 20 seeds of distributed for an individual assay on Petri dishes (90 x 15 mm), containing the respective treatments. Germination was daily evaluated over ten days under the same growth conditions described above. Germination (%) was obtained by the daily verification of the accumulated number of germinated seeds starting on the first day post exposure in the light. Radicle emergence was used as the criterion of seed germination in each evaluated conditions. At the end of the experiment, the germination speed index (IVG) was calculated by the formula: IVG = 100 / N (Σn / j), where N corresponds to the number of seeds per treatment, j to the number of days after sowing number of seeds germinated on day j, according to A-As-Saqui and Corleto (1978).

Determination of gas exchange and chlorophyll *a* fluorescence parameters

Gas exchange parameters were determined using an infrared gas analyser (IRGA) system (LI-6400XT; LI-COR USA), adapted for the *A. thaliana* chamber. Instantaneous gas exchange data were obtained after steady-state was reached under growth light 150 µmol photons m^{-2} s^{-1}, 10 % of blue light, 400 µmol CO_2 mol^{-1} air of reference CO_2, leaf chamber block temperature of 25°C and leaf-to-air vapour pressure deficit of 1.5 to 2.5

kPa. The flow rates were kept from 300 mmol air min^{-1} to ensure maximum precision in the equipment following previous recommendations. The determination of the photosynthetic parameters was performed in 5-week-old plants. Then, photos of the plants were used for the calculation of the leaf area, and for the correction of the values of respiration and photosynthetic rate by area.

In vivo chlorophyll *a* fluorescence parameters were also estimated using dark adapted plants, using a portable chlorophyll meter Mod. CL-01 (Hansatech Instruments Ltd., King's Lynn Norfolk, UK). The maximum quantum yield of photosystem II (PSII) ($F_v/F_m=(F_m-F_o)/F_m$) was obtained following the F_m and F_o parameters which correspond to the maximum and minimum fluorescence of dark-adapted leaves, respectively.

Biometric parameters

Whole rosettes from 5-week-old plants were harvested and the leaf dry weight, total leaf area and specific leaf area were determined. Leaf area was measured by digital image method using a scanner (Hewlett-Packard Scanjet G2410) and the images were after processed using the ImageJ software, whilst specific leaf area was calculated using the classical approach described by Hunt et al. (2002) using dry weight per unit leaf area (mg cm^{-2}).

Determination of lipid peroxidation

Lipid peroxidation was indirectly determined by measuring the content of malondialdehyde (MDA) as described by Cakmak and Horst (1991) with modifications. Briefly, 0.5 g of leaves were homogenized in 750 µL of 1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 13.300 rpm for 15 min, collecting the supernatant. It repeated the process with the pellet, collecting the supernatant again. To 500 µL of the supernatant was added 750 µL of 0.5 % thiobarbituric acid (TBA) (made in 20 % TCA). The samples was then incubated at 95ºC, 30 min and 400 rpm. Absorbance of the supernatant was read at 532 and 600 nm. The content of the MDA was estimated using the extinction coefficient of 155 nM^{-1} cm^{-1}, and expressed as nmol g^{-1} FW.

Detection of ROS (O_2^- and H_2O_2) species

Qualitative evaluation of Reactive Oxygen Species (ROS) in leaves was performed by histochemical test for peroxide (H_2O_2) and superoxide(O_2^-) as previously described (Kong et al., 2011). Briefly, 10-days-old seedlings cultivated in presence of different B levels were harvested for *in situ* determination of the accumulation of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). For H_2O_2 detection, seedlings were treated with 3,3'-Diaminobenzidine (DAB), 1 mg ml^{-1} prepared in 200 mM Na_2HPO_4 solution, pH 3.8. For the $O^{2-\cdot}$ species it was used Nitrobluetetrazolium (NBT) 0.1 mg mL^{-1}, as previously described by Kong et al. (2011). For O_2^- detection, the leaves were immersed in NBT 0.1 mg ml^{-1} (prepared in 25 mM HEPES, pH 7.6).

After the exposure time with the aforementioned reagents, the removal of pigments was necessary, so the staining solution (*i.e.* DAB or NBT) was removed and a distaining solution (ethanol: acetic acid: glycerol - 3:1:1) was added to 50 mL falcon tube until all the samples were completely covered and further cooked in a water bath for 15 minutes at 90 ± 5°C. Next, the distaining solution was removed, and a new distaining solution was added allowing storage until samples were photographed. Photos were analyzed using ImageJ software.

Biochemical analyzes

Whole rosettes from 5-weeks-old plants were harvested in the middle of the day, immediately flash frozen in liquid nitrogen and stored at -80 ºC until further analysis. Metabolite extraction was performed by rapid grinding of tissue in liquid nitrogen an immediate addition of an ethanol series as described by Gibon et al. (2004). The levels of starch, sucrose, fructose, and glucose were determined exactly as described previously by Fernie et al. (2001). Photosynthetic pigments were determined exactly as described by Porra et al. (1989). Protein and total amino acids levels were determined as described previously (Bradford**,** 1976; Cross et al., 2006). Malate and fumarate were determined exactly as detailed by Nunes-Nesi et al. (2007).

Determination of boron concentration

Whole rosettes samples were collected and dried at 65 °C until constant weight was reached. The samples were submitted to dry digestion (incineration) and the content of B was quantified by using the azomethine-H method (Tedesco et al., 1995).

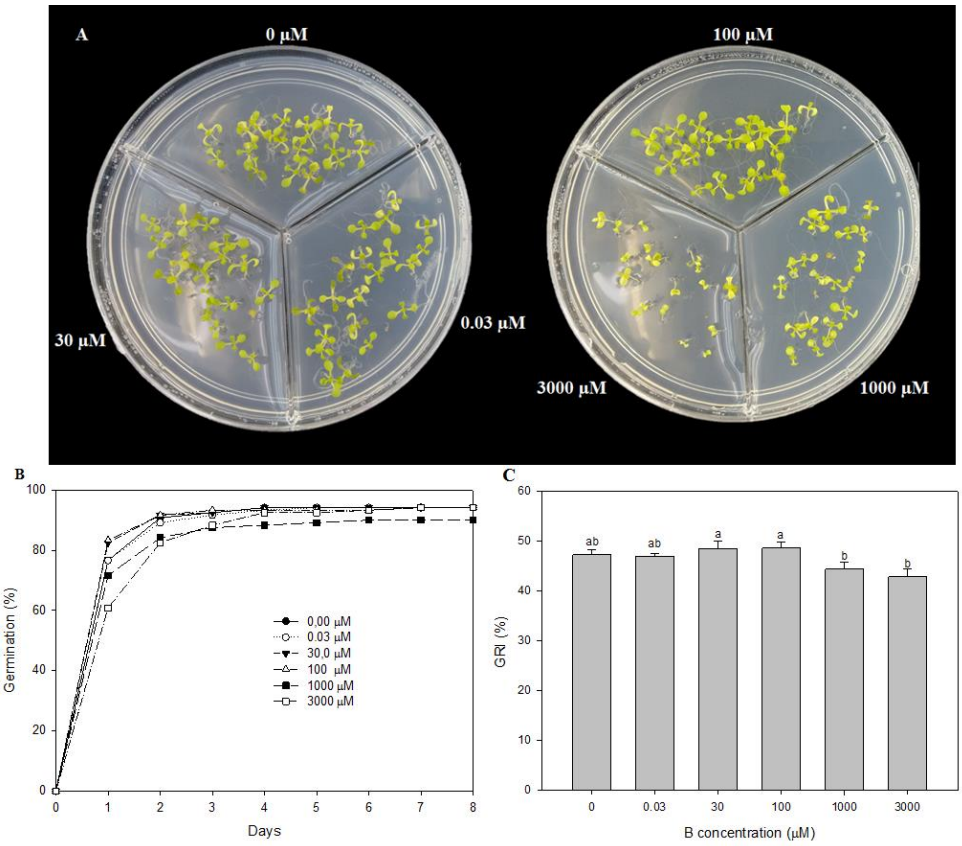
Experimental design and statistical analysis

The data were obtained from experiment conducted in a randomized block design with at least six replicates and six levels of B (0, 0.03, 30, 100, 1000 and 3000 µM B). The levels (30 and 100 µM of B) are those recommended as optimal for the cultivation of *Arabidopsis thaliana* (Takano et al., 2001). Additionally, the complete setup was repeated at least three times with similar phenotypes observed each time. Data was expressed as means ± standard error (SE). The results were submitted to analysis of variance (*F* test) after ANOVA assumptions (additivity, independence and normality of the residues, and homoscedasticity of the variance). In the absence of restrictions, the data was submitted to analysis of variance ($p<0.05$) and comparison of means by the Tukey test *(*$p<0.05$) and Pearson's linear correlation analysis using the SAS statistical program (SAS, 2007).

Results

Root development and germination index are altered by nutrition

Elevated B concentrations caused a decrease in the germination (**Fig. 1A**). At the beginning of the treatments, the germination at the level of 3000 µM was even lower than in the optimal levels (30 and 100 µM). However, at the end of the experiment, the germination was the same as in other treatments (**Fig. 1B**). We also observed reductions in the germination velocity index (GVI) at higher B concentrations (**Fig. 1C**).



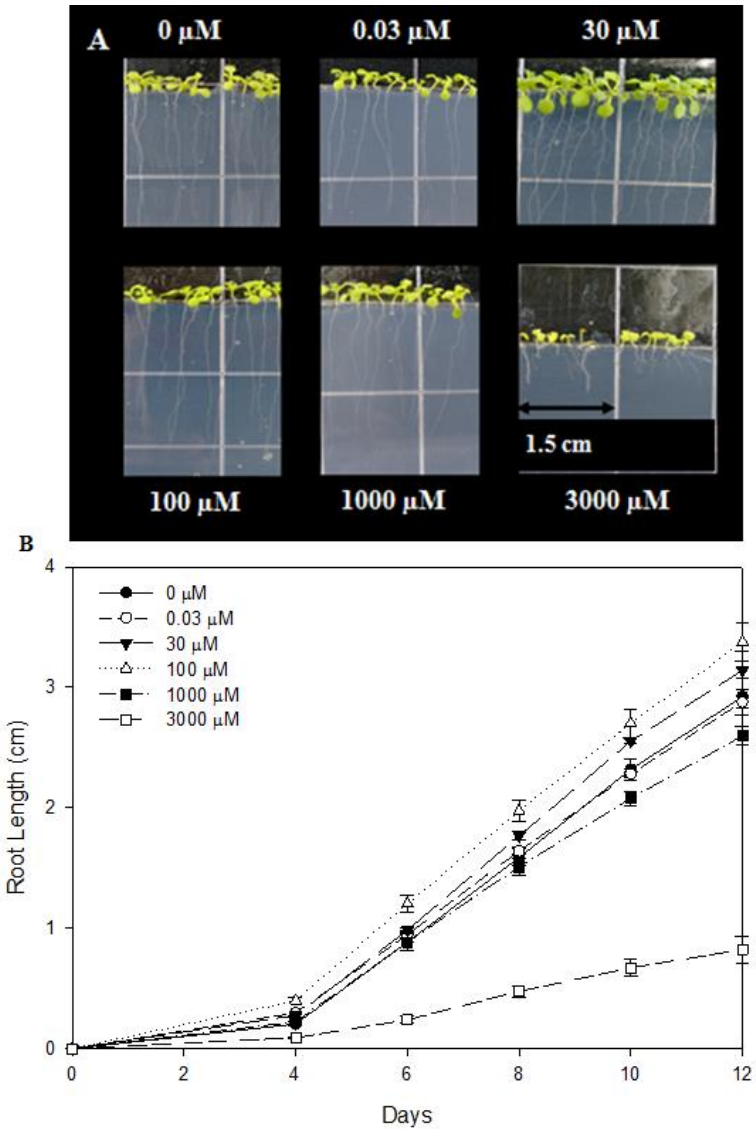
255

Figure 1. Germination is affected by different concentration of B. (**A**) Representative image showing the germination in response to different B concentration (0, 0.03, 30, 100, 1000 and 3000 µM B) in *Arabidopis thaliana* wild-type (WT) seedlings; (**B**) Germination rate, (**C**) Germination rate index. Bars represent means ± SE. Means were compared by Tukey test (p<0.05).

256

257 **B toxicity levels impact root growth**

258 The seedlings under optimal B levels were characterized by well-developed root
259 length, with lateral roots while high levels of B culminated with root inhibition coupled with
260 a yellowish coloration on the leaves (**Fig. 2A**). Root lengths also decreased significantly
261 with the increase in B concentration (**Fig. 2A**), and culminated with a negative correlation
262 between these variables (**Table S1**).



263

Figure 2. **Root growth is affected by different B concentration.** Changes in root growth in response to different B concentration (0, 0.03, 30, 100, 1000 and 3000 µM B) in *Arabidopis thaliana* wild-type (WT) seedlings were monitored over 12 days; (**A**) Representative images of the root phenotype observed in response to different concentration of B (**B**) Root length was determined as daily elongation in different treatments. Bars represent means ± SE. Means were compared by Tukey test (p<0.05).

264

265 **Reduced growth in response to excess B**

266 We next investigated whether the vegetative plant growth is affected by different B
267 concentrations. To this end, *Arabidopis thaliana* plants were cultivated for over 5 week in
268 presence of different B levels. No usual symptoms of either B deficiency or toxicity were

observed (**Fig. 3A**), however, toxic (high) levels of B culminated with significant decrease in leaf area (**Fig. 3A, 3B**) despite no differences in specific leaf area (**Fig. 3C**).

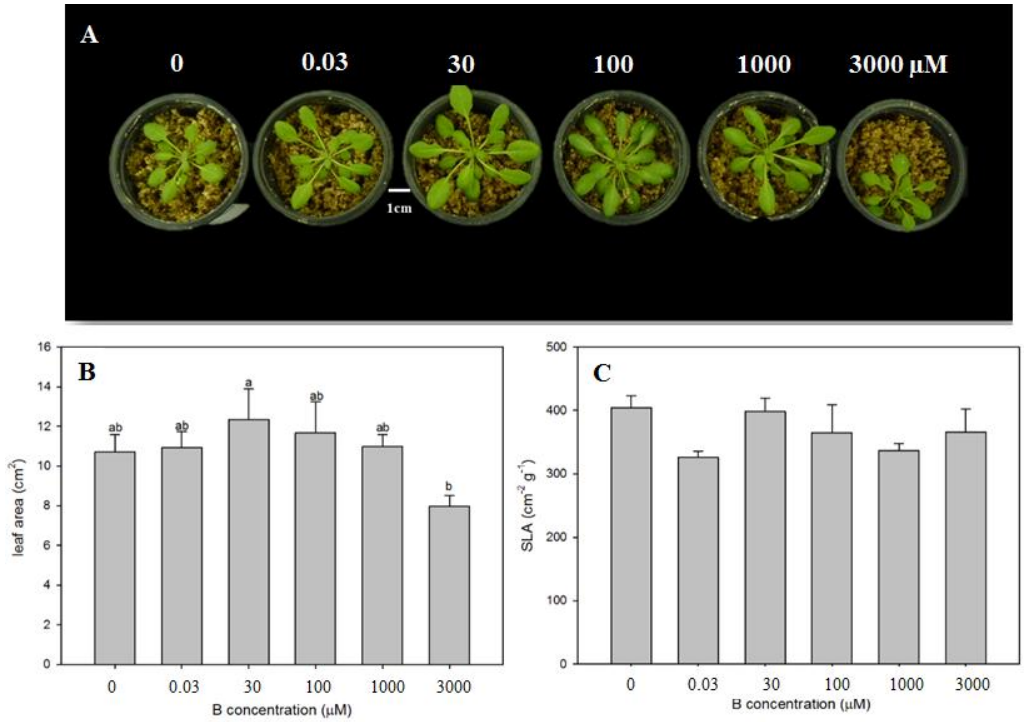


Figure 3. Effects of stress on leaf area growth of *Arabidopis* plants. (A) WT phenotype over concentrations other than B; **(B)** Leaf area; **(C)** Specific leaf area. Bars represent means ± SE. Means were compared by Tukey test (p<0.05).

B toxicity decreased gas exchange without impacting overall plant growth

Regardless of the deficiency or toxicity of B, decreased CO_2 assimilation rate was observed when compared with optimal levels of B (**Fig. 4A**). Moreover, high levels of B also lead to increased dark respiration rate in comparison with the adequate levels of B (**Fig. 4B**). Although no correlation between B doses and CO_2 assimilations rates were observed, a positive and significant correlation was observed between B and dark respiration showing that higher is the B levels higher is the dark respiration (**Table S1**). No changes in the maximum photosynthetic efficiency (F_v/F_m) were observed in response to different B concentrations (**Fig. S1**).

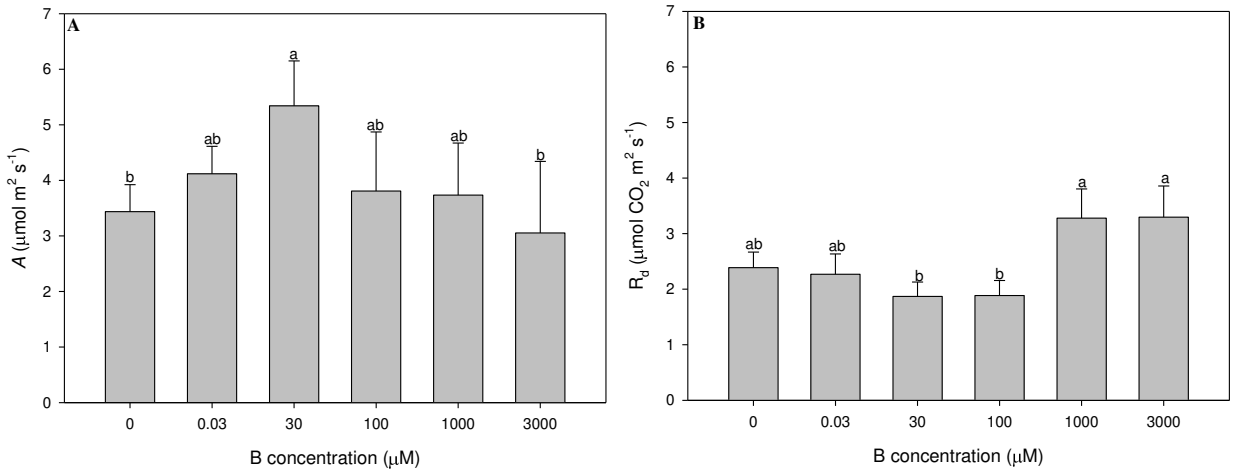


Figure 4. Effect of the B concentration on the gas exchange and fluorescence parameters. (**A**) Net CO_2 assimilation rate, (**B**) Respiration in darkness. Bars represent means ± SE. Means were compared by Tukey test (p<0.05).

Homeostasis in the accumulation of B in leaves

The concentration of B was analyzed in whole rosette of *Arabidopsis* plants cultivated for over 5 weeks in presence of different B concentration. Our results clearly demonstrate that plant grown at B levels equal to or greater than 30 μM do not differ in B accumulation (**Fig. 5**).

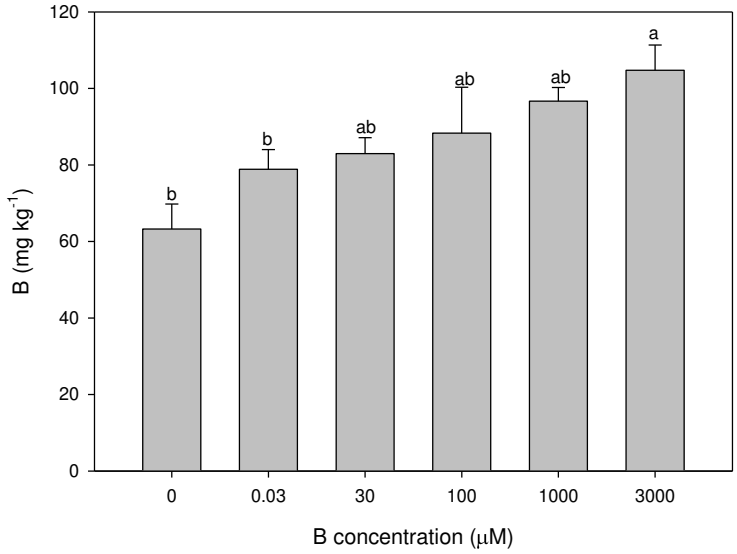
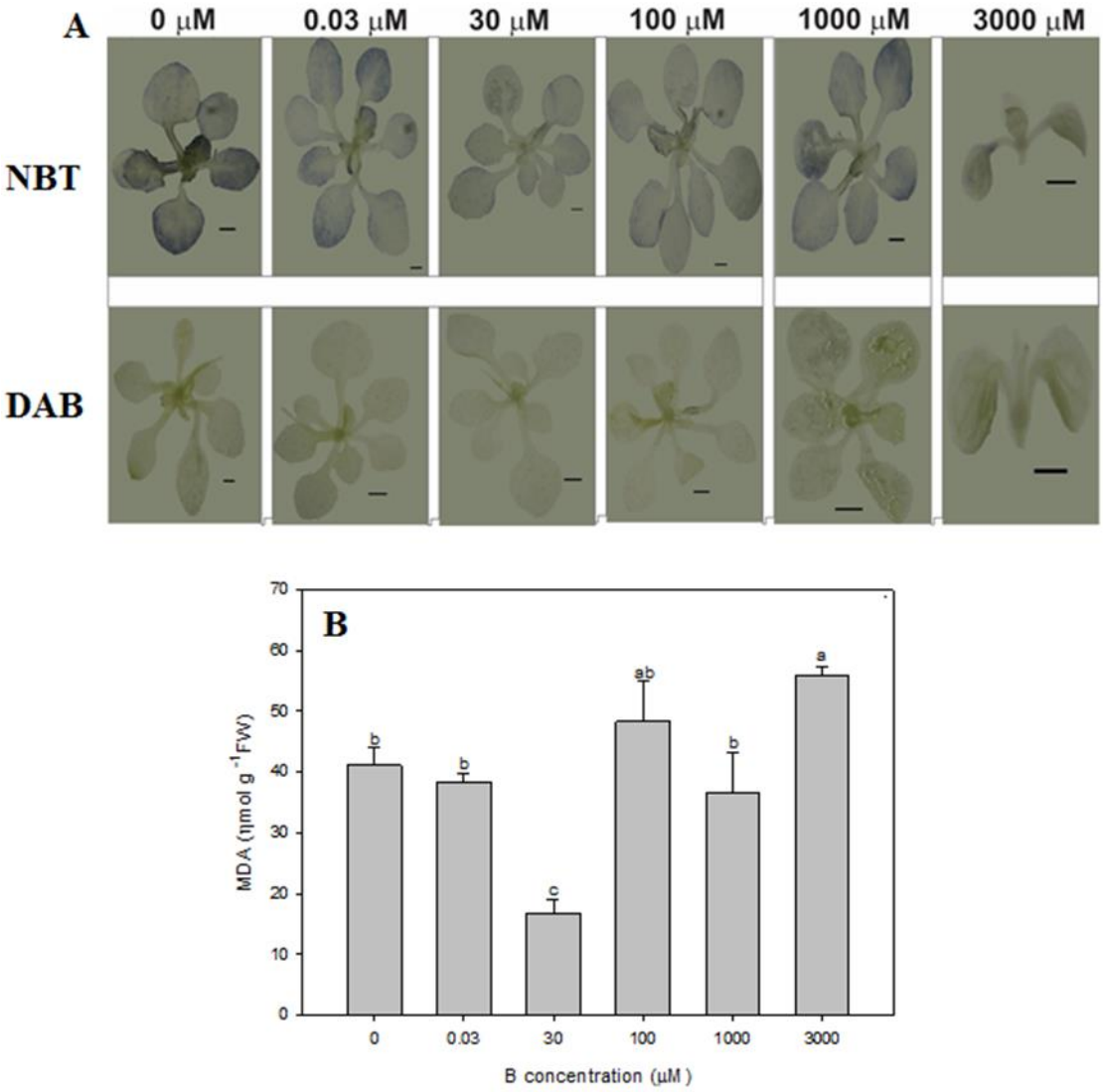


Figure 5. The B concentration in rosettes in response to the levels of B Bars represent means ± SE. Means were compared by Tukey test (p<0.05).

ROS changes and membrane damage in response to B stress

To further evaluate the structural integrity of the membranes and to analyze the degree of oxidative stress that occurs in response to either deficiency or excess of B, the concentrations of MDA as well as the presence of H_2O_2 and $O_2^{\cdot}$ were analyzed . It is important to mention that the levels of MDA significantly increased under both B deficiency and toxicity yet the greatest changes occurred followed toxic levels of B (over 3 x-fold), compared to the optimal condition (30 µM) (**Fig. 6B**). By performing histochemical staining, we could observed that both deficiency and toxicity increased the intensity of the blue ($O_2^{\cdot}$) and brown (H_2O_2) staining in the leaves compared to the optimal conditions (30 and 100 µM) (**Fig. 6A**). Furthermore, both deficiency and toxicity led to oxidative damages, reflecting an increase in membrane lipid peroxidation, through MDA levels (**Fig. 6B**). Remarkably, higher accumulation of H_2O_2 and especially $O_2^{\cdot}$ can be observed on the margins of leaves treated under such conditions, deficiency and toxicity, as indicated by the intensity of the staining (**Fig. 6A**). In summary, the results obtained here demonstrated that despite the relatively minor changes in growth it is highly likely that the plants suffered from oxidative stress in response to low or high levels of B.



306

Figure 6. Effects of stress on accumulation of ROS levels. (A) O_2^- and H_2O_2 in rosettes; (B) Malondialdeyde (MDA).Bars represent means ± SE. Means were compared by Tukey test ($p<0.05$).

307

308 **The relationship between changes carbon and nitrogen metabolism in response the**
309 **B stress**

310 Similar patterns of reductions for photosynthetic pigments namely Chlorophyll *a*,
311 Total Chlorophyll, Chlorophyll *a/b* and carotenoid (**Fig. 7A**, **7C**, **7D** and **7E,** respectively),
312 were observed in responses to high levels of B. In addition, reduced contents of
313 Chlorophyll *b* (**Fig. 7B**) under conditions of deficiency (0.03 μM) were observed.

Regardless of B levels, no differences in the levels of protein and amino acids (**Fig. 8A** and **8B,** respectively) were observed. By contrast, B levels affected the accumulation of nitrate, thus the dose of 0.03 µM culminated with lower nitrate levels, while the highest levels of this nutrient were verified at 30 µM and 3000 µM (**Fig 8C**). At 30 µM of B the lower amount levels of malate and fumarate were observed (**Fig. 8D** and **7E**, respectively), whilst B deficiency caused an increased fumarate concentration.

Starch content was significantly lower at 30 µM B (**Fig. 9A**), while the deficiency (0.03 µM) resulted in starch accumulation. Regarding sugars, we did not observed any difference in the levels of sucrose independently of B levels. Surprisingly, the highest levels of fructose were observed in the absence of B, while the lowest glucose levels were obtained at the higher B concentration (30 µM) (**Fig. 9B**).

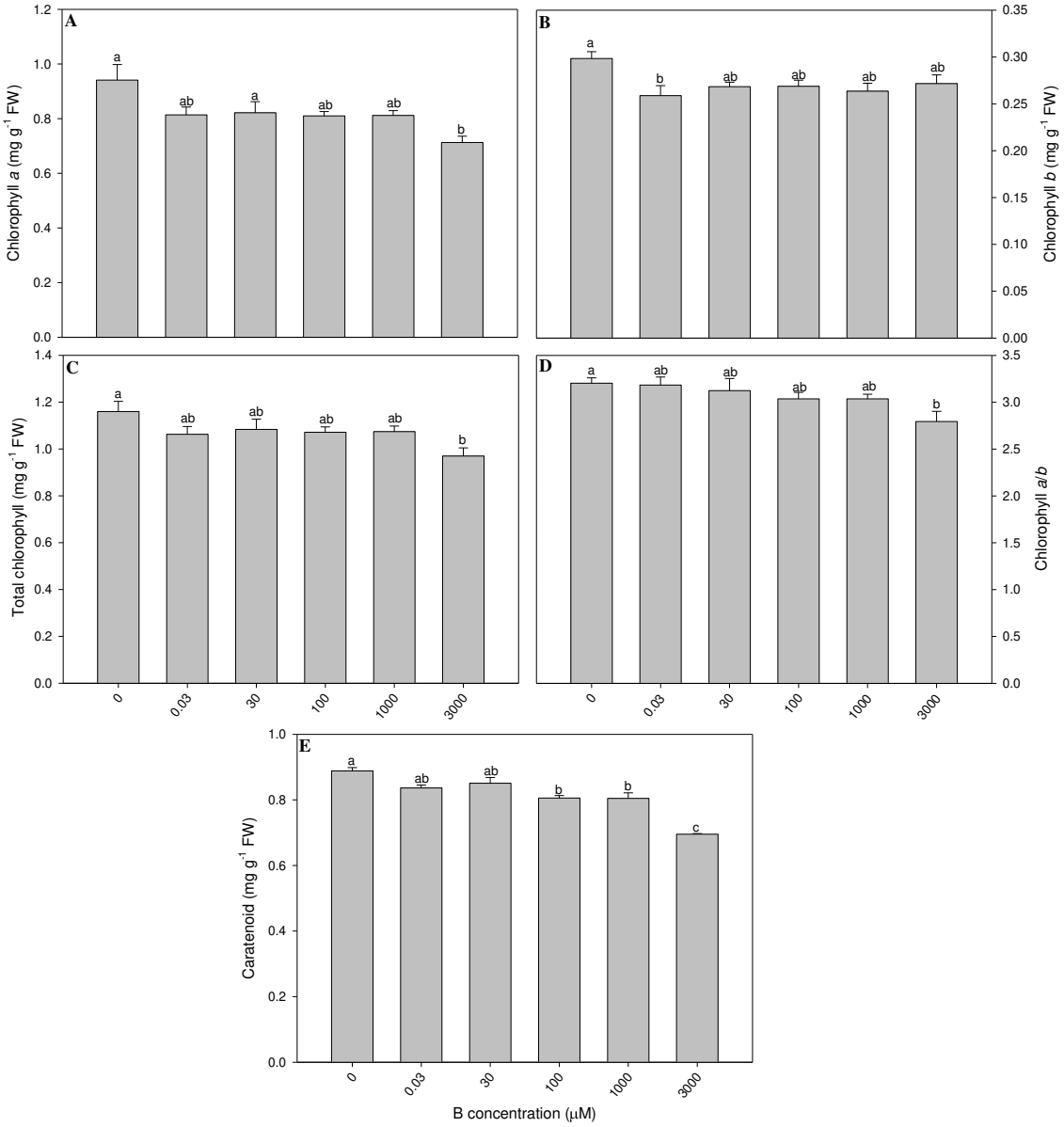


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Figure 7. Photosynthetic pigments in *Arabidopsis thaliana* leaves. (**A**) Chlorophyll *a*, (**B**) Chlorophyll *b*, (**C**) Total Chlorophyll, (**D**) Chlorophyll *a*/*b*, (**E**) Carotenoids. Bars represent means ± SE. Means were compared by Tukey test (p<0.05). FW: fresh weight.

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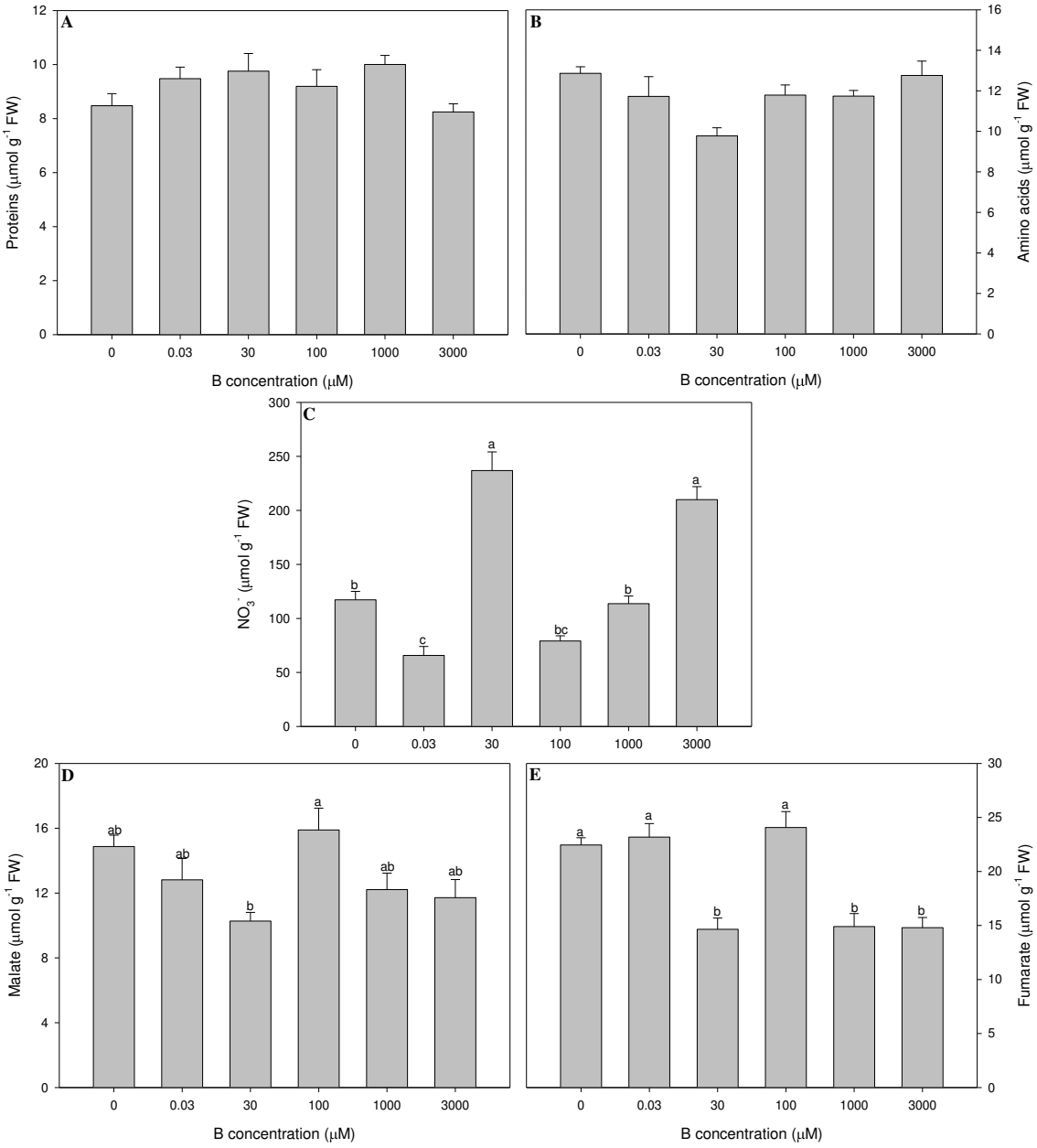

327

Figure 8. Changes in the key leaf metabolites content in response to B concentrations. (**A**) Proteins, (**B**) Amino acids, (**C**) Nitrate, (**D**) Malate, (**E**) Fumarate. Sample were taken from mature source leaves. Bars represent means ± SE. Means were compared by Tukey test (p<0.05). FW: fresh weight.

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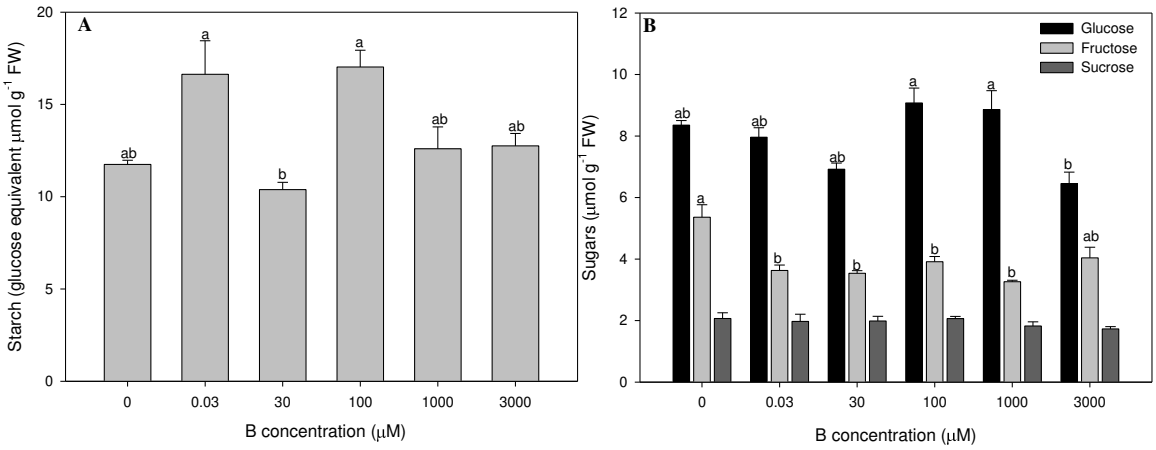

Figure 9. Changes in the key metabolites content in leaves of B concentrations. (**A**) Starch, (**B**) Soluble sugars. Bars represent means ± SE. Means were compared by Tukey test (p<0.05). FW: fresh weight.

Discussion

Here, we investigated the changes in seed germination, accumulation of B, growth, and metabolism in leaves of *A. thaliana* under either B toxicity or deficiency. Our results suggest that excessive B supply significantly influenced not only the antioxidant response but also primary metabolism. Our findings additionally provide novel insights into the mechanisms of different B-toxic symptoms that are most likely mediated by the differential regulation of B transporters. The accumulation of B clearly demonstrates the importance of specific transporters in B homeostasis, since under B deficiency and excess the accumulated levels of B increased and decreased, respectively (**Fig. 5**). In consonance with previous results (Miwa et al., 2010, 2013; Sutton et al., 2007; Schnurbusch et al., 2010; Yoshinari and Takano, 2017), it appears that B transporters are efficiently expressed in response to extreme B availability conditions in order to maintain levels close to that suitable for cell maintenance and consequently plant growth (**Fig. 3**).

Our results also demonstrated that the initial growth and establishment of *A thaliana* were affected exclusively at high concentrations of B (**Fig. 2** and **3**), with relatively minor impacts under B deficient conditions. In fact, high concentrations of B inhibited germination while low concentrations stimulated the initial germination (**Fig. 1**). Toxic B concentration are usually associated with different physiological effects during the life cycle of vascular plants, and thus the consistent decrease in percentage and rate of seed

germination beyond 3000 µM in the present study is in line with several independent findings (Yau and Saxena,1997; Metwally et al., 2012 and Muhammad et al., 2013). Overall, our results indicated that B deficiency is not able to affect the initial vegetative development of *A. thaliana*, given that it did not promote damage or inhibit vegetative growth. Most likely the B stored in *A. thaliana* seeds is most likely capable to sustain adequate growth of the roots and seedlings under deficiency of B. Further studies should investigate whether the translocation of B from the seeds to the aerial part is required during initial growth.

Roots are the first to undergo signs of deficiency, developing corresponding symptoms. These symptoms mainly include inhibition of root tip splitting and elongation (Dell and Huang, 1997) and suppression of growth of the lateral root and main root, which results in root branching or even necrosis (Martín-Rejano et al., 2011). Although such signs were not investigated in our study, we observed that excessive B supply inhibited seed germination and seedling growth. In rice, this phenotype has been associated with an increased ROS production (Wang et al., 2016), similarly to what was observed here (**Fig. 6**). Notwithstanding, these authors also demonstrated that hydrogen gas (H_2) is able to mitigate B toxicity in rice seedlings most likely the maintenance of an adequate ROS balance and water status. Although we cannot provide such mechanistic explanation at this moment we cannot rule out that *A. thaliana* seedlings are able to increase overall ROS production in response to fluctuations in the levels of B and that this is likely enough to sustain initial growth.

In line with our results, the strong impact of excess B on growth has been demonstrated in several species such as tomato (Cervilla et al., 2009), wheat (Turan et al., 2009) and grapevine (Gunes et al., 2006). Collectively, it also indicates that since the root system is in direct contact with the soil solution it is responsible for the uptake process. Therefore, the major phenotypic effect of toxicity B is associated with inhibition of root growth (Reid et al., 2004; Turan et al., 2009). Work has shown that B toxicity causes abnormal cell division in the root meristem, as well as hypodermis formation, along with progressive suberin deposition in the cortical cell wall of soybean roots (Ghanati et al., 2005). Leaving aside this fact, lignification was not considered an essential factor for B-

induced root growth inhibition in tomato (Cervilla et al., 2009). In addition, Reid et al. (2004) reported a localized inhibitory response to high B concentration at the root tips of wheat but not at the mature root zones. By using a mathematical model, Shimotohno et al. (2015) predicted that the highest B concentration is near the tip and the lowest in the proximal region of the meristem zone. In addition, this model predicted that root-tip absorbed B was not efficiently translocated into shoots, suggesting that root-tip absorption of B was probably used for local root growth, while more mature roots were responsible for transportation to the shoot (Shimotohno et al., 2015). In this line of study, it is necessary to explore in more detail the impact of B at root level and a possible relationship of this nutrient in the change of "phase" of the root system, modulating the development of roots as a way to tolerate the stress condition.

B toxicity has been associated with impacts in several plant developmental processes (Reid et al., 2007; Guo et al., 2014). Our work further revealed that B toxicity leads to a significant and negative effects on gas exchange. Reductions in CO_2 assimilation by excess B, as observed here (**Fig. 4**) are probably caused by a combination of factors such as oxidative damage (ROS production) (Han et al., 2009). Although it has been reported that B toxicity induces alterations in photosynthesis (Sheng et al., 2010; Ruuhola et al., 2011; Chen et al., 2012; Landi et al., 2013), photosynthesis was not particularly sensitive to B excess on barley leaves as it can be deduced by the lack of impacts at 50 mM of B coupled with the relatively low inhibition (23 %) in a concentration of 100 mM of B (Reid et al., 2004). Our results aid more complexity to this response since, despite the lower photosynthesis observed at excess B supply, increased dark respiration (**Fig. 4**) coupled with increased overall ROS (**Fig. 6**) yet no changes in final growth (**Fig. 3**) were observed. Since leaf area is correlated with the photosynthetic rates, we also investigated it and noticed that excess B reduce the rate of CO_2 assimilation and consequently impacted the rate of leaf area expansion (**Fig. 3** and **4**). Due to the low requirement of B, high doses may reduce the development of the plant, and thus, decrease the leaf area. In addition, the different levels of B did not promoted changes in the specific leaf area (**Fig. 3**). Thus, it seems reasonable to assume that the absence of changes in leaf density and leaf thickness in response to different doses of B might be an

410 important response to sustain photosynthetic rates, particularly following low levels of B.
411 Future analysis of the interplay of leaf anatomy with B will be clearly required to uncover
412 the regulatory mechanism of B response. Further efforts to develop cultivars having high
413 B usage flexibility should aid in the generation of high-yield performance crops under
414 different B conditions.

415 Our results do not indicate a close relationship between photosynthesis and B
416 deficiency, implying that plants growing under this condition had to cope with low levels
417 of B by adjusting their photosynthetic machinery at the level of photosynthesis since no
418 photochemical limitation was observed (**Fig. S1**). B deficiency has been associated with
419 an indirect reduction of photosynthesis via changes in biochemical and physiological
420 processes (Broadley et al., 2012; Bogiani et al., 2013; Mukhopadhyay et al, 2013).
421 Notably, our results revealed that both stresses, deficiency and toxicity, cannot be
422 associated with photoinhibitory effects on PSII since no changes in F_v/F_m values in
423 fluctuations in B levels were observed.

424 Stress caused by both deficiency and excessive nutrient supply promotes changes
425 in the plants oxidizing system, inducing lipid peroxidation and the overproduction of ROS
426 such as $O_2^{\cdot}$ and H_2O_2. As observed here, plants exposed to B deficiency showed
427 increased MDA content that results in oxidative stress and membrane peroxidation
428 (Tewari, 2004; Ardic et al., 2009; Cervilla et al., 2009). One of the most used analyzes to
429 measure the damage caused by increased ROS production is lipid peroxidation (Erdal
430 and Demirtas, 2010) and MDA is one of the best biomarkers for this purpose (Erdal and
431 Demirtas, 2010). B toxicity led to the formation of ROS (**Fig. 6**), which can damage the
432 metabolism, modify the membrane, and ultimately cause cell death in plants (Mitter,
433 2002). Histological analysis of ROS via NBT and DAB revealed that adequate levels of B
434 maintained less ROS, indicating a reduced potential for oxidative damage resulting from
435 B homeostasis. On the other hand, toxicity and deficiency generated greater O_2 reaction
436 and H_2O_2. Results described by Pandey and Archana (2013) and Siddiqui et al. (2013),
437 indicate that exposure to deficiency and to excess B resulted in ROS accumulation. It
438 should be noted that this accumulation together with higher levels of MDA may be not only

detrimental to membranes in general but also impact activities of antioxidant enzymes in plants under extreme B conditions (Pandey and Archana, 2013).

High intracellular concentrations of B negatively affect chlorophyll content, rates of photosynthesis and cell growth (Miwa and Fujiwara, 2011). To cope with this situation, plants must to acclimate to stress, especially by modifying their metabolism. Thus, plants in B-toxicity condition have visible symptoms such as leaf chlorosis, similar to what we observed in this work (**Fig. 2A**) This response is explained by the degradation or inhibition of chlorophyll synthesis resulting from the formation of ROS (Papadakis et al., 2004). Paparnakis et al. (2013) reported a decrease in chlorophyll content in plants submitted to excess B, but observed that the chlorophyll *a/b* ratio was higher than in control conditions. Here we observed that the absence of B increased both chlorophyll and carotenoid concentrations. Given that carotenoids contribute to the mechanism of protection of chlorophyll in plants, an increased carotenoid content are generally related to an enhancement of tolerance to oxidative stress (Valladares et al., 2003).

Plants require large amounts of nitrogen (N) for the biosynthesis of amino acids, nucleic acids, proteins and secondary metabolites. Accordingly, adequate levels of B have been associated with positive effects on N uptake and metabolism (Lopez-Lefebre et al., 2002), whereas deficiency and toxicity of B affect plant N metabolism. Our results demonstrated that B deficiency conditions reduced the nitrate content, which may be a consequence of increased nitrate reductase activity and/or decreased nitrate uptake by the roots. Camacho-Cristóbal et al. (2008) found that B deficiency decreased nitrate content in tobacco, most likely associated with the lower rate of net nitrate uptake due to the reduction of levels of root plasma membrane H^+ATPase (PMA2) transcript. In contrast, excess B alters N metabolism, causing nitrate accumulation in leaves (**Fig. 8C**), possibly by altering N-cycle activity. Moreover, Cervilla et al. (2009) found that glutamine synthetase, glutamate synthetase and glutamate dehydrogenase activity increased in tomato leaves under B toxicity, while a decrease in nitrate reductase and nitrite reductase activity was observed. Furthermore, nitrate reductase activity tended to decrease (15-17 %) after B toxicity in the root and leaf tissues of barley cultivars (Mahboobi et al., 2002).

The concentration of proteins and amino acids remained stable regardless of the concentrations of B. Several authors have reported similar results in different species such as tobacco, tomato and tea (Camacho-Cristóbal and González-Fontes, 2007; Matas et al., 2009; Hajiboland et al., 2011). During nutrient limitation, amino acids are recycled and allocated for the synthesis of specific proteins required by plants, when these proteins are degraded concomitantly the oxidation of amino acids causing the energy production necessary to meet the need of plants. In addition, the role of amino acids during signaling in plants can provide an energetic connection enabling plants to cope with stress conditions. Thus, we verified an efficient regulation of amino acid synthesis and catabolism, keeping the amino acid and protein levels similar to the 30 µM optimal condition, being important in the N status and energy generation, considering that the defense expenses of plants is too high.

Changes in carbon (C) metabolism are common in plant responses to various environmental stresses in order to maintain plant growth. Deficiency and excess B influence carbon allocation, therefore our results show that *A. thaliana* grown under low B accumulates more starch without changing sucrose accumulation, possibly inducing lower assimilation of CO_2 by a negative feedback process (Paul and Foyer, 2001; Zhao and Oosterhuis, 2002; Camacho-Cristóbal et al., 2004; Fuentes et al., 2011; Burnett et al., 2016; White et al., 2016). In fact, several studies have demonstrated that B had a variable effect on sugar biosynthesis, including sucrose. In our current study, sugar concentrations were higher in B deficient plants. The content of the sugars was higher (glucose and fructose), whereas the non-reducing sugar content (sucrose) did not change. Therefore, we provide circumstantial evidence that the difference in photosynthesis under conditions of low B supply is likely mediated by significant changes in primary metabolism. Further evidence for this assumption comes also from the crucial role mitochondrial respiration plays in maintaining the optimal rate of photosynthesis (Araújo et al., 2014; Nunes-Nesi et al., 2008), indicating that plants optimize carbon and nutrient allocation to maximize photosynthesis and growth (Plaxton and Podesta, 2006; González-Meler et al., 2009). In this work, B toxicity culminated with an enhanced dark respiration, possibly associated

with the provision of energy to maintain metabolic processes, especially given the low photosynthetic rate.

Conclusions

The results presented here underscore the importance and/or implication of fluctuations of B levels in physiological modifications and reprogramming of primary energy metabolism. Initial growth and plant establishment were affected exclusively in B toxicity, while deficiency was not able to affect early development. However, both conditions modified both physiology and metabolism and as such extreme levels of B reduced photosynthesis and increased oxidative damage despite no changes in plant growth. We additionally obtained evidence that the regulation of photosynthesis under conditions of either B deficiency or toxicity cannot be associated with photochemical limitations but are likely rather mediated by a feedback regulation coupled with an efficient energy provision via amino acid metabolism and respiration, which ensures the maintenance of plant growth. Therefore, to the detriment of the contrasting levels of B (deficiency and toxicity), proper fertilization management (via soil and leaf) and/or the use of more tolerant species are of utmost importance in order to improve the productive capacity and quality of the plants.

Supplemental Data

The following supplemental materials are available

Supplemental Figure S1. Effect of the B concentration on the fluorescence parameters. The maximum quantum yield of PSII chemistry.

Supplemental Table S1 Intensity and direction of the linear relationship between doses of B and variable responses.

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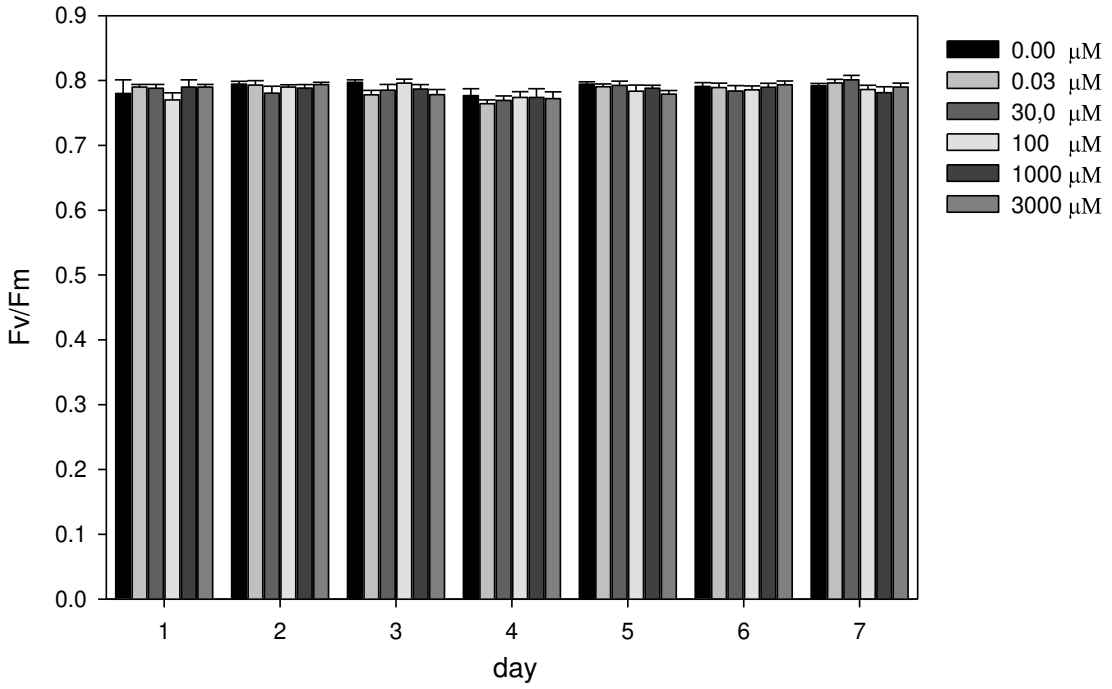
Supplemental Data



Supplementary figure 1: **Effect of the B concentration on the fluorescence parameters. The maximum quantum yield of PSII chemistry.** Evaluations were performed daily, starting measurements at 40º days after germination. Bars represent means ± SE. Means were compared by Tukey test ($p<0.05$).

Supplementary table 1: Intensity and direction of the linear relationship between doses of B and variable responses. Pearson's linear correlation coefficients and level of significance between the doses of B applied and the variables analyzed

Variables	Linear equation	Correlation coefficient	*p*-value
B * Germination	y=94.48-0.0039x	-0.32	0.051
B * Root length	y=3.14-0.00075x	-0.94	<0.0001
B * Photosynthesis	y=4.18-0.00038x	-0.2	0.2418
B * Dark respiration	y=2.17+0.00032x	0.42	0.01
Photosynthesis * Dark respiration	y=4.97-0.44x	-0.17	0.309
B * Leaf area	y=11.4-0.00041x	-0.14	0.821
B * SLA	y=372-0.0002x	-0.003	0.988
B * Chlorophyll *a*	y=0.85-0.000055x	-0.61	<0.0001
B * Chlorophyll *b*	y=0.27-0.0000029x	-0.14	0.42
B * Total chorophyll	y=1.12-0.000058x	-0.59	0.0002
B * Chorophyll *a/b*	y=3.17-0.000165x	-0.51	0.0014
B * Caratenoid	y=0.85-0.000052x	-0.87	<0.0001
B * Protein	y=9.39-0.00028x	-0.25	0.24
B * Amino acid	y=11.5+0.0004x	0.27	0.19
B * Nitrate	y=118.65+0.027x	0.43	0.045
B * Malate	y=13.37-0.0005	-0.22	0.3
B * Fumarate	y=20.58-0.0023x	-0.51	0.011
B * Starch	y=13.81-0.00041x	-0.14	0.52
B * Glucose	y=8.27-0.00048x	-0.42	0.043
B * Fructose	y=4-0.000065x	-0.08	0.7
B * Sucrose	y=2.011-0.0001x	-0.55	0.006

Article type: Research article

Physiological and metabolic changes in response to Boron levels are mediated by ethylene in tomato plants

Greice Leal Pereira[1], William Batista da Silva[1], Dimas Mendes Ribeiro[1], Adriano Nunes-Nesi[1] and Wagner L. Araújo[1*]

[1]Departamento de Biologia Vegetal, Universidade Federal de Viçosa, 36570-900 Viçosa, Minas Gerais, Brazil

Running title: Differential responses to Boron levels

***Corresponding author**
Wagner L. Araújo
Departamento de Biologia Vegetal,
Universidade Federal de Viçosa,
36570-900 Viçosa, Minas Gerais, Brazil
E-mail: wlaraujo@ufv.br
Phone: +55 31 3612-5358

ABSTRACT

Boron (B) is an essential microelement for plant growth and development, but its deficiency and toxicity are major problems that severely affect agricultural production. Plants may respond differently to B availability through ethylene signaling. To better understand the connections between ethylene and B here, we investigate the underlying growth function of ethylene and the physiological and metabolic responses to B deficiency and excess in tomato (*Solanum lycopersicum*) plants using ethylene mutants namely *nor* (*non-ripening*), *rin* (*ripening inhibitor*), *Nr* (*never ripe*) and *Epi* (*epinastic*). Our results show that B deficiency and excess do not inhibit plant growth, but both B deficiency and toxicity severely affected photosynthesis, stomatal conductance and chlorophyll *a* fluorescence. Under excess B, visible symptoms of toxicity appeared in the roots and margins of the older leaves through necrosis, caused by the accumulation of B which stimulated ethylene biosynthesis in the shoot. Both *nor* and *rin* mutants presented similar responses, being more sensitive as observed by physiological and metabolic alterations in response to toxic conditions. Our results suggest that physiological and metabolic changes in response to B fluctuations are at least partially mediated by ethylene.

Keywords: Ethylene; boron deficiency; boron toxicity; central metabolism

INTRODUCTION

Boron (B) is an essential micronutrient which plays crucial roles during both plant growth and plant development (Marschner, 2012). The essentiality of this element was first reported in 1923 by Warington who showed that the growth of *Vicia faba* (field bean) and other plants was reduced in the absence of B, but it was rescued following the resupply of B. Afterwards, it has been widely accepted that B is a necessary and beneficial element for different organisms, and in plants, the beneficial functions of B, from a nutritional and physiological perspective, have been extensively studied and reviewed elsewhere (Camacho-Cristóbal et al., 2018; Landi et al., 2019). Accordingly, B is an important element for cell wall formation and stability as well as maintenance of plasma membrane functions (Brown et al. 2002; Wimmer et al., 2009). Furthermore, B influences also the development of reproductive organs, including pollen germination and both fruit set and development (Tanaka et al., 2013). Moreover, B has been also associated with seed formation and development by directly impacting seed germination and seedling establishment (Zohaib et al., 2018). Notably, the influence of B on secondary metabolites biosynthesis such as antioxidants polyphenols has been also suggested due to the fact that B nutrition is likely an important agent regulating reactive oxygen species (ROS) levels (Brown et al., 2002; Goldbach and Wimmer, 2007; Camacho-Cristóbal et al., 2008; Landi et al., 2019). B is also involved in metabolism of RNA (pyrimidine biosynthesis) and indole-3 acetic acid oxidase root elongation (González-Fontes et al., 2008) phenolic metabolism, carbohydrate metabolism and translocation (Graham and Webb, 1991). It is important to mention that the molecular mechanisms behind the majority of these functions remains largely unknown.

In soil, B exists in three compartments - soil, minerals and organic matter. Thus, B can occur naturally in soil and groundwater or be added to the soil via fertilizer and irrigation water (Nable et al., 1997). In soil solution, B occurs mainly in the undissociated form of boric acid (H_3BO_3), which greatly influences its availability (Hu and Brown, 1997). Thus, generally two extremes, deficiency and B toxicity, are observed in arable lands drastically affecting crop production, yield and quality, leading to significant economic losses (Camacho-Cristóbal et al., 2008; Camacho-Cristóbal et al., 2018; Landi et al.,

2019). Noteworthy, studies on B fertilization have shown that the limits between deficiency and toxicity are very narrow and that the ideal doses are relatively variable across plant species (Gupta et al. 1985; Camacho-Cristóbal et al., 2018; Landi et al., 2019). In fact, among micronutrients, B deficiency is the most common worldwide in several cultures (Shorrocks, 1997; Yan et al., 2006). It usually occurs in areas commonly related to both sand soils highly leached and low organic matter content (Yan et al., 2006). On the other hand, B toxicity occurs predominantly in regions characterized by alkaline and saline soils, along with low rainfall and lack of leaching (arid and semi-arid regions) (Tanaka and Fujiwara, 2008; Camacho-Cristóbal et al., 2008; Smith et al., 2013).

Plants use distinct mechanisms to maintain B homeostasis in their tissues due to the amplitude in B levels usually found in the soil solution (Ozhuner et al., 2013). Usually, this occurs at the root uptake stage. Thus, B tolerance mechanisms include differential regulation of B level detection and response (Yoshinari and Takano, 2017). Indeed, significant advances have been made in understanding the regulation of B transport in plants and the mechanisms related to the maintenance of B levels in plants (Chatterjee et al., 2014; Hanaoka et al., 2014). Our understanding of the mechanisms associated with B absorption regulation has been greatly facilitated by the identification and characterization of different transporters involved in this process. In *Arabidopsis thaliana*, several B transporters have been identified and physiologically characterized (Yoshinari and Takano, 2017). Additionally, their function has been associated with efficient root uptake of B and transport to the aerial part and B exclusion to soil solution (Miwa and Fujiwara, 2011; Miwa et al., 2013; Yoshinari and Takano, 2017).

A growing body of evidence indicates that plant hormones likely play important roles in the primary sensory cascades associated with B stress response (Pommerrening et al., 2019). Recent studies indicate possible interactions between B availability and ethylene signaling pathways, auxin polar transport and reactive oxygen species (ROS), culminating in significant root growth inhibitions (Martín-Rejano et al., 2011; Oiwa et al., 2013; Camacho-Cristóbal et al., 2016). Notably, the ethylene plays a significant role in plant responses to different abiotic stresses such as hypoxia, heavy metals, salinity, heat, low soil pH, drought, ozone, aluminum toxicity, as well as phosphorus and B deficiency (Sun

et al., 2007; 2010; Lei et al., 2011; Khan et al., 2013; Habben et al., 2014; Ludwikow et al., 2014; Steffens, 2014; Li et al., 2015; Yang et al., 2016; Brito et al., 2018). Accordingly, B deficiency seems to rapidly induce ethylene biosynthesis, which leads to inhibition of root elongation (Sun et al., 2007; Martin-Rejano et al., 2011; Tian et al., 2014). It has been recently demonstrated that moderate toxic levels of B increased both jasmonate (JA) and ethylene leading to a differential regulation of microRNAs such as miR159, miR172 and miR319 related to JA and ethylene and it was associated with an oxidative stress-acclimatizing response of Arabidopsis to B toxicity (Kayihan et al., 2019).

Ethylene inhibition of root growth occurs mainly by stimulating auxin biosynthesis and modulating the transport of basipetal auxin toward the elongation zone (Swarup et al., 2007). Nevertheless, our knowledge of how exactly B levels act together with ethylene impacting primary metabolism and plant growth in general remains highly fragmented. Here, we hypothesized that extreme conditions (deficiency and toxicity) of B availability directly alter the synthesis and/or signaling of ethylene which is responsible for the process associated with B homeostasis by modifying plant growth and primary metabolism and plant growth. To further investigate the mechanisms underlying B and ethylene response, tomato ethylene mutants were fertilized with different B concentrations (0, 25 and 640 µM) during both the vegetative and reproductive phase.

MATERIALS AND METHODS

Plant material and experimental conditions

Seeds of tomato (*Solanum lycopersicum* cv Micro-Tom - MT) and four mutants in the same genetic background namely *non-ripening* (*nor,* NAC transcription factor that repress the normal ripening), *ripening inhibitor* (*rin*, MADS box family of transcriptional regulators that inhibit fruit ripening), *never ripe* (*Nr,* defective for the LeETR3 ethylene receptor blocking ethylene perception) and *epinastic* (*Epi,* ethylene over-productive) (Carvalho et al., 2011) were used in this study. The seeds were kindly provided by Prof. Dr. Lázaro EP Peres from the Universidade de São Paulo, Brazil.

Plants were grown in a glasshouse located in Viçosa (20°45′S, 42°15′W, 650 m above sea level), southeastern Brazil, with a minimum of 400 µmol photons m^{-2} s^{-1}.

Throughout the entire growth period, plants were maintained under naturally fluctuating conditions of light intensity, temperature and relative air humidity. Seeds were surface-sterilized with 5 % sodium hypochlorite for 10 min, then washed with running distilled water and subsequently sowed in sterile Petri diches. After germination, seeds were transferred to plastic cups (200 mL) containing filter paper. The cups were initially watered with 20 mL nutrient solution ½ strength (Hoagland and Arnon, 1950) without B. The roots systems received water every day at the beginning and at the end of the day with 1 mL nutrient solution ½ strength. Seedlings containing the first pair of true leaves were transplanted to Styrofoam boxes (2 seedlings per box) containing 5 L of half-strength Hoagland's solution (supplemented with 0, 25 and 640 µM B, provided as H_3BO_3) renewed each week, from the second week the complete Hoagland solution was used. All physiological, biochemical and molecular parameters analyzed in the experiments were performed on the third and/or fourth fully expanded source leaves from 4-week-old plants. Additionally, the experiment was repeated at least three times (even in different growth facilities) with similar phenotypes observed each time.

Growth analysis and B concentration

Growth parameters were determined in 4-week-old plants by measuring root length, height and stem diameter, plant height, and stem diameter. The 4th leaf from the apex were collected, then dried at 65°C until constant weight. The samples were submitted to dry digestion (incineration) and the B contents quantified by the azomethine-H method (Tedesco, 1995).

SPAD Index

The SPAD (Soil Plant Analyzer Development) index, which represents the green color intensity of the plant, was measured using a portable chlorophyllometer (SPAD-502 Minolta Corp.). Readings were performed between 8-10 a.m. in the upper, middle and lower third of the plant and two readings for each segment of the plant, totaling six readings per plant were performed. From these readings (6 leaves), the average of each leaf sample was calculated, using the SPAD meter itself.

Measurements of gas exchange and chlorophyll fluorescence

Gas exchange parameters were determined simultaneously with chlorophyll *a* (Chl *a*) fluorescence measurements as described in Batista-Silva et al. (2019). The analysis was performed using an open-flow infrared gas exchange analyser system (LI-6400XT; LI-COR Inc., Lincoln, NE) equipped with an integrated fluorescence chamber (LI-6400-40; LI-COR Inc.). Instantaneous gas exchanges were measured after 1 h illumination during the light period under 1,000 μmol m^{-2} s^{-1} at the leaf level (light saturation) of photosynthetically active photon flux density (PPFD) as determined previously by Batista-Silva et al. (2019). The reference CO_2 concentration was set at 400 μmol CO_2 mol^{-1} air. All measurements were performed using the 2 cm^2 leaf chamber at 25 °C, as well as a 0.5 stomatal ratio (amphistomatic leaves), and leaf-to-air vapour pressure deficit was kept at 1.2 kPa whereas the amount of blue light was set to 10% PPFD to optimize stomatal aperture. The initial fluorescence emission (F_0) was obtained by illuminating dark-adapted leaves (2 hours) were initially exposed to weak modulated measuring beam (0.03 - μmol of photons m^{-2} s^{-1}. A saturating white light pulse (8,000 μmol m^{-2} s^{-1}) was applied for 0.8 second to obtain the maximum. The variable-to-maximum Chl fluorescence ratio was calculated: $F_v/F_m=[(F_m-F_0)/F_m)]$. The capture efficiency of excitation energy by open photosystem II reaction centers (F_v'/F_m') was estimated according Logan et al., (2007) and the actual PSII photochemical efficiency (φPSII) was estimated as $\varphi PSII=(F_m'-F_s)/F_m'$ according Genty et al. (1989).

Dark respiration (R_d) was measured after 2 h in the dark period (at night), using the same gas exchange system described above, and it was divided by two ($R_d/2$) to estimate the respiration rate in the dark (R_d; Niinemets et al., 2009).

Electrolyte leakage

Cellular damage was analyzed through electrolyte leakage assayed immediately after leaf sampling using a conductivity meter (Lima et al., 2002).

Root characterization by WinRHIZO

Roots harvested at the end of the experiment were stored in 30 % (v/v) ethanol. The roots were scanned using a flated scanner and analyzed using the image-processing software WinRhizo Pro (Regent Instr. Inc; Québec, Canada) as described by Zhu et al. (2005).

Image capture, documentation and analysis of the total length of root (cm); surface area of the total root (cm^2); root mean diameter (mm); total root volume (cm^3) and root ramifications (Bouma et al., 2000).

Biochemical analyzes

Leaf samples were harvested in the middle of the light period and immediately frozen in liquid nitrogen and stored at -80ºC until further biochemical analysis. Metabolite extraction and analysis were performed as previously indicated in Batista-Silva et al., (2019). Briefly, the extraction was performed by rapid grinding of tissue in liquid nitrogen and immediate addition of a methanol. Photosynthetic pigments were determined exactly as described by Porra et al. (1989). The levels of starch, sucrose, fructose, and glucose in the leaf tissue were determined exactly as described previously by Fernie et al. (2001). Malate and fumarate were determined according to Nunes-Nesi et al. (2007). Total protein were determined as previously described by Bradford**, (**1976) and total amino acids levels were determined as Yemm. (1955).The metabolites profile was carried out as described by Lisec et al. (2006) with modifications. Briefly, approximately 25 mg of homogenized leaf were aliquoted in tubes and extracted in 100 % methanol and internal standard (0.2 mg ribitol mL^{-1} water). The derivatization and sample injection steps were carried out exactly as previously described (Lisec et al., 2006). Peak detection, retention time alignment, and library matching were performed using Target Search R-package (Cuadros-Inostroza et al., 2009). Metabolites were identified in comparison to database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005). Identification and annotation of detected peaks followed the recommendations for reporting metabolite data described in Fernie et al. (2011).

Expression Analysis by qRT-PCR

The qRT-PCR analysis was performed exactly as described by Zanor et al. (2009) with total RNA isolated from at least three biological replicates. The samples were harvested and snap frozen in liquid nitrogen. The total RNA extraction was perfomated using Trizol® reagent (Ambion, Life Technology) following the manufactures's manual. Digestion with DNAse I (Ambion; http://www.ambion.com) was used according to the manufactirer's instructions. The integrity of the RNA was checked on 1 % (w/v) agarose gels, and the concentration was measured before and after DNase I digestion using a Nanodrop ND-1000 spectrophotometer (http://www.nanodrop.com/). DNAse-treated RNA (1 µg) was used for cDNA synthesis using High- Capacity reverse transcriptase (Applied Biosystems, CA, USA), according to the manufacturer's instructions. The expression of genes was assessed using PCR in real time (Step One Plus TMReal Time PCR System). The reactions were performed in a 96-well microtitre plate, using Power SYBR Green PCR Master Mix according to Piques et al. (2009).

The primers used here were designed using the open-source program QuantPrime-qPCR primer designed tool (Arvidsson et al., 2008) and detailed primer information are described in the **Table S1** Data analyses were performed as described by Caldana et al. (2007). The relative expression levels were normalized using the constitutively expressed genes Ubiquitin 3 (Wang et al., 2008).

Ethylene measurements

The production of ethylene was determined in different tissues (roots, leaves and fruits). Briefly, leaves and roots of 4-week-old plants were harvested, weighted, and placed in Erlenmeyer's (25 mL) completely sealed and incubated for 8 and 24 h, respectively, under room temperature. We used roots collected 2 cm from the apex following removing excess water. Fruits were harvested at different developmental stages namely at 15, 30, 40 and 60 days after anthesis (DAA), weighed, measured the fruit volume and incubated for 6 h exactly as described above. For control samples, it was consisted of air-sealed bottles. Ethylene was measured exactly as described by Ribeiro et al., 2010) by taking gas sample (1 ml) from each Erlenmeyer flask and injecting into a

gas chromatograph (Hewllett Packard 5890, Series II). Ethylene concentrations were quantified with authentic ethylene standards.

Fruit fresh water loss measurements

Three fruits per genotype and treatment were detached at the red ripe stage (60 days), and kept at room temperature for over a 12-d period. Water loss per unit fruit surface area was calculated after measuring the weight decrease over time and measuring fruit dimensions.

Agronomic characterization of fruits

To further understand B and ethylene influences we have analyzed important agronomic traits after harvesting time. The total number of fruits and the fresh mass of the fruits per plant were quantified. Ten fruits per plant were used to evaluate length (distance between the base (insertion of the peduncle) and the apex); diameter of the fruit (taken perpendicular to the height in the larger region of the fruit); thickness of the pericarp (measured in the equatorial region of the fruit) and number of seeds per fruit. Productivity was estimated by multiplying the total number of fruits by the mean mass of these fruits (g $plant^{-1}$).

Experimental design and statistical analysis

The experiments were arranged in a completely randomized block design following a 5 x 3 factorial [five genotypes (MT, *nor*, *rin*, *Nr*, *Epi*) and three levels of B (0, 25, 640 µM)], with four replicates. Data was expressed as means ± standard error (SE). The results were submitted analysis of variance (*F* test), after ANOVA assumptions (additivity, independence and normality of the residues, and homoscedasticity of the variance). To compare the difference between genotypes and B levels, the means were compared by the Tukey test *($p \leq 0.05$)*. All statistical analyzes were performed by using SAS statistical software (SAS, 2007).

RESULTS

Vegetative growth is affected in response to boron level

To investigate the ethylene contribution in B responses, we here investigated physiological and metabolic impacts using previously characterized tomato mutants. In an attempt to show this differential response we first analyzed plant growth and observed that, overall, the distinct tomato genotypes with different ethylene levels subjected to excess B were characterized by an inhibition of stem diameter and root length (**Fig. 1A and 1C; Table S2**). Briefly, WT, *Nr* and *Epi* genotypes were more sensitive to B toxicity, while *nor* and *rin* were characterized by smaller yet not significant stem diameter when exposed to high levels of B (**Fig. 1A**). Reduction in root length is one of the earliest symptoms of B toxicity, and the *nor* genotype was most sensitive (**Fig. 1C**). B deficiency display no significant effect on plant growth in any genotype. The results obtained can be in fact associated with the foliar concentration of B, since it increased substantially following B toxicity and it was observed that higher accumulations occurred in WT and *nor,* while *Epi* accumulated less B in leaves (**Fig. 1D**).

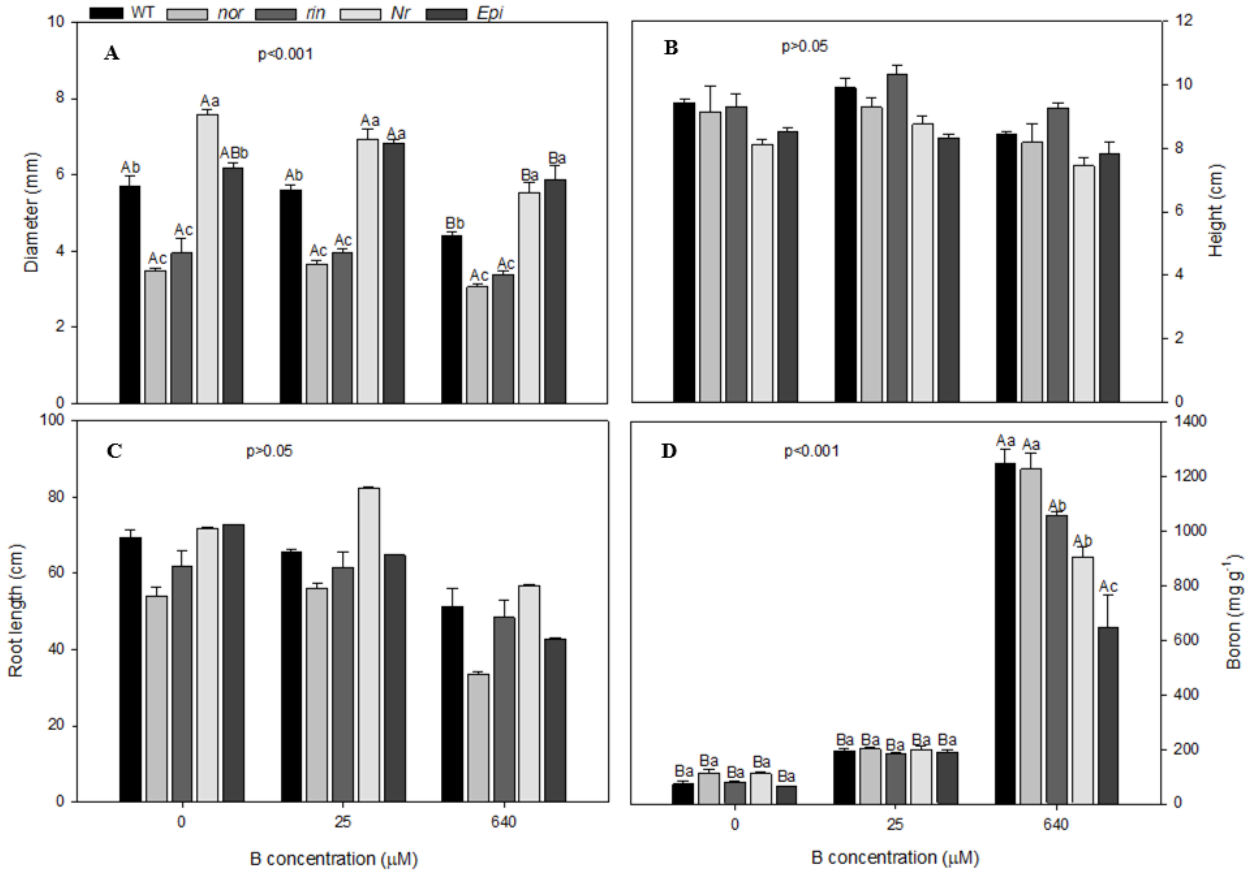


Figure 1. **Differential growth and Boron accumulation in tomato ethylene mutants under contrasting conditions of Boron availability.** (**A**) Stem diameter, (**B**) Plant height, (**C**) Root length, (**D**) foliar B concentration in tomato plants submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), nor (non-ripening), rin (ripening inhibitor), Nr (never ripe), Epi (epinastic).

Ethylene mutants modify root morphology in response to distinct boron levels

To further investigate the significance of ethylene under distinct B conditions, we analyzed a root morphology approach. Notably, there was significant interaction between factors (Genotype and B) for all parameters evaluated (**Fig. 2**). Accordingly, reduced root length and surface area under B toxicity conditions was observed for both WT and *nor* genotypes (**Fig. 2A and 2B**). On the other hand, *nor* and *rin* were statistically lower than the other genotypes under both adequate and deficient B conditions (**Fig. 2**). Smaller root diameter and root volume were found for all genotypes in response to B excess. By

contrast, under adequate B levels, *Nr* performed better than the other genotypes, yet significantly reduced all parameters under extreme B conditions (**Fig 2C and 2D**). In addition, the *Nr* genotype showed higher forks under adequate level of B compared to both B omission and excess (**Fig. 2E**).

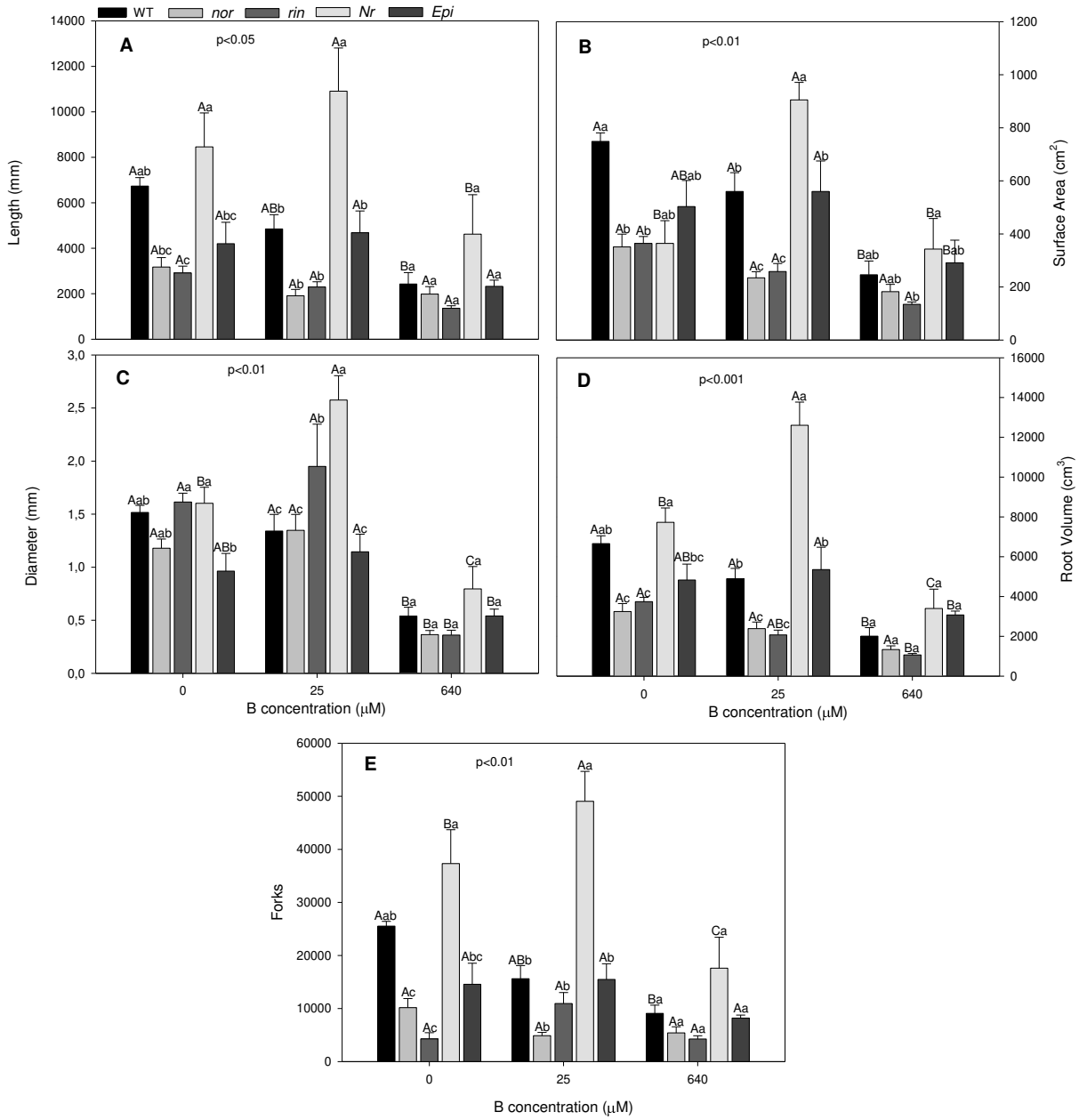


Figure 2: Contrasting Boron levels modulate differential root morphology in tomato ethylene mutants. (A) Length, **(B)** Surface area, **(C)** Diameter, **(D)** Root volume, **(E)** Forks, in tomato plants submitted

to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

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319 Further analysis of root morphology by diameter class also showed significant
320 differences as it can be observed by the smallest diameters (0-1mm) and the largest
321 diameters (> 4mm. In presence of B excess the formation of thick roots (> 4mm) in *Epi*
322 appears to be stimulated (**Fig. 3A and 3E**). Overall, the other root diameter classes were
323 reduced under excess B and the *Nr* appears to be the more sensitive to changes in B
324 conditions at the root level (**Fig. 3B, 3C and 3E**).

325

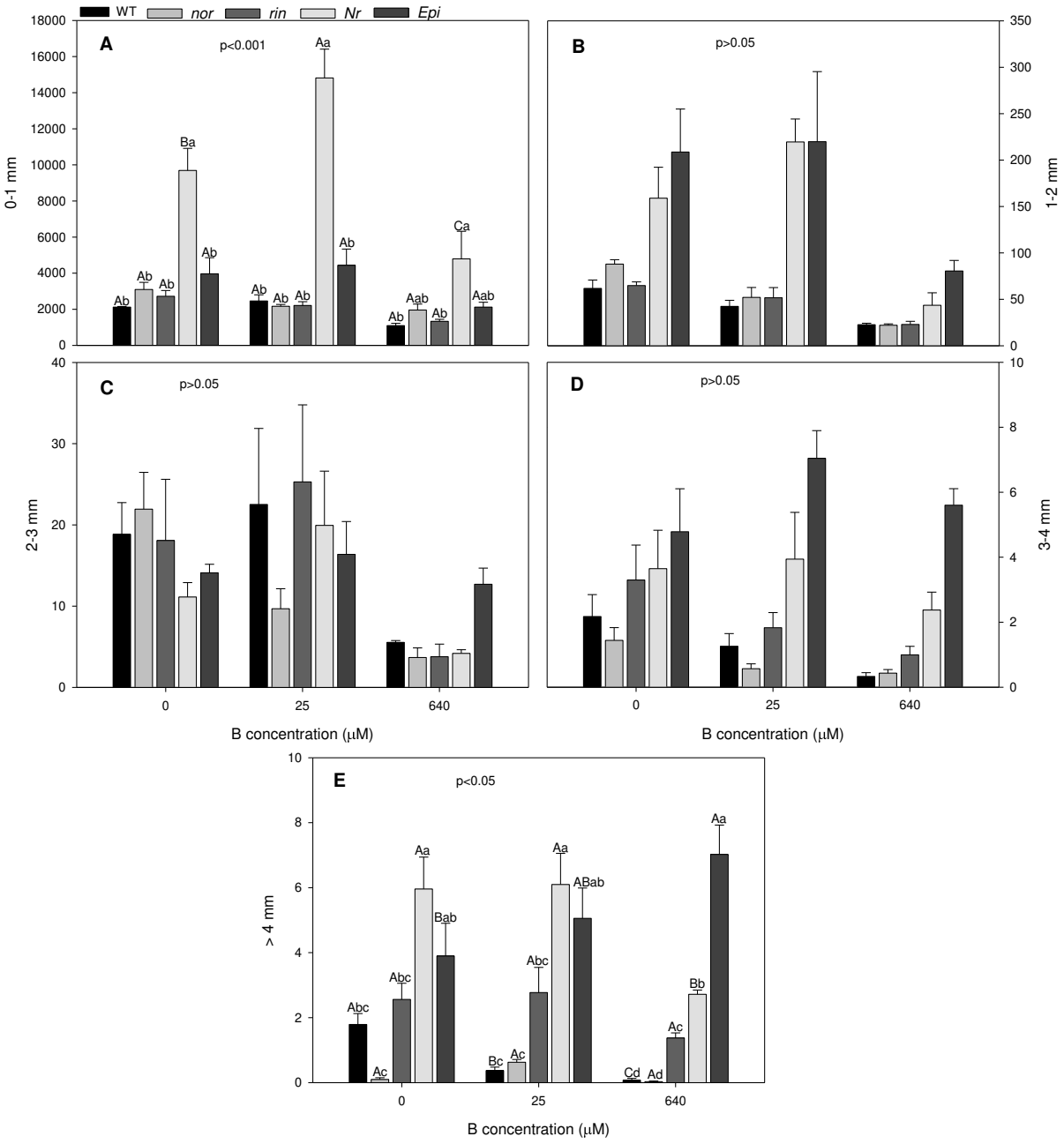

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Figure 3: Root diameter is altered in tomato ethylene mutants under contrasting Boron conditions. **(A)** Diameter 0-1 mm, **(B)** Diameter 1-2 mm, **(C)** Diameter 2-3 mm, **(D)** Diameter 3-4 mm, **(E)** Diameter >4 mm, in tomato plants submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further

submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

Photosynthetic and photochemical efficiency of ethylene mutants in response to different levels of boron

We next investigated the photosynthetic performance, through gas exchange and chlorophyll *a* fluorescence. We observed that plants under either B deficiency or toxicity exhibited reductions in gas exchange parameters (**Fig. 4**). Both CO_2 assimilation and stomatal conductance shown drastic reductions under B stress conditions (**Fig. 4A** and **4B**), highlighting the *nor* and *rin* genotypes, as the more sensitive to changes in B levels, emphasizing that adequate B doses are essential for maintaining plant photosynthetic capacity. We further noticed that C_i/C_a, E, A/E and R_d are characterized by significant interaction in relation to genotypes (**Fig. 4C, 4D, 4E** and **4F**; **Table S2**). Thus, both *Nr* and *Epi* genotypes presented higher C_i/C_a, E and R_d, however they presented lower water use efficiency.

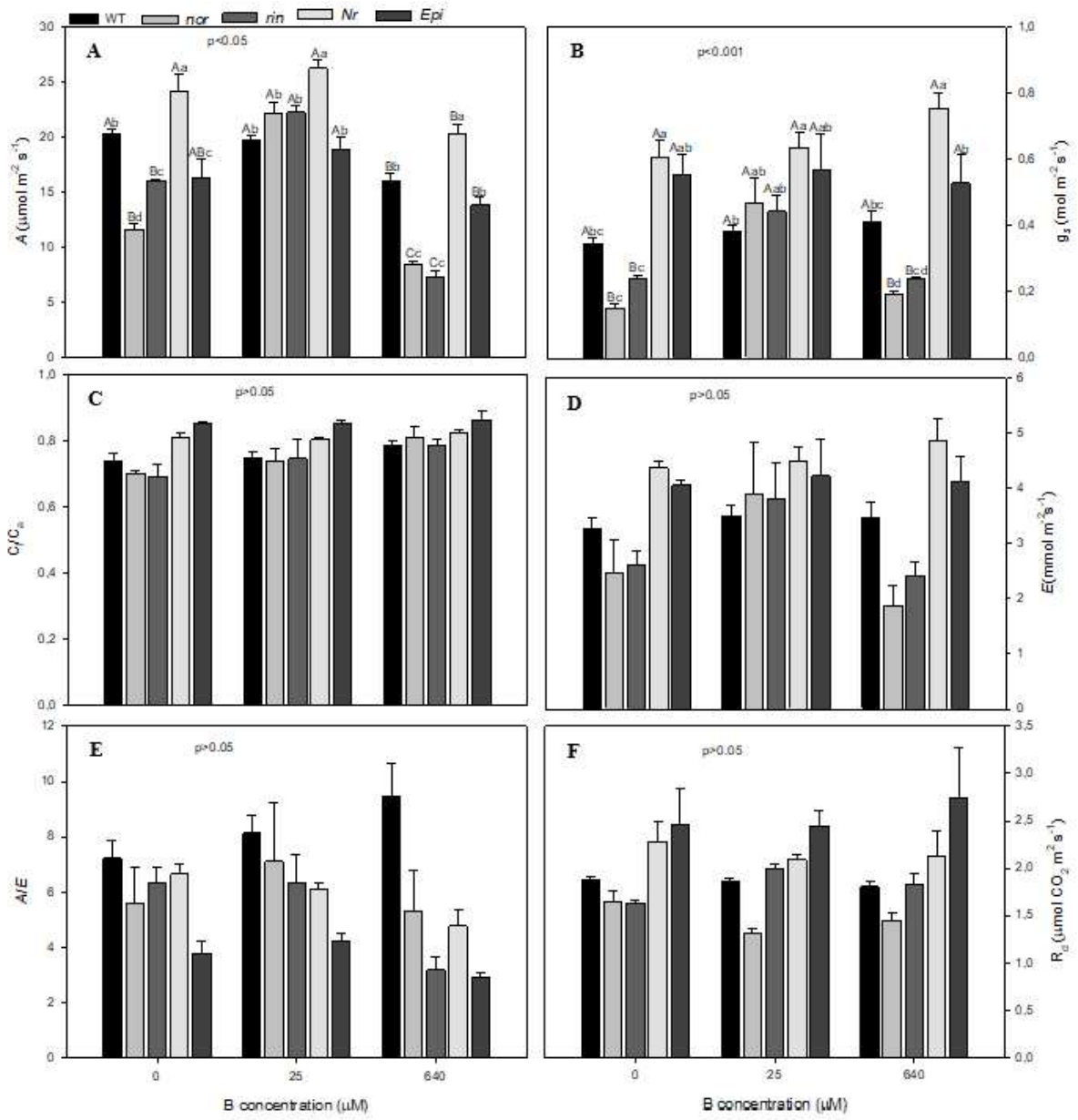

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Figure 4: Boron deprivation and excess reduces CO_2 assimilation in WT and tomato ethylene mutants. (A) Net CO_2 assimilation rate, **(B)** Stomatal conductance, **(c)** C_i/C_a ratio, **(D)** Transpiration rate, **(E)** Water use efficiency, **(F)** Respiration in darkness, in tomato plants submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

340

341 Despite the changes aforementioned, photochemical extinction coefficient (q_p),
342 Non-photochemical extinction coefficient (q_n), Non-photochemical quenching (NPQ),
343 Regulated energy dissipation Y(NPQ) remained virtually invariant under experimental
344 conditions with no differences between genotypes and B levels. (**Fig. 5D, 5E, 5F** and **5H**).
345 By contrast, F_v/F_m, F_0, ETR, YNO, YII and q_L were characterized by significant changes
346 in genotypes cultivated under different B levels, highlighting the similar behavior in
347 response to B. Briefly, F_v/F_m, ETR and YII were reduced in both *nor* and *rin* under both
348 deficiency and toxicity conditions (**Fig. 5A, 5C** and **5I**). Under B excess significant
349 reductions in F_0 were observed for the *Epi* (**Fig. 5B**). In the absence of B, reductions in q_L
350 were observed for both *nor* and *rin* whereas in *Epi* it increased (**Fig. 3J**). Notably, following
351 excess of B, qL increased only in *nor* (**Fig. 5J**). Both B deficiency and toxicity increase
352 YNO in both *nor* and *rin* (**Fig. 5G**).

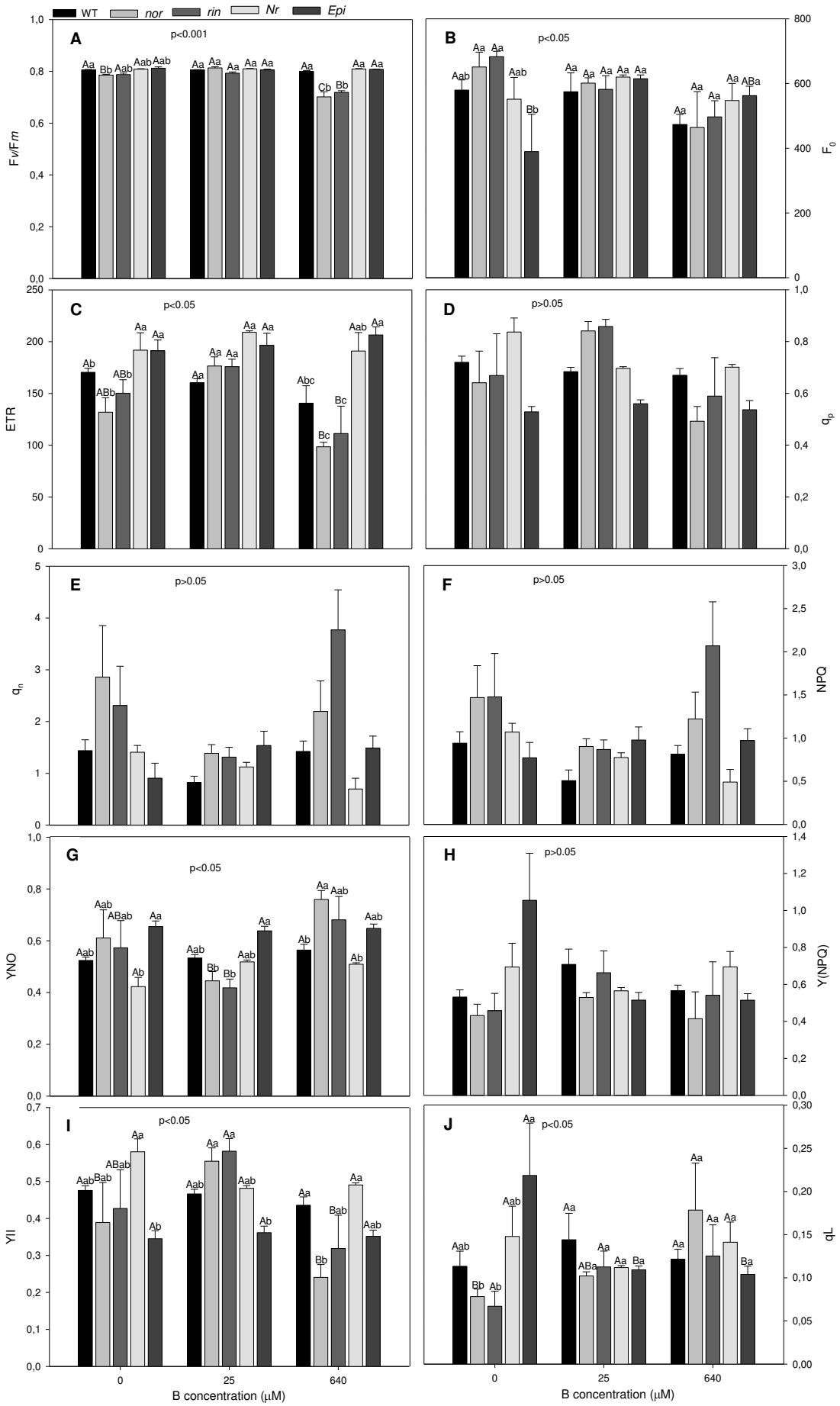

WT
nor
rin
Nr
Epi
A
p<0.001
Fv/Fm
B
p<0.05
F0
C
p<0.05
ETR
D
p>0.05
qp
E
p>0.05
qn
F
p>0.05
NPQ
G
p<0.05
YNO
H
p>0.05
Y(NPQ)
I
p<0.05
YII
J
p<0.05
qL
0
25
640
B concentration (μM)

Figure 5: Photochemical limitation in response to Boron is mediated by ethylene. (A) Maximum quantum yield of the FSII, **(B)** Minimum fluorescence, **(C)** Electron transport rate, **(D)** Photochemical extinction coefficient, **(E)** Non-photochemical extinction coefficient, **(F)** Non-photochemical quenching, **(G)** Unregulated energy dissipation, **(H)** Regulated energy dissipation, **(I)** Quantum yield of photochemical energy conversion in photosystem II **, (J)** Fraction of PSII centers that are open, in tomato plants submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

Boron influences ethylene-mediated nitrogen accumulation

There was significant interaction between genotype and SPAD index as it can be deduced by the reduction of this parameter in all genotypes under B excess. The most significant reductions were observed for *nor* and *rin* (**Fig. 6A**). The reduction observed in the SPAD index seems to be, at least partially, caused by lower leaf NO_3^- levels as a result of the reduction uptake efficiency N through membrane disruption (**Fig. 6B; Table S1**). Following excess of B increased membrane damage as observed by the greater electrolyte leakage (**Fig. 6C, Table S1**).

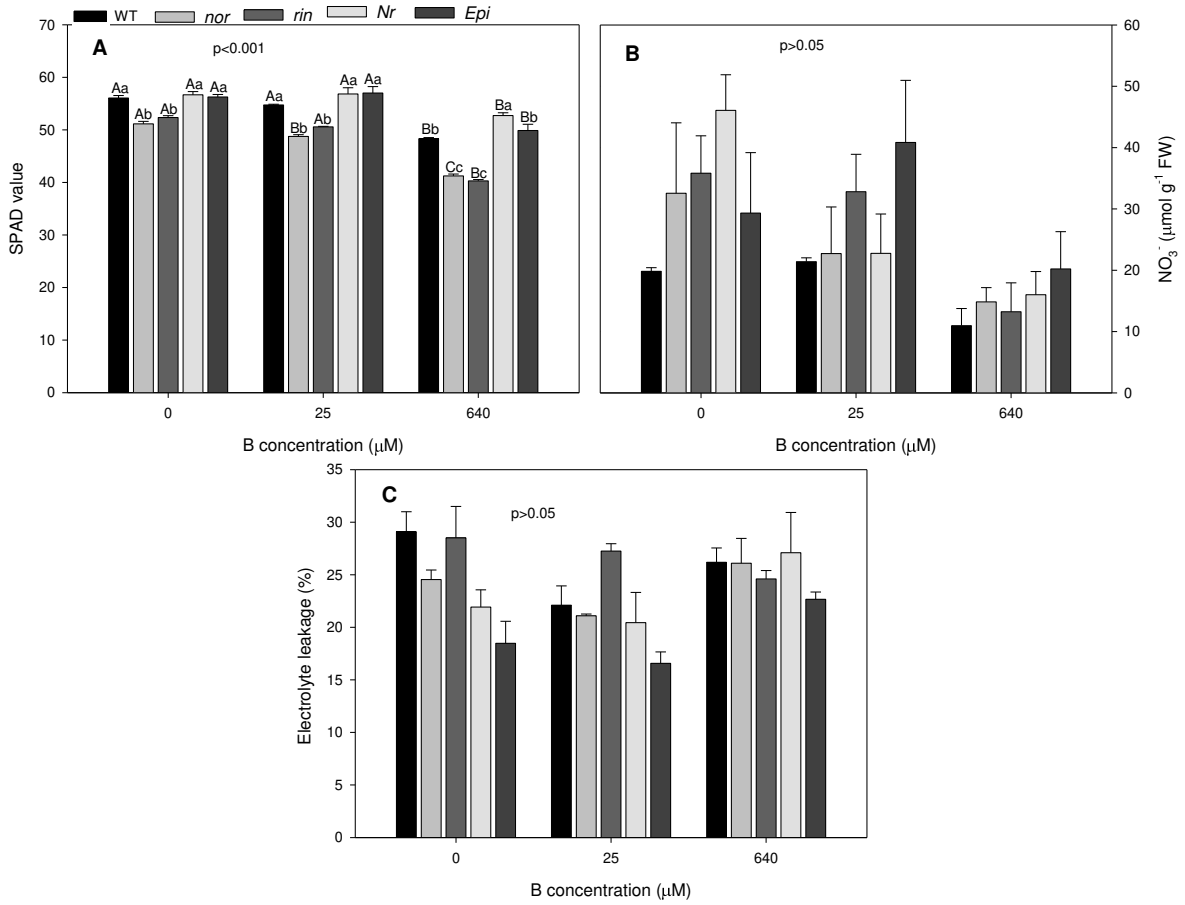


Figure 6: Boron excess seems to affect N uptake and assimilation with oxidative stress. (A) SPAD index, **(B)** Nitrate, **(C)** Electrolyte leakage, in tomato plants submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

Sugar and amino acid metabolism is affected by the level of boron

To investigate the link between ethylene and B in growth and photosynthesis responses, a detailed metabolic characterization was performed. First, we analyzed the pigment levels and observed that the chlorophyll *a* and total chlorophyll were reduced under toxicity conditions for *Epi* genotype, and the same behavior was verified for chlorophyll *b* (**Fig. 7A, 7B and 7C**). Chlorophyll *a*/*b* ratio did not change in response to B and the different genotypes (**Fig. 7D**).

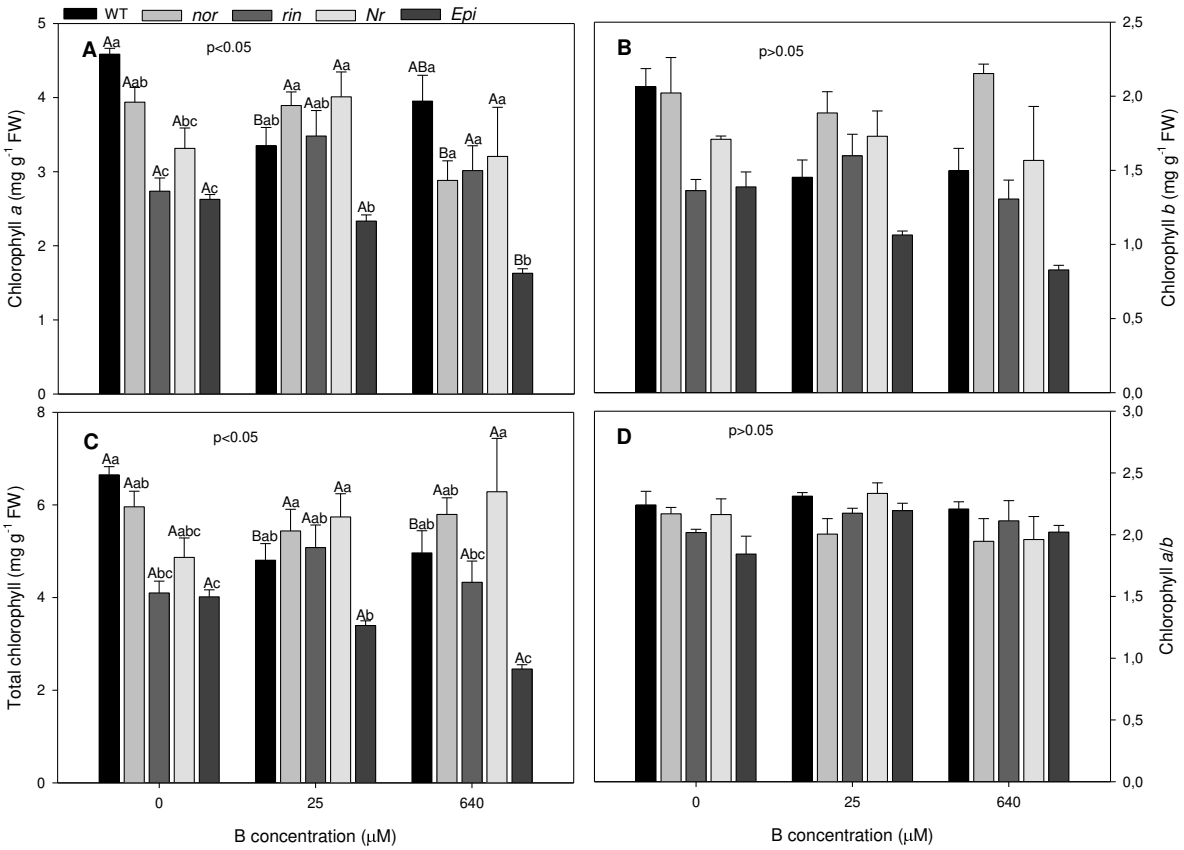


Figure 7: Chlorophyll content of WT and ethylene mutants under contrasting conditions of Boron. (A) Chlorophyll a, **(B)** Chlorophyll b, **(C)** Total chlorophyll, **(D)** Chlorophyll a/b, submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

Total soluble protein content did not differ with respect to B levels, however the lowest content was observed for *Epi* (**Fig. 8A, Table S2**). There was a reduction in amino acids for the WT genotype under extreme conditions of B availability compared to the adequate level (**Fig. 8B)**. Lower starch content was observed for *nor* and *rin* at all B levels (**Fig. 8C**). In general, sugars (Glucose, Sucrose and Fructose) behave similarly and thus excess B increases while deficiency reduces sugar content for WT and *Nr* relative to adequate availability of B. Notably, *nor* and *rin* genotypes maintained sugar content

relatively unvariant regardless of the B level, yet it is the genotypes that accumulate lower amounts of sugars (**Fig. 8D, 8E and 8F**).

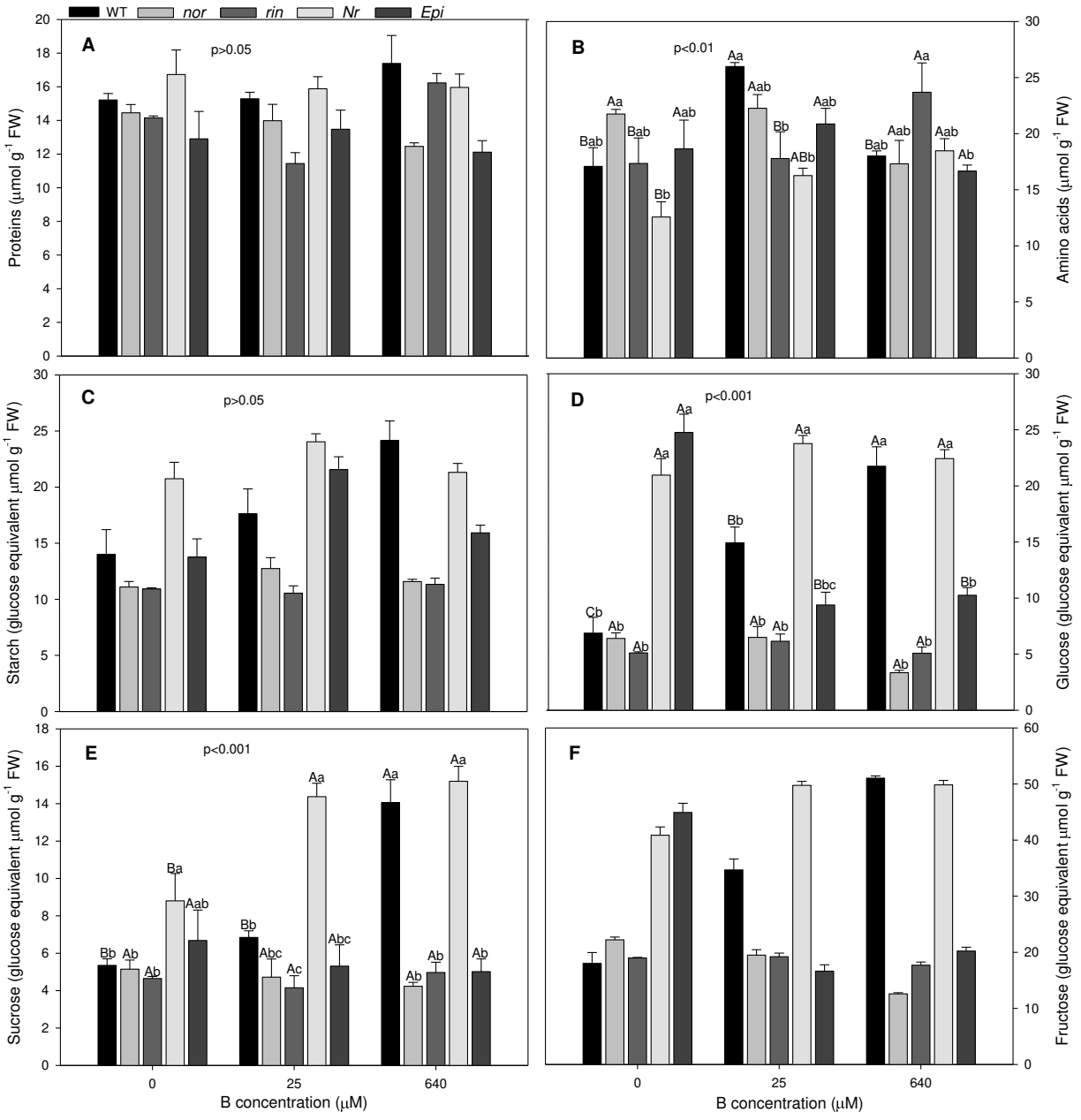
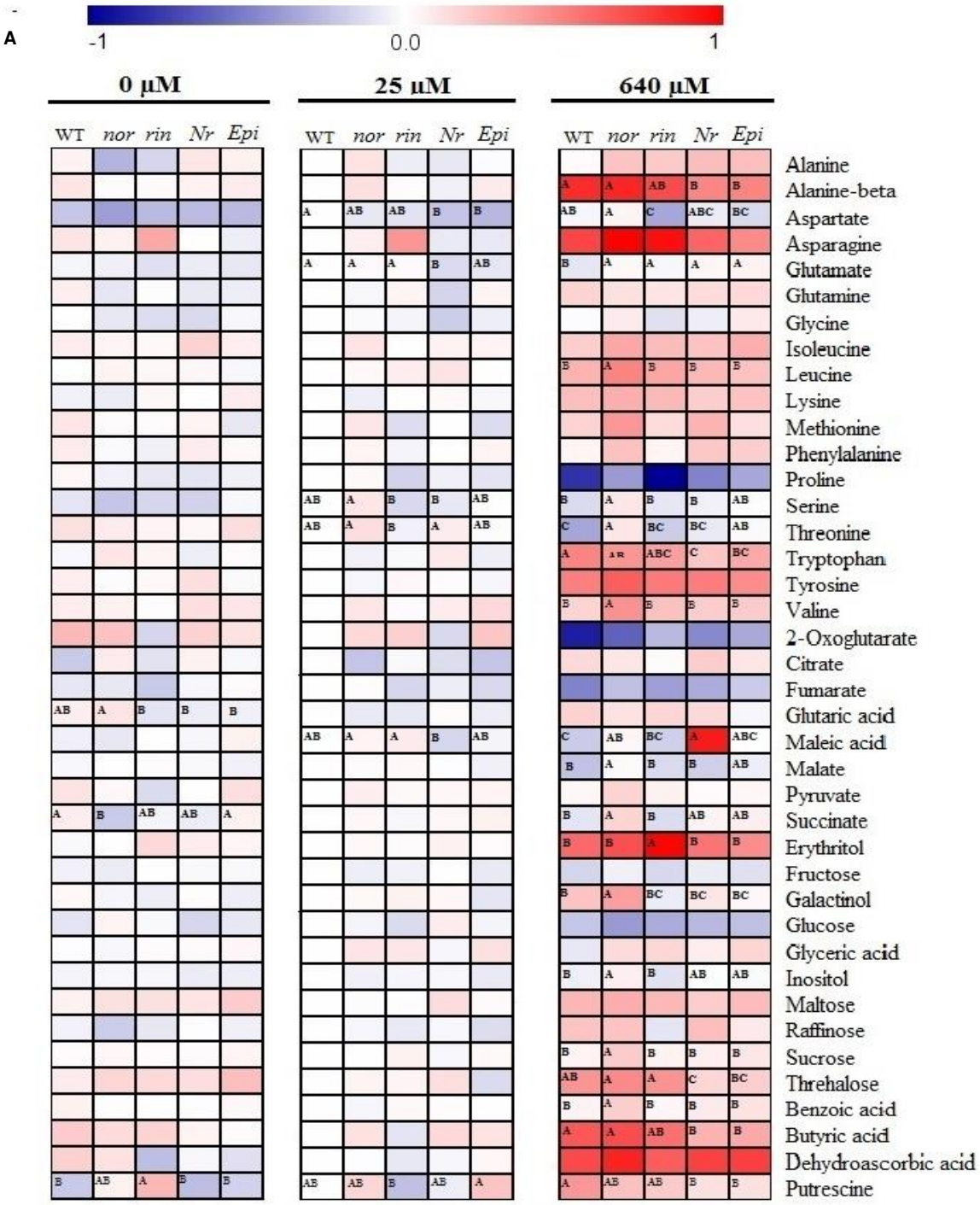


Figure 8: Boron affects ethylene-mediated sugar metabolism. (A) Proteins, **(B)** Amino acids, **(C)** Starch, **(D)** Glucose, **(E)** Sucrose, **(F)** Fructose, in submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

To further explore the consequences of fluctuations on B levels and ethylene on primary metabolism we extended the analysis by studying the major primary metabolic pathways, by means of an established GCMS protocol (Lisec et al., 2006) on leaves harvested at the middle of light period. These analyses revealed that among the 37 successfully annotated compounds related to primary metabolism, considerable changes occurred in the levels of a wide range of amino acids, organic acids, and sugars (**Fig. 9**). The heat map generated indicates significant changes following either B deficiency or excess conditions. It was possible to observe increased levels of Alanine, Asparagine, Erythritol, Butyric acid and Dehydroascorbic acid, while Proline, 2-oxoglutaric and Fumaric acid were less abundant metabolites. In the comparison between the investigated genotypes no major differences were observed, indicating that metabolic changes are dependent greatly on B levels. By comparing the genotype effect for each level of B, it appears that the response is seemingly relevant especially with respect to amino acids (Alanine-beta, Aspartate, Glutamate, Leucine, Serine, Thronine, Tryptofan and Valine), following B excess condition. Noteworthy, other metabolites that showed differences between genotypes under B excess were accumulated predominantly in *nor* (**Fig. 9A**), highlighting the largest accumulations of the amino acids aforementioned for *nor*.

Principal component analysis (PCA) was also performed to explore more deeply the contribution of changes in the levels of ethylene and B in the leaf metabolite composition (**Fig. 9B**). This analyzes revealed that the first two components explained, together 70 % of the data variability observed (Fig. 9B; CV1 covers 54.6 % of the total variance and CV2 16 %). This fingerprint analyzes showed that indeed the dominant source of variation in the combined dataset is the differential contribution of the metabolite composition in each genotypes. The metabolites that accounted for the main changes observed in primary metabolism following B toxicity were amino acids namely branched chain amino acids (Valine, Leucine, Isoleucine), in addition to the great contribution of Putrescine. Notably, sugars (glucose and fructose) contributed to the separation following B levels (**Fig. 9B; Table S3**). This fingerprint analysis resulted in five clusters, of which three were clustered under B toxic conditions namely the first containing both *rin* and WT

429 genotypes, the second *Epi* and *Nr* and the third *nor*. In summary, this analysis
430 demonstrated that *nor* was the genotype suffering greater metabolic changes when
431 subjected to elevated B levels. Accordingly, the genotypes *nor*, *rin* and *Nr* formed another
432 cluster under B deficiency. Furthermore, another cluster was obtained by the other
433 genotypes (e.g. WT and *Epi*) under both adequate B conditions and B deficiency (**Fig.**
434 **9B**).


A
-1
0.0
1
0 µM
25 µM
640 µM
WT nor rin Nr Epi
Alanine
Alanine-beta
Aspartate
Asparagine
Glutamate
Glutamine
Glycine
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Proline
Serine
Threonine
Tryptophan
Tyrosine
Valine
2-Oxoglutarate
Citrate
Fumarate
Glutaric acid
Maleic acid
Malate
Pyruvate
Succinate
Erythritol
Fructose
Galactinol
Glucose
Glyceric acid
Inositol
Maltose
Raffinose
Sucrose
Threhalose
Benzoic acid
Butyric acid
Dehydroascorbic acid
Putrescine

435

B

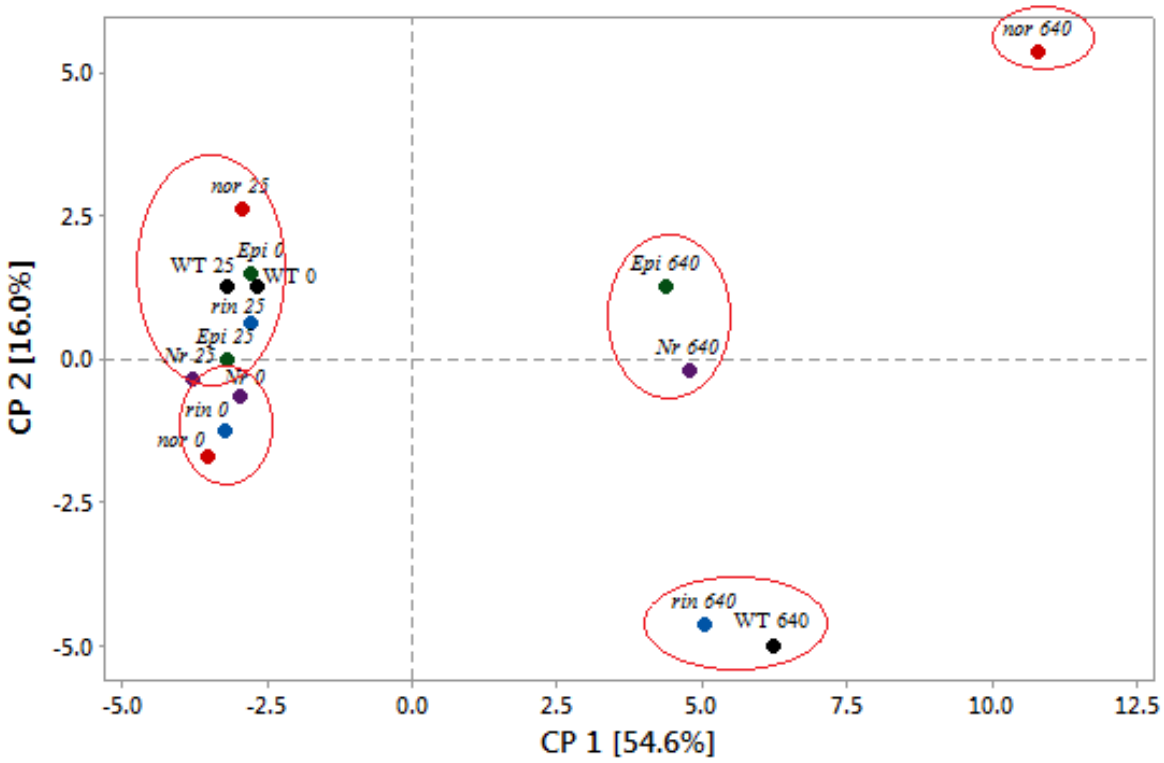


Figure 9: Global comparison of metabolic profiles between WT and different ethylene mutants submitted to distinct treatments with Boron. (A) Heat map representation metabolites, the samples were normalized according to the control condition (WT 25) **(B)** Clustering analysis and principal component analysis (PCA) of metabolic profiles in the leaves. PC 1, the first principal component; PC 2, the second principal component. WT (wild-type) (●); *nor* (non-ripening) (●); *rin* (ripening inhibitor) (●); *Nr* (never ripe) (●); *Epi* (epinastic) (●).

Boron affect ethylene production and perception in WT and ethylene mutants

Ethylene production was evaluated in leaves and roots as well as in fruits at different times in order to verify the influence of B on this hormone by using different genotypes related to ethylene sensitivity and production. In leaves, it was clearly observed that high levels of B stimulated ethylene synthesis in all genotypes (**Fig. 10A, Table S2**). Interestingly, ethylene levels in roots was lower than in leaves and B excess lead to significative reductions in ethylene levels in WT, *nor* and *rin* (**Fig. 10B**).

By further investigating the impact of distinct B levels in ethylene production using tomato fruits harvested at 15, 30, 40 and 60 days after anthesis (DAA). 15 DAA fruits of WT and *nor* were characterized by reduced and increased ethylene production,

447 respectively, when subjected to excess B (**Fig. 10C**). At 30 DAA, fruits of *Epi* were
448 characterized by reductions in ethylene levels under B deficiency however, adequate
449 levels and excess B lead to greater accumulation of ethylene than the observed for other
450 genotypes (**Fig. 10D**). At 40 DAA, WT reduced and increased ethylene levels following B
451 toxicity and deficiency, respectively, compared to the appropriate B level. Under stress B
452 conditions, ethylene levels were higher for *Nr* (**Fig. 10D**). At 60 DAA, ethylene levels were
453 higher for *Nr* under B deficiency while higher accumulation of ethylene under conditions
454 of B toxicity was observed for *Epi* (**Fig. 10E**). Overall, *nor* and *rin* accumulated less
455 ethylene compared to the other genotypes regardless of B level (**Fig. 10**). Notably,
456 ethylene production over time was generally similar between genotypes and different B
457 levels. Thus, the ethylene peak occurred at 40 DAA, followed by a reduction in its
458 production, except for *Epi*, where the ethylene peak was verified at 60 days under B
459 toxicity (**Figure S2**).

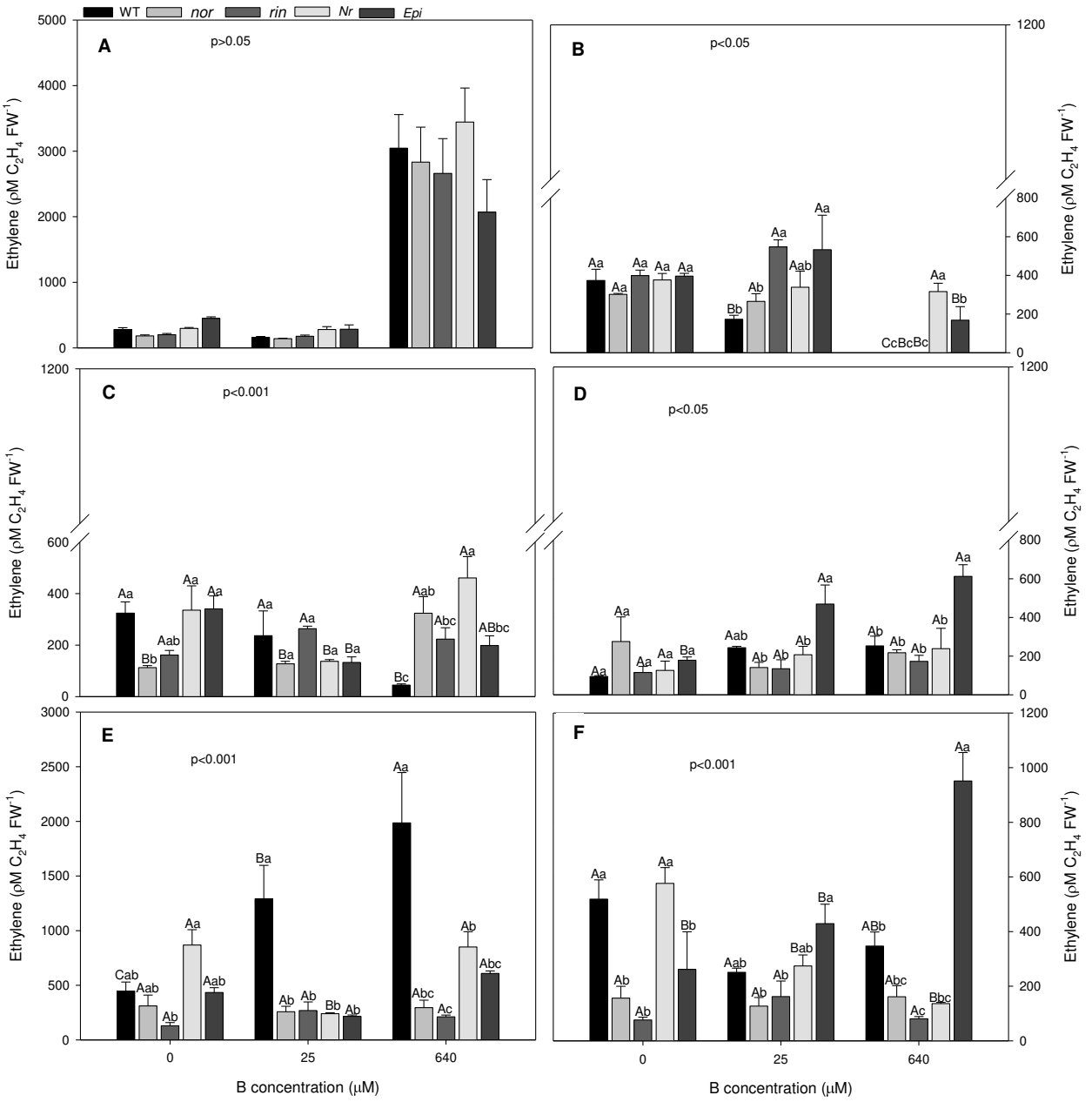


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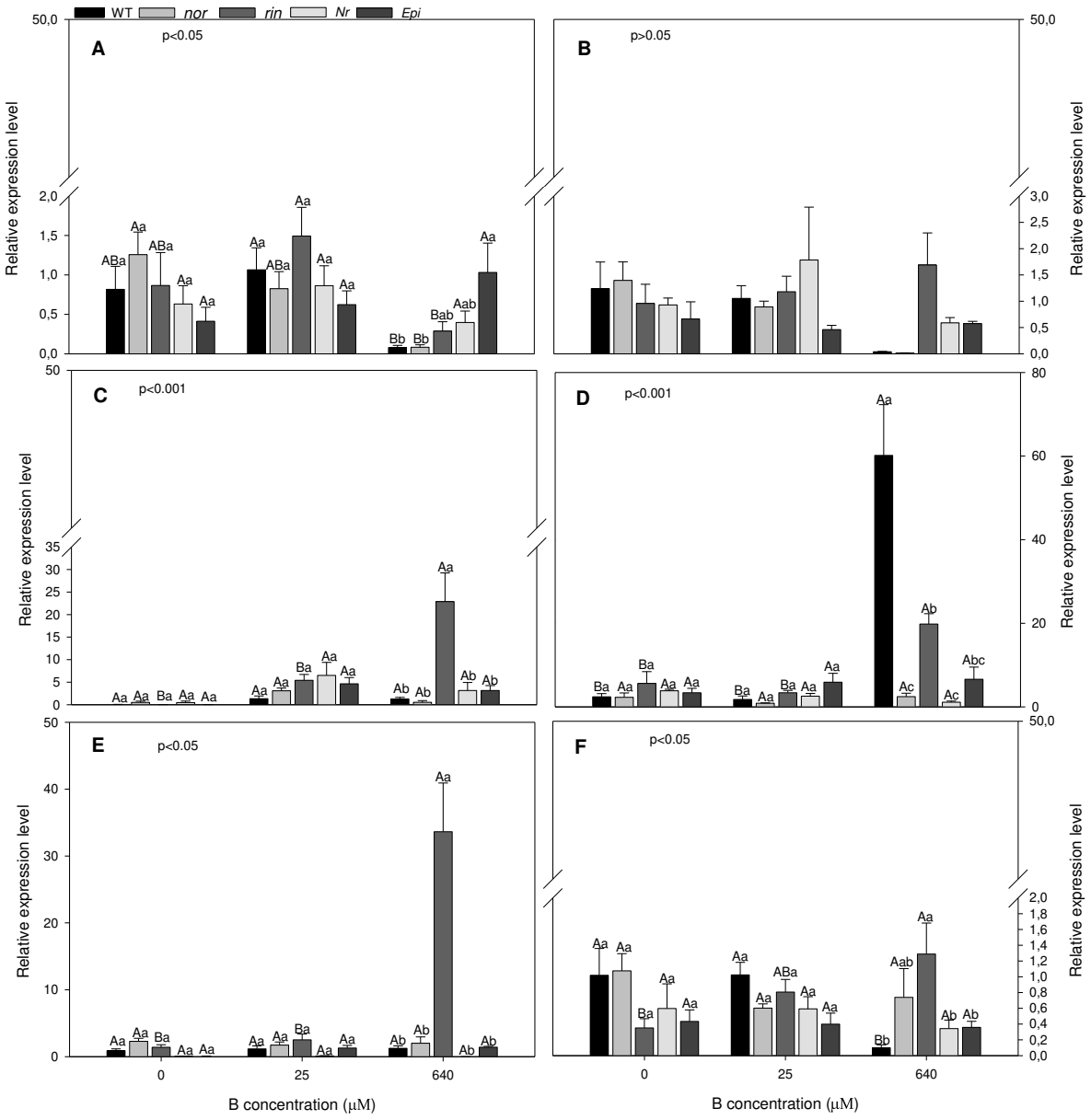
Figure 10: Elevated levels of Boron increased ethylene. (A) Leaves, **(B)** Roots, **(C)** Fruits of 15 days, **(D)** Fruits of 30 days, **(E)** Fruits of 40 days, **(F)** Fruits of 60 days, in tomato plants submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

461

462 To further understand the impacts on ethylene production in response to B, we next
463 evaluated the expression of genes related to the ethylene biosynthesis signaling pathway
464 in leaf samples. Briefly, the expression of ETR3, ETR4, ETR5 and ETR6 increased
465 significantly for *rin* under B toxicity conditions, highlighting a significantly higher
466 expression in *rin* compared to the other genotypes (**Fig. 11C, 11D, 11E and 11F**). This
467 fact aside, the expression of the ethylene receptor ETR1 was smaller in *rin*, WT and *nor*
468 under B excess (**Fig. 11A**); whereas there was no difference in ETR2 receptor expression
469 between genotypes and B doses (**Fig. 11B, Table S2**). Under appropriate and excess B
470 conditions, the expression of EIN3 increased in *rin*. In addition, the expression of this gene
471 was lower in *nor* and WT under B excess (**Fig. 11G**). The expression of the ACO1 reduced
472 under B toxicity in both WT and *nor* (**Fig. 11H**). High level of ACO4 expression was verified
473 in *rin* compared to other mutants (**Fig. 11I**). Regardless of the genotype, under B excess
474 conditions, significantly increased expression of ACS2 was observed (**Fig. 11J**).



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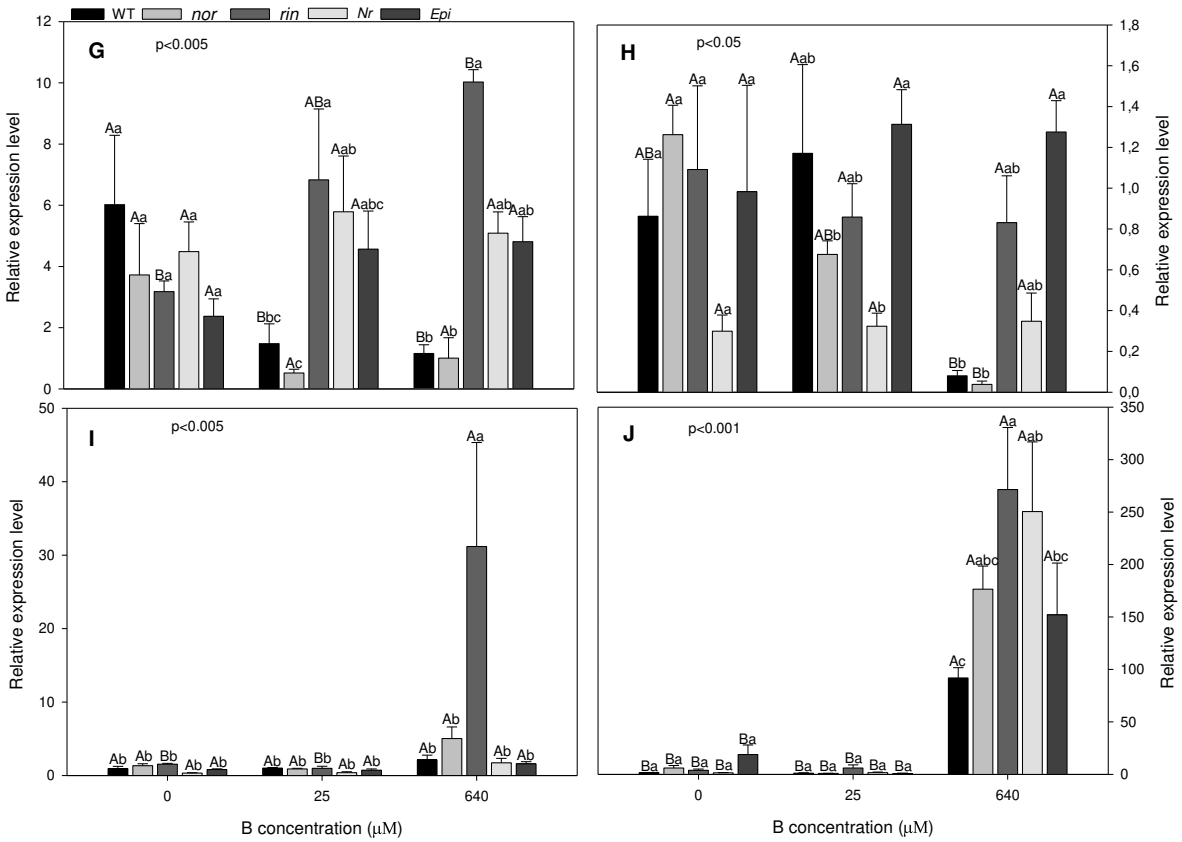

Figure 11: Changes caused by Boron levels in genes related to biosynthesis and perception of ethylene. (A) ETR1, **(B)** ETR2, **(C)** ETR3, **(D)** ETR4, **(E)** ETR5, **(F)** ETR6, **(G)** EIN3, **(H)** ACO1, **(I)** ACO4, **(J)** ACS2, in tomato plants submitted to different B availability. Bars represent means ± standard error (SE). Average values were compared by Tukey test ($p \leq 0.05$). When the interaction was significant, the factors were dismembered. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were further submitted to principal factor analysis (**Table S2**). WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).

Ethylene overexpression and boron excess genotype induce mass loss in post-harvest quality of tomato fruits

During storage, the response of genotype and B dose to the weight loss percentage of tomato fruits is clearly verified. The behavior of the different treatments was similar, that is, the weight loss is gradual as a function of time. Excess B emphasizes weight loss over time for WT and *Nr* (**Fig. 12A and 12D**). *nor* and *rin* genotype remained more stable over time, and they had less weight loss regardless of B level (**Fig. 12B and 12C**). Of the genotypes evaluated, *Epi* presented the highest percentage of loss (**Fig, 12E Fig S.**).

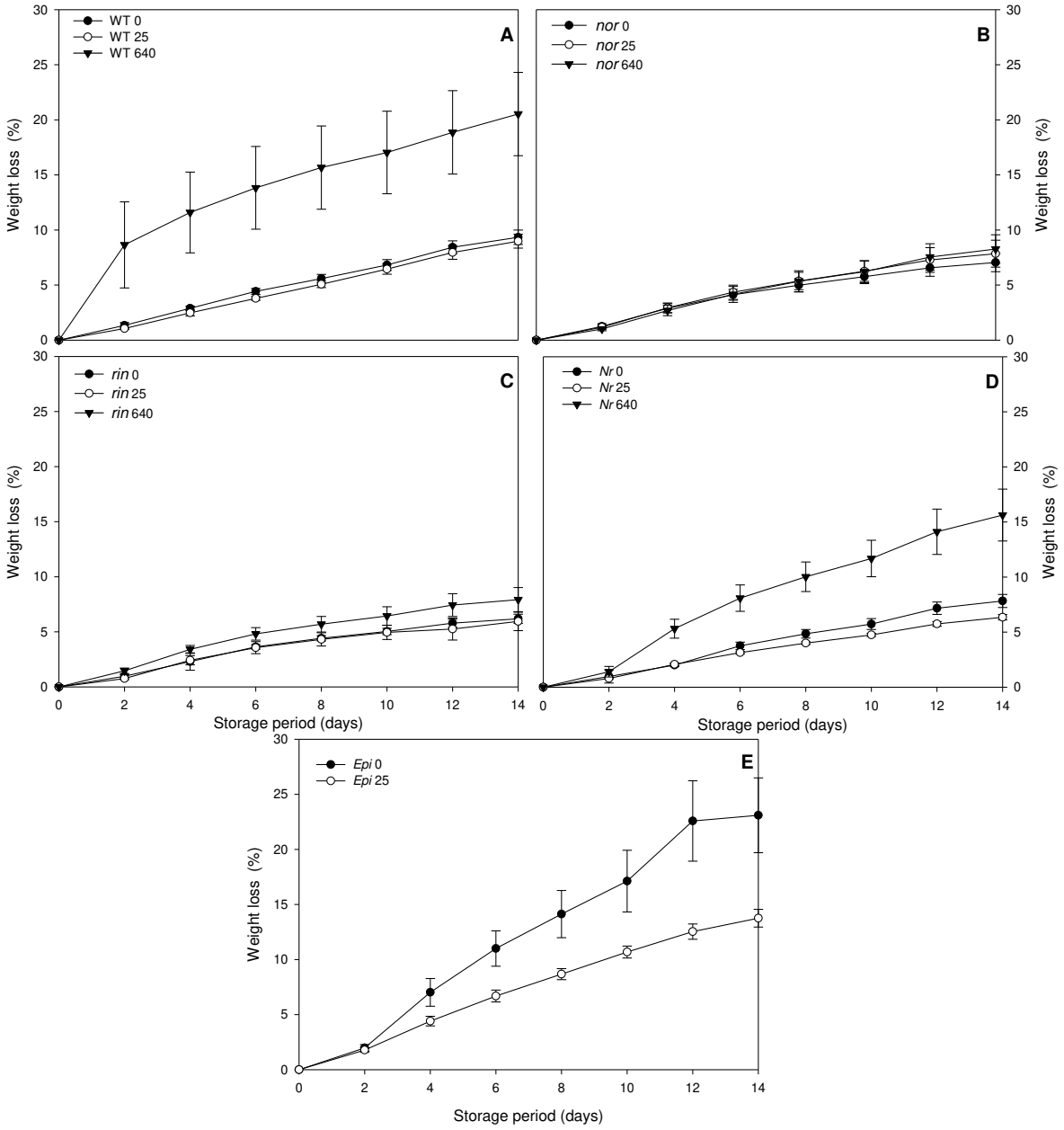

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Figure 12: Boron levels alter the conservation of tomato fruits intermediated by ethylene. (A) WT (wild-type), **(B)** *nor* (non-ripening), **(C)** *rin* (ripening inhibitor), **(D)** *Nr* (never ripe), **(E)** *Epi* (epinastic), in tomato plants submitted to different B availability. Bars represent means ± standard error (SE).

488 **Boron excess compromises fruit morphology drastically affecting crop yield**

489 To further understand the impacts of B we next evaluated fruit morphology by its
490 intrinsic characteristics due to the economic importance of tomatoes. Our results

demonstrated that under adequate B conditions fruits were characterized by greater pericarp thickness compared to B toxicity particularly in the genotypes *rin*, *Nr* and *Epi* and it was followed by fruit growth as a whole (**Fig. 13**).

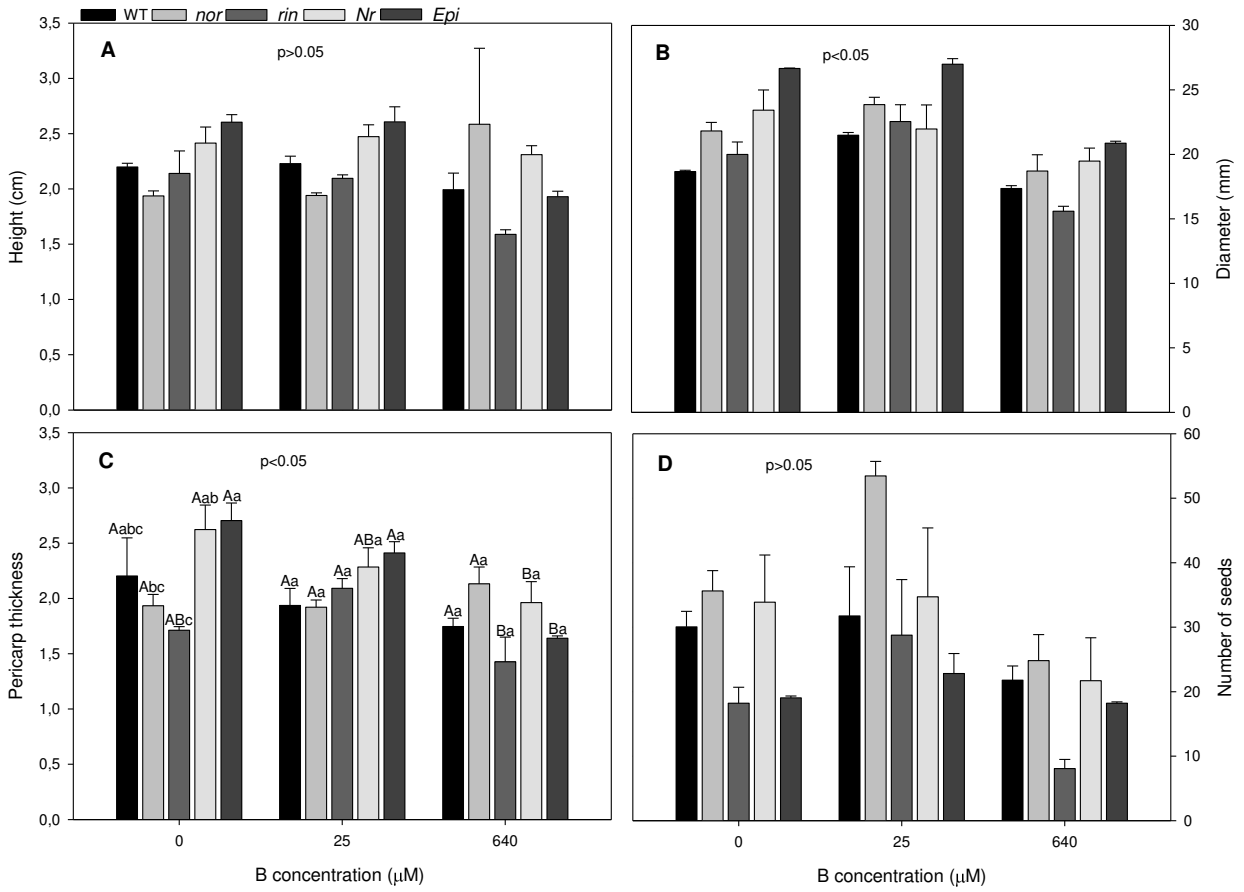

Figure 13. The significance of Boron for tomato fruit shelf life. (A) Length**, (B)** Diameter, **(C)** Pericarp thickness, **(D)** Number of seeds in five tomato genotypes submitted to different B availability in hydroponic system. Bars represent means ± SE. Means were compared by Tukey test ($p \leq 0.05$). When the interaction was significant it dismembered the factors. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were submitted to principal factor analysis (**Table S2**).

In order to understand whether the effect of B and ethylene was maintained until the end of the tomato cycle, we further evaluated the fruit yield. Our result clearly demonstrated that the absence of B did not lead to significant impact on yield, yet a reduction in *Epi* was observed. (Fig. 14). Nevertheless, the impact of excess B on fruit

yield for all genotypes was considerable leading to reductions in all genotypes, being *rin*, WT and *nor* more sensitive to B excess (**Fig. 14**).

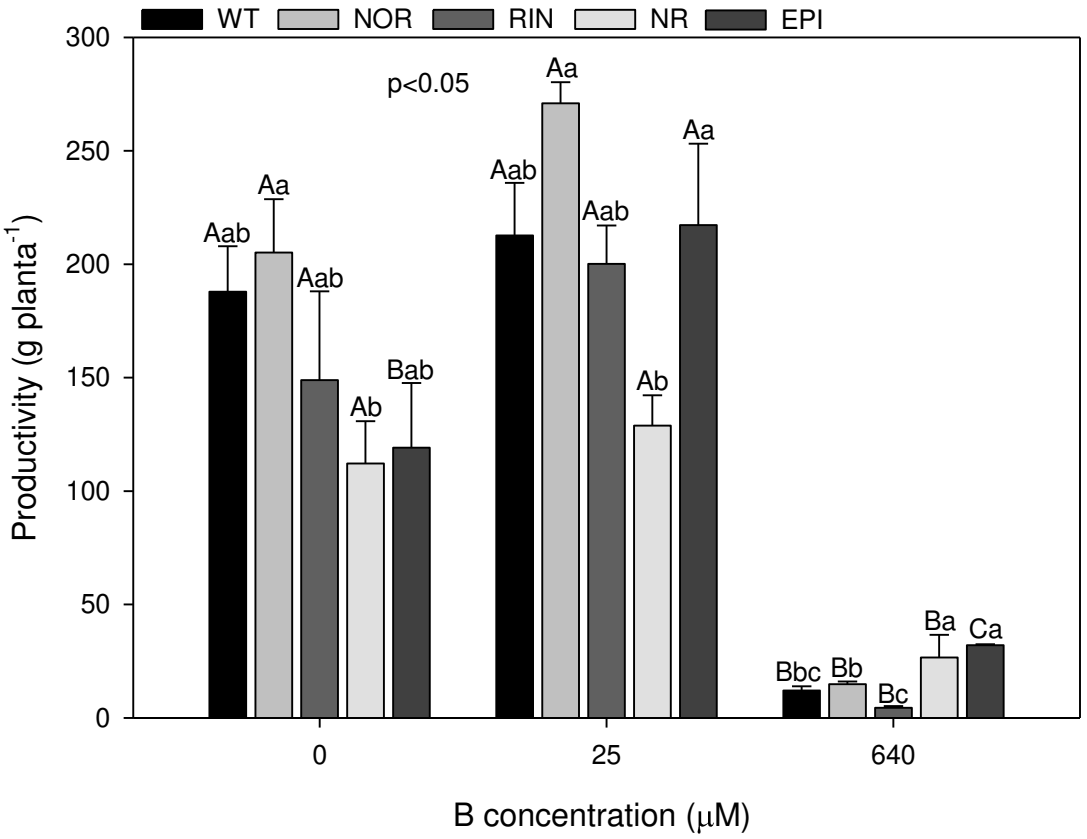

Figure 14. Boron compromise tomato final productivity. Five tomato genotypes submitted to different B availability in hydroponic system. Bars represent means ± SE. Means were compared by Tukey test ($p \leq 0.05$). When the interaction was significant it dismembered the factors. Thus, uppercase letters compare B concentrations within each genotype and lowercase letters compare genotypes within each concentration. Variables that did not show interaction were submitted to principal factor analysis (**Table S2**).

DISCUSSION

Fluctuations in the availability of mineral nutrients may affect hormone level and signaling, and hormones in turn may alter mineral homeostasis, biomass production and metabolism in plants (Song and Liu, 2015). However, the significance of B in both plant growth and metabolism and its connection with ethylene remains largely unknown. Our work demonstrate that there is an intrinsic interaction between ethylene signaling in response to B in tomato. Our results showed that regardless of the tomato genotypes investigated here, when subjected to excess B, all were able to maintain their growth

511 despite the reduction in diameter and root length coupled with greater accumulation of B
512 in leaves compared to the control condition (**Fig. 1**). It is important to mention that despite
513 these results all genotypes clearly presented the typical symptoms of B toxicity (**Fig. S1**),
514 namely mature-older leaves with chlorosis followed by necrosis in lamina tip and margins
515 (Landi et al., 2019).
516 Although the genotypes used in this study did not appear to be hyperaccumulative,
517 our data indicate that they able to maintain a relatively high growth rate despite
518 accumulating B concentrations above the appropriate condition (25 µM) showing that
519 tomato does not have B exclusion mechanism. Similar results were also previously
520 observed (Cervilla et al., 2012). Since the root system is in direct contact with the soil
521 solution it is responsible for the uptake process, it is not surprisingly that to stop or inhibit
522 root elongation is the most immediate response to B toxicity (Sakamoto et al., 2011).
523 Therefore, the major phenotypic effect of toxicity B is associated with inhibition of root
524 growth (Reid et al., 2004; Turan et al., 2009), and compeling evidence suggest that this
525 inhibition results from DNA damage (Sakamoto et al., 2011).
526 We also performed a detailed analysis of the impact of B and ethylene signaling
527 manipulation on root morphological characteristics (**Fig. S2**). Our results demonstrate that
528 all analyzed attributes were affected in response to both B and genotypes (**Fig. 2**). The
529 *Nr* genotype strongly responded to changes in B availability, with reduction of all
530 parameters under either deficiency or toxic B conditions. Ethylene interaction with many
531 developmental processes has been demonstated (Van de Poel et al., 2015). In both
532 Arabidopsis and tomatoes, ethylene caused a reduction in primary root elongation and
533 inhibited initiation and elongation of lateral roots (Negi et al., 2008, 2010). It has been
534 previously proposed that B deficiency increases root ethylene levels by inhibiting root
535 growth via an enhanced auxin levels in certain root areas (Stepanova et al., 2007,
536 González-Fontes et al., 2015). Our results also demonstrated that plants are more
537 sensitive to B excess as it can be deduced by the reduction of most root morphological
538 characteristics. Plants can cope with poor nutrient availability conditions by triggering
539 physiological and developmental responses designed to increase nutrient acquisition that,
540 in many cases, alter the morphology and metabolism of the entire plant (Lopez-Bucio et

al., 2002). Our results demonstrated that under B deficient conditions tomato plants maintain normal root growth and increase root volume in relation to the adequate B supply condition and as such there is an increase in the total absorbent surface of the roots (Lopez-Bucio et al., 2003). Such adjustments can be attributed to root morphological plasticity and allow plants to adapt to unequal distribution of soil resources (Sultan, 2000). Root diameter decreased under B excess, indicating that B toxicity significantly inhibits B growth (Choi et al., 2007; Sheng et al., 2010; Reid, 2007).

The maintenance of growth observed in response to fluctuation in B is seemingly not related to impacts on photosynthesis once reductions in CO_2 uptake under B deficiency and toxicity conditions were observed (**Fig. 4A**), while dark respiration remained unvariant regardless of B levels (**Fig. 4F**). It seems reasonable to assume that the maintenance of growth under stressful conditions is most likely related to the generation of energy from respiration providing further evidence for the respiratory carbon as an important component in the entire plant carbon balance (Pyl et al., 2012, Ribeiro et al., 2016). Further evidence for this assumption comes also from the crucial role that mitochondrial respiration plays in maintaining the optimal rate of photosynthesis (Araújo et al., 2014; Nunes-Nesi et al., 2008), indicating that plants optimize carbon and nutrient allocation to maximize photosynthesis and growth (Plaxton and Podesta, 2006; González-Meler et al., 2009). Our results also revealed that *nor* and *rin* plants are more sensitive to either deficiency or toxicity of B associated with lower photosynthetic capacity and transpiratory rates, which are probably due to their reduced stomatal conductance (**Fig. 4A, 4B, 4D**).

Mechanisms dedicated to dissipating excess excitation energy from photosystems are required under stress conditions to protect the chloroplast from oxidative damage. Our results shown that both *nor* and *rin* increased NPQ (**Fig. 5F**). NPQ is an estimate of the PSII heat loss rate, mainly representing the dissipation of LHCII thermal energy via zeaxanthin (Gallé et al., 2007; Li et al., 2010). The increase in q_h indicates higher efficiency of heat dissipation due to the increase in the proton gradient between the lumen and chloroplast stroma (Genty et al., 1989; Maxwell and Johnson, 2000). This process was likely enough to decrease the electron transport rate (ETR) in these genotypes (Han et al., 2009) and this might be important for maintain growth .

High levels of B reduced SPAD index and NO_3^- concentration while deficiency increases NO_3^- concentration (**Fig. 6A and 6B**). We further demonstrated here that we B toxicity compromises the integrity of the membranes. In fact, it has been suggested that lower NO_3^- concentration in tomato plants (Cervilla et al., 2009) can be due to electrochemical changes in the plasma membrane. On the other hand, increased nitrate and nitrite reductase activity reducing NO_3^- has been also observed (Cervilla et al., 2009). The preservation of the integrity and function of the cell membrane under stress is considered as plant tolerance mechanisms (Niu and Xiang et al., 2018). Accordingly, we found that B deficiency did not affect membrane integrity unlike toxicity (**Fig. 6C, Table S2**).

To further understand the connection between ethylene and B in modulating plant growth responses we performed a detailed analyzes of primary metabolism. No abrupt changes in chlorophyll content were found, although the *nor* and *Epi* reduce the chlorophyll *a* content under B excess, which may be indicative of reduced synthesis or increased oxidative processes (Landi et al., 2013). Reductions in total chlorophyll can be attributed to a decrease in chlorophyll *a* with no changes in chlorophyll *b* (**Fig. 7**). Given that chlorophyll *b* function is mainly to gather light energy and transfer it to chlorophyll *a* (Duysens, 1951; Tanaka and Tanaka 2011), our result cannot explain the damage to the PSII by reducing F_v/F_m following B excess (**Fig. 5A**), as there is no reductions in chlorophyll *b*.

Small changes in amino acid levels in plants cultivated with B excess were observed despite no changes in protein levels. Proteins are amino acid reservoirs, so amino acids are subsequently recycled and allocated for protein synthesis (Hildebrandt et al., 2015). Therefore, the maintenance of protein levels could be able to supply energy sustaining overall plant growth. Moreover, proline acts as an osmoregulator and is accumulated under stress conditions (Szabados and Savoure, 2010). Surprisingly, in this work proline is reduced and not sensitive to changes in B levels. The amino acids Serine and Threonine are reduced under excess of B, most likely due to the fact that their synthesis are metabolically expensive and that probably will not play a role during amino acid catabolism (Hildebrandt et al., 2015).

Regulation of carbon metabolism is important as it is known that synthesized and accumulated starch must be remobilized to allow continued sucrose export and growth (Gibon et al., 2009; Pilkington et al., 2015). Thus, the amount of carbon available for growth is the result of a balance between net photosynthesis, starch accumulation and various carbon-containing metabolites and their remobilization (Sulpice et al., 2014). We observed that despite no change in starch content there was change in soluble sugars, possibly inducing lower assimilation of CO_2 by a negative feedback process. Intriguingly, *Nr*, *nor* and *rin* did not modify the sugar content in response to B levels. Therefore, it seems reasonable to suggest that in response to B there is an exquisite metabolic adjustment and that this change is likely modulate by the ethylene perception. Our work therefore reveals that modification in ethylene are responsible for changes in plants responses under B excess conditions and that *nor* is quite contrasting to with the others mutants (**Fig. 8B**).

Given that ethylene production may be influenced by nutrient deficiency or toxicity conditions (Maksymiec, 2007; Tian et al., 2009; García et al., 2015), we next asked whether increasing ethylene can mediated tolerance to extreme conditions of B. To this end, we further evaluated ethylene production and the expression of genes related to signaling and biosynthesis of this phytohormone. In leaves, B excess stimulated ethylene production while led to inhibition in the roots. Nevertheless, the levels of ethylene increased in roots of *Nr* under B excess. Interestingly, we observed that this ethylene-insensitive mutant is less sensitive to high B concentration than the WT and the other ethylene mutants, as it can be deduced by the relatively minor morphological changes of the root system of *Nr* (**Fig. 2**). It seems tempting to suggest that the roots of *Nr* are not able to effectively sense B excess conditions.

A potential model proposed previously (González-Fontes et al., 2015) explained how *A. thaliana* seedling roots respond to B deprivation for a relatively short time and how ethylene is associated with this response. This model indicated that B deficiency triggers an increase in ACS gene expression (ACC synthase) and consequently in ACC and ethylene levels. This increase leads to an auxin response in roots, which in turn results in a decrease in root length (González-Fontes et al., 2015). By sharp contrast to our results,

631 we did not verify such increase in ethylene in the roots, possibly due to the prolonged
632 condition of B deficiency, which may have caused other significante changes in plant
633 response as a means to tolerate B deficiency, which is in good agreement with the
634 maintenance of growth root.
635 The highest ethylene production reported under B toxicity was supported by up-
636 regulation of ethylene signaling and synthesis genes. Thus, we found changes in
637 expression levels in mature leaves of the receptor (ETR family) and of the ethylene
638 signaling cascade component (EIN3). In agreement, *rin* plants incresed the expression of
639 these genes under B excess (**Fig. 11**), suggesting the existence of redundancy or
640 compensation mechanisms between the ethylene receptor. In addition, these results
641 coupled with other evidence that the observed physiological changes may be due to
642 ethylene. By further analyzing the expression of genes associated with ethylene
643 biosynthesis we have shown that there is increased expression in ACS4 for *rin* and ACS2
644 for all genotypes subjected to B excess. It seems reasonable to assume that high leaf
645 ethylene production is positively regulated by increased ACS2 expression (**Fig. 11**).
646 In the vast majority of plant species, the need for B during reproductive growth is
647 much higher than during vegetative growth and as such extreme conditions of B
648 availability may compromise fruit quality and productivity (Loomis and Durst, 1992; Günes
649 et al., 2003; Shaaban et al., 2006). Thus, we further evaluated the agronomic
650 characteristics of tomato fruit and overall plant productivity. B toxicity strongly affected
651 tomato fruit quality and yield, while minor changes in response B deficiency were
652 observed. These results revealed that the thickness of the pericarp of mature fruits of *Nr*
653 and *Epi* decreased in excess of B compared to that of control fruits (**Fig. 13C**). This
654 difference may well explain the water loss in these fruits, which was observed after harvest
655 (**Fig. 12D, 12E**) (Czerednik et al., 2012). In addition, excess B caused a drastic reduction
656 in yield, indicating a toxic effect as observed in the present study for the vegetative phase,
657 being *Nr* and *Epi* less sensitive to excess B compared to other genotypes (**Fig. 14**) (Keren
658 and Bingham, 1985; Papadakis et al., 2003; Landi et al., 2013).
659
660

CONCLUSIONS

Our results demonstrate that to cope with high B levels tomato plants require an exquisite reconfiguration of metabolism to maintain the necessary energy production and thus sustain overall vegetative growth. We also demonstrate that ethylene appears to be involved in this response by showing that following B excess ethylene is seemingly part of the response to leaf toxicity. Thus, under prolonged conditions of B excess, plants suffer toxicity favoring ethylene biosynthesis, which is associated with cell death and induction of leaf necrosis. However, the absence of changes in plant growth is most likely due to the respiration coupled with the reduction in photosynthesis caused by a negative feedback associated with the accumulation of starch in the leaves. It is equally important to mention that the distinc genotypes investigated here differently respond to the toxic conditions of B and that both *nor* and *rin* are more sensitive to such changes. We cannot assume that the mechanisms that regulate cellular homeostasis following excess of B are mediated by transporters given the large accumulation of B in leaves. Remarkably, the impacts of toxicity were able to drastically reduce productivity and fruit quality. When considering reproductive characteristics, both *Nr* and *Epi* produced more ethylene at 40 and 60 DAA, which favored the loss of mass through the lower thickness of the pericarp (**Fig. S3**). It seems reasonable, therefore, to assume that ethylene is involved in B responses increasing ACS expression and consequently leaf ethylene expression. Thus a complete understanding of the interaction between ethylene and B would provide new strategies for qualitatively and quantitatively improving crops in an environment of B-level fluctuations. Due to the significance in plant fitness, understanding the mechanism of which plants accurately maintain the balance of defence and growth under distinct B conditions will strengthen our knowledge on plant development and will facilitate optimization of agricultural strategies for crop yield improvement.

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Supplemental Data

Supplemental Table 1. Primers utilized to qRT-PCR

SGN ID	NAME	FORWARD	REVERSE
Solyc12g01330	**ETR1**	GGCATTCCTGGACGTGCAAATG	TTTGGTTACCATCCTGCTAACACC
Solyc07g056580	**ETR2**	TGGCATTCCTGGTCGCTTAAATG	CTTCGTTACCATCCTGCTAAACCC
Solyc09g075440	**ETR3 (Nr)**	TGTTCCCTATGCCAAGTGATGGC	ACACCCAGTATCTCTAACCTGGAC
Solyc06g053710	**ETR4**	TGTGCAGAAAGCTGGTTCAGTTG	AGTTGAAGCCCAAGAACGACAGC
Solyc11g006180	**ETR5**	AGGAAGTAACGGAGGGCTTGAG	TCCCTGCATCATCTGAACAAGC
Solyc09g089610	**ETR6**	GCAGGAAGTTGGTTCATTTGATGC	GGGAATTAACGGCTGCCTTTGG
Solyc01g014480	**EIN3**	TTGCCTCCTAGGCAGCTATGTG	TGGTGCAGCTTTCAGGTACATTC
Solyc02g036350	**ACO1**	AAATCATGAAGGAGTTTGCTGATAAA	TTTTCACACAGCAAATCCAACAG
Solyc07g049550	**ACO3**	ACGGGAAGTACAAGAGCGTGAT	CTAGTGACATCCGAGTCCCATCT
Solyc01g095080	**ACS2**	TGGAGAAAACAAGAGGAGGAAGA	GGCACCACCAGCCATAACA

Supplemental Table 2. Analysis of (ANOVA) of parameters involving five genotypes under deficiency, adequate and B toxicity

F value of ANOVA	Boron	Diameter	Height	Root lenght	*A*	gs	C_i/C_a	*E*	*A /E*
Genotypes	14.99***	41.55***	5.71^{ns}	14.55***	55.31***	29.94***	13.77***	10.11**	5.19**
Boron	850***	184.31***	6.77^{ns}	46.96***	122.54***	6.56**	7.56***	3.66*	1.28^{ns}
Boron x Genotypes	13.12***	4.32***	3.59^{ns}	1.46^{ns}	11.43**	2.88*	0.99^{ns}	1.47^{ns}	0.77^{ns}
C.V (%)	18.02	7.39	10.81	12.38	9.94	24.48	5.88	23.81	43.57

F value of ANOVA	R_d	F_v/F_m	F_0	ETR	q_p	q_n	NPQ	YNO	Y(NPQ)
Genotypes	11.30***	37.20***	0.69^{ns}	15.13***	3.75*	5.54**	4.35**	5.11***	2.06^{ns}
Boron	0.11^{ns}	53.07***	3.48*	8.62***	4.52*	3.12^{ns}	2.69^{ns}	8.21***	0.79^{ns}
Boron x Genotypes	0.69^{ns}	18.74***	2.20*	2.26*	1.86^{ns}	2.16^{ns}	1.58^{ns}	2.90*	2.39*
C.V (%)	21.34	1.6	19.67	15.6	20.69	55.38	50.01	16.9	37.25

F value of ANOVA	YII	qL	SPAD value	NO_3^-	Electrolyte leakage	Root lenght	Surface Area	Root diameter	Root Volume
Genotypes	5.16**	1.04^{ns}	120.04***	4.17*	7.94***	22.07***	20.50***	14.67***	47.98***
Boron	8.22***	0.57^{ns}	256.44***	16.14***	6.24**	11.04***	25.26***	92.71***	52.57***
Boron x Genotypes	2.92*	2.79*	7.34***	1.40^{ns}	1.86^{ns}	2.20*	3.55**	4.45***	11.01***
C.V (%)	22.11	43.05	2.38	40.59	15.12	31.5	30.52	23.56	26.6

F value of ANOVA	Forks	0-1 mm	1-2 mm	2-3 mm	3-4 mm	>4 mm	Chlorophyll *a*	Chlorophyll *b*	Total Chlorophyll
Genotypes	46.73***	66.71***	18.61***	0.58ns	20.45***	44.41***	15.81***	14.16***	0.53^{ns}
Boron	20.17***	24.18***	17.56***	11.54***	2.95^{ns}	2.62^{ns}	4.67*	2.97^{ns}	15.25***
Boron x Genotypes	6.11***	8.63***	0.78^{ns}	1.40^{ns}	1.10^{ns}	4.58**	2.71*	1.69^{ns}	0.86^{ns}
C.V (%)	37.53	35.74	53.19	65.47	58.05	45.95	17.97	20.09	18.98

F value of ANOVA	Chlorophyll *a /b*	Proteins	Amino acids	Starch	Glucose	Fructose	Sucrose	Ethylene Leaves	Ethylene Roots
Genotypes	2.48^{ns}	4.01**	4.17**	17.03***	47.52***	60.71***	83.40***	0.893^{ns}	6.13***
Boron	3.05^{ns}	0.59^{ns}	4.71**	6.39*	0.19^{ns}	0.95^{ns}	19.10***	132.24***	33.44***
Boron x Genotypes	1.25^{ns}	1.18^{ns}	4.20**	1.89^{ns}	10.29***	19.54***	15.05***	1.20^{ns}	3.51**
C.V (%)	9.88	17.88	17.19	25.43	28.9	18.38	18.18	52.37	43.75

F value of ANOVA	Ethylene Fruits 15 days	Ethylene Fruits 30 days	Ethylene Fruits 40 days	Ethylene Fruits 60 days	ETR1	ETR2	ETR3	ETR4	ETR5
Genotypes	2.83*	9.82***	20.97***	24.85***	0.45^{ns}	1.75^{ns}	8.73***	16.19***	22.30***
Boron	3.60*	6.97**	7.97**	2.73^{ns}	7.12**	2.70^{ns}	11.41***	30.68***	19.59***
Boron x Genotypes	6.66***	2.81*	5.74**	12.36***	2.49*	1.90^{ns}	6.99***	17.60***	18.31***
C.V (%)	43.64	51.69	56.16	41.31	62.38	72.1	98.86	73.53	99.07

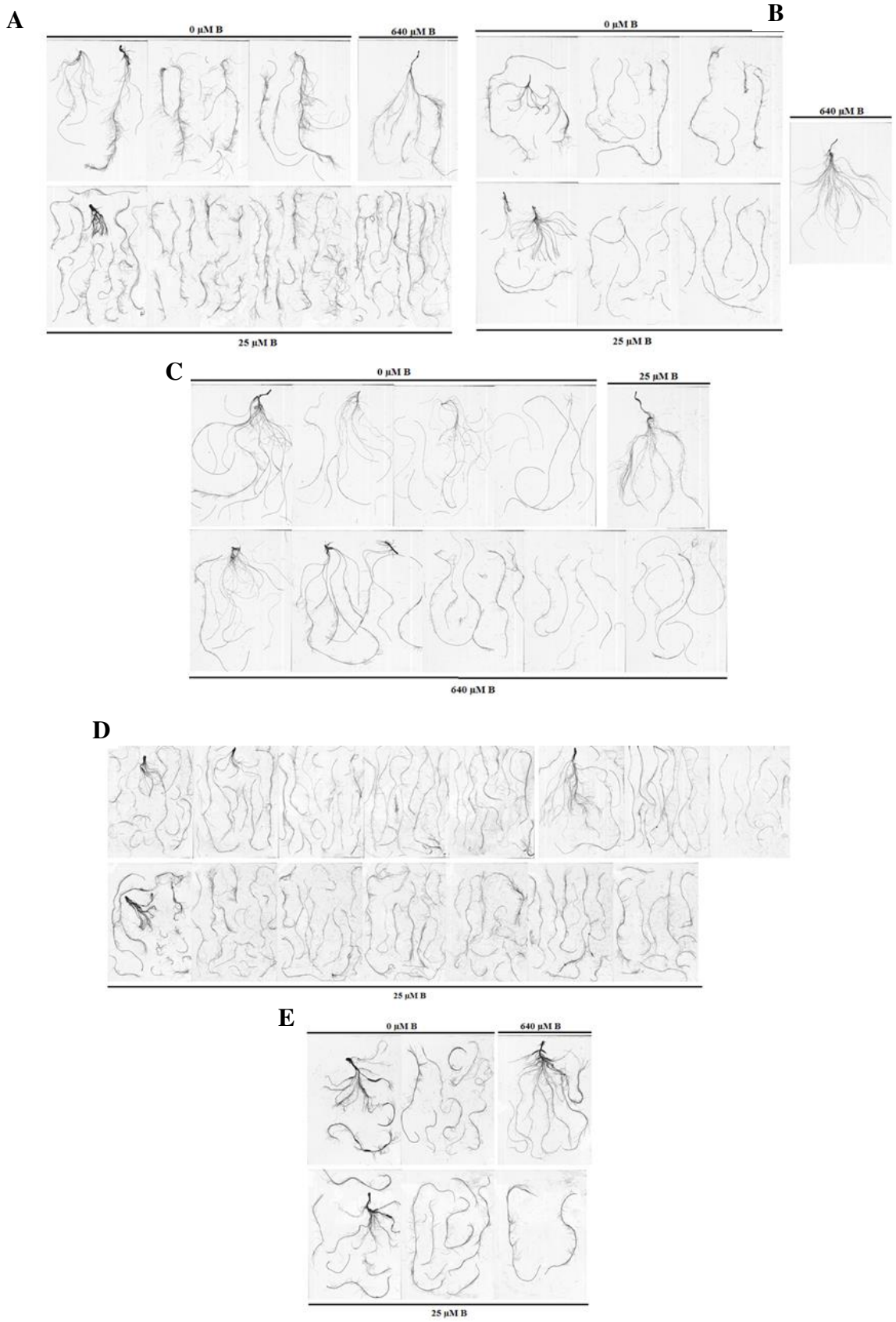
F value of ANOVA	ETR6	EIN3	ACO1	ACO4	ACS2	Fruit Height	Fruit diameter	Pericarp thickness	Number of seeds
Genotypes	2.10^{ns}	7.23***	5.57*	4.40*	2.38^{ns}	2.96*	20.08***	7.53***	7.47***
Boron	0.53^{ns}	0.31^{ns}	4.07*	6.86*	74.52***	1.55^{ns}	43.36***	13.26***	12.00**
Boron x Genotypes	2.81*	3.91*	2.30*	3.96*	2.40*	2.40^{ns}	1.96^{ns}	3.59*	1.01^{ns}
C.V (%)	59.5	52.9	53.95	98.18	72.69	17.47	8.25	14.59	37.47

***, **,* *significant at the level 0.1; 1 e 5 %, respectively by the F test* ^{ns}*, not significan*

Supplemental Table 3. Results of the principal component analysis carried out to synthesise the metabolic variation

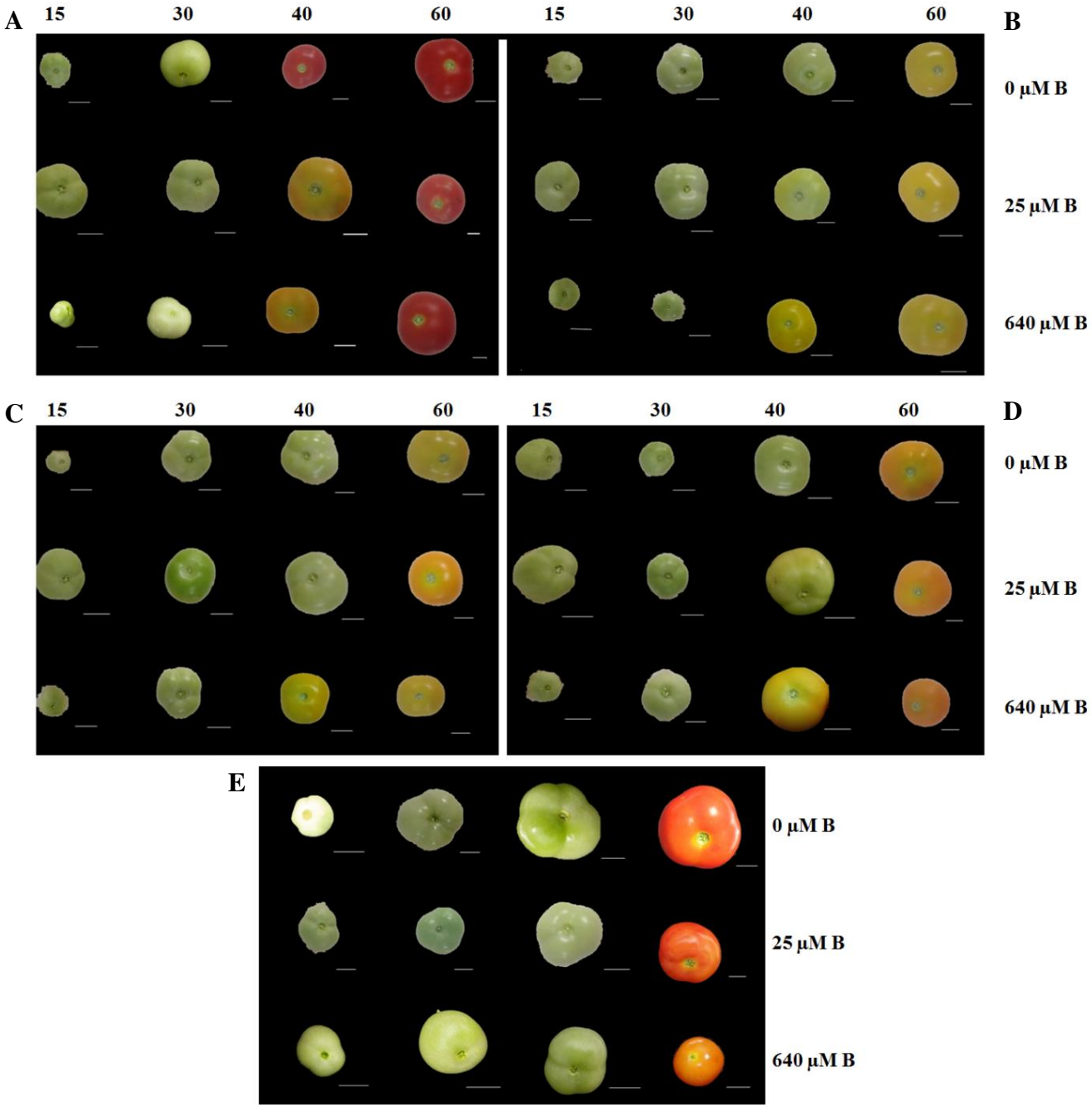
Variable	CP1	CP2
Alanine	0.16	0.09
Alanine, beta	0.20	-0.06
Aspartate	0.11	0.18
Asparagine	0.19	-0.03
Glutamine	0.10	0.19
Glutamine	0.17	-0.05
Glycine	0.12	0.22
Isoleucine	0.19	0.04
Leucine	0.20	0.01
Lysine	0.20	-0.04
Methionine	0.18	0.11
Phenylalanine	0.16	0.17
Proline	-0.18	0.18
Serine	0.07	0.28
Threonine	-0.09	0.28
Tryptophan	0.19	-0.11
Tyrosine	0.21	-0.02
Valine	0.18	0.12
2-Oxoglutarate	-0.16	0.07
Cirate	0.14	-0.09
Fumarate	-0.16	0.19
Glutaric acid	0.14	-0.16
Maleic acid	-0.03	0.29
Malate	-0.10	0.25
Piruvate	0.09	0.20
Succinate	0.07	0.32
Erythritol	0.15	-0.15
Fructose	-0.13	0.17
Galactinol	0.16	0.13
Glucose	-0.17	-0.01
Glyceric acid	0.09	0.13
Inositol	0.10	0.25
Maltose	0.18	-0.06
Raffinose	0.17	0.04
Sucrose	0.17	0.17
Trehalose	0.17	-0.08
Benzoic acid	0.18	0.17
Butyric acid	0.19	-0.06
Dehydroascorbic	0.21	0.02
Putrescine	0.18	-0.08

Supplemental Figure 1. Phenotypic chances of WT and ethylene mutants in response to different levels of Boron. WT (wild-type), *nor* (non-ripening), *rin* (ripening inhibitor), *Nr* (never ripe), *Epi* (epinastic).


A
0 µM B
640 µM B
25 µM B
B
0 µM B
640 µM B
25 µM B
C
0 µM B
25 µM B
640 µM B
D
25 µM B
E
0 µM B
640 µM B
25 µM B

1096

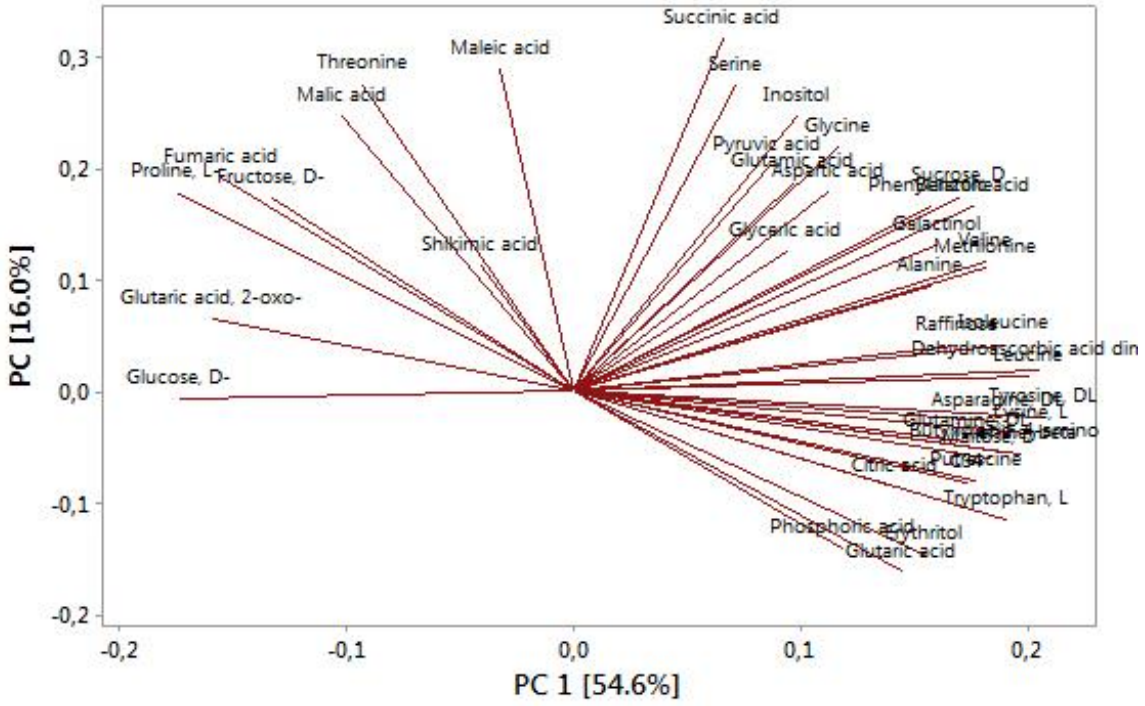
Supplemental Figure 2. Boron alters root system architecture characterization ethylene mediated. Root images obtained by WinRHIZO software. (**A**) WT (wild-type), (**B**) *nor*, (non-ripening) (**C**) *rin* (ripening inhibitor), (**D**) *Nr* (never ripe), (**E**) *Epi* (epinastic).



1097

Supplemental Figure 3. Fruit ripening phenotype WT and ethylene mutants under different availability conditions of Boron. Expression profiles in tomato genotypes. (**A**) WT (wild-type), (**B**) *nor*,

(non-ripening) (**C**) *rin* (ripening inhibitor), (**D**) *Nr* (never ripe), (**E**) *Epi* (epinastic). Fruits are shown at 15, 30, 40 and 60 days after anthesis (DAA).



Supplemental Figure 4. Principal component analysis (PCA) graph showing data distribution in relation to measured metabolic variables.

CONCLUDING REMARKS

The ability of plants to properly respond to fluctuations in the soil concentrations of nutrients is of essential relevance for plant survival. The work presented here was largely focused in understanding how Boron (B), an essential micronutrient for plant growth and development (Warington 1923) impacts plant growth and leaf carbon metabolism as well as to investigate the functional role of the ethylene in mediating this response. Given that plant development largely depend on the adequate availability of B, to further understand the plant responses to B deficiency and toxicity coupled with physiological and metabolic tolerance mechanisms are of interest. This is particularly true in the case of micronutrients, such as B, especially considering the narrow range of the optimal concentration. Thus, the last part of this thesis synthesizes the main findings of this work and the main challenges and perspectives in understanding B function during plant development are briefly discussed.

Recent years have witnessed a growing body of exciting research that clearly improve our understanding of the role of B in plants. This fact aside, Lewis (2019) recently questioned the essentiality of B by revisiting current experimental evidence and finally argued that B is a rather toxic element and thus not essential, with indirect positive effects. Notably, the obvious symptoms of B deficiency usually described are, in fact, toxic effects of phenolic compounds that are not complexed under low supply conditions of B. Nevertheless, compelling evidence supports an essential role of B in mediating plant development programs. Consequently, the presence of such dual point of view should actually stimulate new experimental focus that would allow further characterization of the biological significance of B in plants. It seems reasonable to posit that the development of new experimental tools to accurately determine B levels and sites of action *in vivo*. It seems clear that significant further development is still required to make such approaches more accessible to plant biologists and soil science community in general.

Significant advances have been made during recent years in an attempt to identify regulatory pathways that control plant responses to extreme conditions of B availability. By using a combination of morphological, physiological, metabolic and genetic

approaches we were to provide several novel aspects of the influence of B on plant growth and primary metabolism. The results obtained indicated that adequate levels of B are required to sustain growth responses once either high or low levels of B significantly altered overall leaf metabolism. Briefly, changes in carbon assimilation, carbohydrates turnover, and an interesting reprogramming of metabolites levels were observed in response to fluctuations in B levels. Thus, we had obtained circumstantial evidence that the difference in photosynthesis under conditions of low B supply is likely mediated by significant changes in primary metabolism.

Yet, our current understanding of how plants sense fluctuations on B levels by regulating growth and development under such conditions remains rather fragmented. We hypothesized that ethylene connect B responses and decided to next investigated the physiological, molecular, and biochemical responses using tomato (*Solanum lycopersicum*) mutant plants. The results described here suggest that ethylene directly participates in the regulation of B deficiency and toxicity responses by triggering morphological, physiological, and metabolic changes. Notably, growth inhibition in response to B is likely associated with decreases in carbon assimilation due to both reduced photosynthesis rates and stomatal conductance, coupled with impairments in carbohydrates turnover. Moreover, B is able to affect ethylene biosynthesis, most likely by acting in enzymes related with its biosynthesis. At the same, B impacted carbon assimilation and culminated with significant changes in central metabolism. We emphasize that these findings must be integrated at the whole plant level to fully understand such mechanism allowing plants to cope with fluctuations in B homeostasis. It is important to mention that the differential behavior of *nor* and *rin* mutants is likely associated with a higher sensitive to changes in B levels. Therefore, ethylene signaling is seemingly used during responses to contrasting B concentration as one way to adjust the tolerance of these plants. Further investigations are clearly required to fully assess the significance of biochemical and molecular changes that allow plants to maintain growth under conditions other than B that would otherwise be fatal. This fact aside, it seems reasonable to assume that results presented here are fundamental for understanding B-mediated signaling responses.

In summary, a comprehensive dataset of plant responses following B toxicity and deficiency is here provided. We have shown that (i) such responses are likely associated with changes in primary metabolism and (ii) that ethylene mediates the regulation of plant growth and development by altering root morphology by attenuating root length under excess B whereas under B deficiency changes in root system architecture likely help to improve B acquisition. Our study sheds light on the intrinsic relationship between B and ethylene. Perhaps more importantly this study not only helps to dissect the response mechanism of plants to B excess or deficiency but also paves the way for a full understanding of the interaction between ethylene and B by providing new strategies for improving crop vigor and development in an environment of changing in B levels. Although future work is required to spatially and temporally characterize B-induced or repressed responses and dissect the connection with ethylene, it seems reasonable to suggest that molecular interactions as well as additional genes involved in B homeostasis are likely present. Dissecting these mechanisms is clearly required to fully understand the key components underlying ethylene responses and its connections with energetic processes in fruit growth and development.

Further investigations on the mechanisms allowing plants to cope with distinct environmental conditions would help clarify how they evolved B-resistance mechanisms. Our hope is that the results presented within this thesis will introduce the current need to involve researchers beyond the soil science and plant physiology (e.g. molecular biologists, cell biologists, evolutionary biology), who may have interest in understand this unique and not boring at all microelement to address long-standing questions and recent developed issues in B stress physiology.