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Antioxidant and Photosynthetic Responses in Plants Under Boron Toxicity: A Review

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ABSTRACT

Boron (B) is a micronutrient that has long been known essential not only for plants and animals. Otherwise, in crop plants the range of B concentration from essentiality to toxicity appears extremely narrow. Although B deficiency has been well investigated, during recent years many researchers have also investigated boron toxicity effects, making progress in the comprehension of boron transport within plants. Moreover, worldwide B is not uniformed distributed and, especially in arid and semiarid regions, high B concentrations are found in the soil. Boron in plants primarily moves following transpiration stream even though recent evidences have also confirmed the involvement of B transporter and channel (BOR and NIP). In general, the species that tolerate B excess tends to accumulate less B compared to sensitive species. Likewise other abiotic stresses, antioxidants may be involved in the scavenging of Reactive Oxygen Species (ROS) triggered by B toxicity. Photosynthesis parameters in plants grown under high B supply can draw the photosystems situation and the reactions to B toxicity drawing the healthy status of the plants evaluated. Although boron deficiency has been well investigated, during recent years many researchers have also investigated boron toxicity effects, making progress in the comprehension of boron transport within plants. This review represents the actual state of knowledge concerning B toxicity.

Keywords: Reactive Oxygen Species (ROS), Adenosine Triphosphate (ATP), Nicotinamide Adenine Dinucleotide Phosphate (NADPH), Glutathione Reductase (GR)

1. INTRODUCTION

1.1. Chemical Identity of Boron

Boron is a metalloid element from Group IIIA of the periodic table with an atomic number of 5, an atomic weight of 10.81 g mol^{-1} and an oxidation state of +3. Boron naturally exists as 19.78% ^{10}B isotope and 80.22% ^{11}B isotope (WHO, 1998). Boron is electron-deficient, possessing a vacant *p*-orbital; it does not form ionic bonds, but it forms stable covalent bonds. Boron compounds often behave as Lewis acids, readily bonding with electron-rich substances. It has intermediate properties between nonmetals and metals.

Elemental B naturally occurs and is found in borax ore or tincal ($Na_2B_4O_7x10H_2O$), boric acid (H_3BO_3), colemanite [$CaB_3O_4(OH)_3$-H_2O], kernite or rasorite ($Na_2B_4O_7x4H_2O$), ulexite ($NaCaB_5O_9xH_2O$) and borates (salt or ester of boric acid). Boric acid is the main compound presents at neutral pH and in this condition it exists as odorless, colorless, translucent crystals or white granules or powder at ambient temperatures (O'Neil *et al.*, 2004). Boric acid is a weak acid with a pKa of 9.2; therefore, in aqueous solution at physiological pH boric acid primarily exists (more than 98%) as the undissociated acid $B(OH)_3$, as do borate salts (Tanaka and Fujiwara, 2008).

1.2. Environmental and Anthropogenic Sources of Boron

In the environment B primarily derives from the weathering of minerals containing this element (Kot, 2009). Moreover, geothermal steams significantly contribute to natural occurrence of B in the environment. High B concentrations, with an average of 4.5 mg L^{-1} as boric acid, are also recorded in sea water that represents the major source of B contamination in coastal areas due to seawater intrusion into fresh water aquifers. Furthermore, seawater volatilization produces boric acid dissolved in aerosol and boric acid is sometimes found in volcanic spring waters and in the material released by an erupting volcan. Global releases of elemental B through weathering, volcanic and

geothermal processes are estimated at approximately 360,000 metric tons annually (Moore, 1991).

Sources of B resulting from human activities are less important than natural processes. Anthropogenic sources are as follows: agriculture, waste and fuel wood burning, power generation using coal and oil, glass product manufacture, industrial and household use of borates/perborates, borate mining/processing, leaching of treated wood/paper, sewage and sludge disposal (HSDB, 2003). The principal uses for B compounds in the United States in 2001 were estimated as follows: 78% glass and ceramics; 6% soaps and detergents; 3% agriculture; 4% flame retardants; and 9% as other boron compounds (USGS, 2009).

1.3. Boron Concentration is Extremely Narrow in Soils and Waters

Boron is an essential micronutrient for crops and its availability in the irrigation water or in the soil represents an important factor for crops production. The range of B concentrations in soil is extremely narrow (Gupta *et al.*, 1985). In many parts of the world including Japan, China, USA and Brazil, natural B levels are insufficient for crop production since this element is present in the soil as boric acid, which is easily leached out by rainfall due to its high solubility (Bolaños *et al.*, 2004). For this reason, B is frequently added as fertilizer in agricultural systems (Gupta *et al.*, 1985).

On the other hand excess of B may naturally occur in soil or as result of over-irrigation with water rich in B. In arid or semiarid countries, the mainly cause of boron accumulation in the agricultural soil is that the evaporation of groundwater, that reaches the topsoil by capillary. Regions affected by high B level in the soil are in South Australia, Iraq, Egypt, Jordan, Libya, Morocco, Syria, Turkey, Chile and California (You *et al.*, 1995).

1.4. Boron and Salinity: Which Consequences?

As mentioned above B toxicity represents an important limiting factor of crop production in many regions of the world. Furthermore, high B concentration is often found in association with high salinity in arid and semiarid environments (Nable *et al.*, 1997). Indeed, simultaneous stress induced by B toxicity and salinity can occur when crop plants either are irrigated with water containing high levels of B and salts or are grown in saline and B-rich soils. Both conditions may occur in semiarid and arid regions characterized by low rainfall and poor drainage (Marschner *et al*., 1995; Nable *et al.*, 1997). Several researches reported crop responses to the simultaneous excess of B and salinity, for instance in: tomato (Alpaslan and Gunes, 2001; Ben-Gal and Shani, 2002; Guidi *et al.*, 2011), melon (Edelstein *et al.*, 2005), carrot (Eraslan *et al.*, 2007a), bell pepper (Yermiyahu *et al.*, 2008), cucumber (Alpaslan and Gunes, 2001), lettuce (Eraslan *et al.*, 2007b) and spinach (Eraslan *et al.*, 2008). In general, it has been reported that combined B toxicity and salinity causes less severe effects on plant growth than what would be expected if the effects of the separate factors were additive. This result was ascribed to a reduction of both B and chloride uptake as a result of antagonism between these two elements (Yermiyahu *et al.* 2008). It has also been proposed that, under simultaneous presence of B and salt stress, boric acid could affect the activity of specific membrane components (Martinez-Ballesta *et al.*, 2008). In fact, at high external B levels, significant B transport occurs through the plasma membrane aquaporins (Dordas *et al.*, 2000; Dordas and Brown, 2001). Interestingly, high B and calcium supply enhances crop tolerance to salinity and increases yield in saline soils (El-Hhamdaoui *et al.*, 2003; Bonilla *et al.*, 2004).

Although differences in B tolerance reported for species and cultivars, some general conclusions can be drawn: (i) salinity may reduce B susceptibility in many species through a sort of osmotic protection, that is by reducing the root water uptake that in turn decreases B accumulation (Ben-Gal and Shani, 2002); (ii) B improves plant response to NaCl salinity by reducing root uptake and shoot accumulation of Cl (Yermiyahu *et al.*, 2008); (iii) growth and symptoms due to B toxicity appear reduced under saline conditions (Ben-Gal and Shani, 2002; Yermiyahu *et al.*, 2008).

1.5. Boron: From Essential to Toxic Element

In 1923 the essential role of B was demonstrated in *Vicia faba* (Warington, 1923). Since then, B has been considered an essential micronutrient for plant growth as it plays an important role in many metabolic processes (Marschner *et al.*, 1995; Goldbach *et al.*, 2001; O'Neill *et al.*, 2004). Boron is essential not only for plants, algae, cyanobacteria, diatoms also required an adequate concentration of this element to their development (Loomis and Durst, 1992) and also animals such as trout and frog (Rowe and Eckhert, 1999). Deprivation of B in animals can influence their growth due to an inadequate bones development or it can cause others micronutrient-dependent effects. However, its essentiality for human has not been established. On the other hand, it seems established that excessive B intake can be responsible for weight loss and anorexia, diarrhea, neurological and cardiovascular effects reported in mice and rats. Furthermore, B can also cause malformations at skeletal and cardiovascular level on fetal body in pregnant animals (Yazbeck *et al.*, 2005). For this reason the European Food Safety Authority (EFSA, 2004) has established a Maximum daily B Intake (MDI) of 10 mg for adult people considering that the main sources of B in human diet are fruits, vegetables and drinking water (Tanaka and Fujiwara, 2008). In many regions of the world, B concentration in groundwater can be as high as 3-13 mg L^{-1} thus exceeding the maximum acceptable B concentrations established for drinking water in some countries (**Table 1**).

Table 1. Maximum acceptable concentrations for boron in drinking water (Kot, 2009), Reference in the Table are in Kot (2009)

Country/ organisation	concentration (mg L^{-1})	Reference
Canada	5.0	Health Canada (2006)
European Union	1.0	Weinthal *et al.* (2005)
Russia	0.5	SanPiN (2001)
USA	0.3	EPA (1996)
WHO	0.3	WHO (1998)

Concerning plants, excess of B exerts different effects such as: reduced root cell division, lower leaf chlorophyll contents and photosynthetic rates; decreased levels of lignin and suberin, among others (Nable *et al*., 1997; Reid, 2007). Accordingly, a reduced growth of both shoot and root is typical of plants exposed to high B levels (Nable *et al.*, 1990). Although the physiological basis for B toxicity are not clear, three main causes have been hypothesized considering the B chemistry: (i) alteration of cell wall structure; (ii) metabolic disruption by binding to the ribose moieties of molecules such as Adenosine Triphosphate (ATP), Nicotinamide Adenine Dinucleotide (NADH) or Nicotinamide Adenine Dinucleotide Phosphate (NADPH); (iii) disruption of cell division and development by binding to ribose, either as the free sugar or within RNA (Reid *et al.*, 2004). Although growth was rapidly inhibited by internal B concentrations in the range 1-5 mm, this inhibition was not attributable to the effects of B on either energy supplies or inhibition of protein synthesis, but the toxicity to mature tissues was rather due to the accumulated retardation of many cellular processes, enhanced in light by photo-oxidative stress (Reid *et al.*, 2004).

Boron was found to inhibit one step of *in vitro* pre-mRNA splicing reaction (Shomron and Ast, 2003), which suggests that B toxicity is primarily due to disruption of RNA splicing (Reid, 2007). Interestingly, several genes that encode transcription factors of ribosomal proteins conferred B tolerance in lupin (*Lupinus albus*) and *Arabidopsis thaliana* (Reid, 2007) as well as in yeast (Nozawa *et al.*, 2006). These proteins could act as splicing sites protectors from B bound, thus suggesting the occurrence of a mechanism that confers genuine B tolerance other than the ability to efflux B from the cells (Reid, 2007).

1.6. Different Susceptibility to Boron Toxicity

In plants, the range between optimal and toxic B concentrations is extremely narrow (Gupta *et al.*, 1985). Plants tolerance to B toxicity is widely variable from species to species and even among varieties of the same species. For instance, safe B concentrations in irrigation water range from 0.3 mg L^{-1} for sensitive plants (e.g., *Phaseolus vulgaris*) to 1-2 mg L^{-1} for semi-tolerant plants (e.g., *Zea mays* and *Solanum tuberosum*), 2-4 mg L^{-1} for tolerant plants (e.g., *Daucus carota* and *Cuminos melo*) and 4-6 mg L^{-1} for high tolerant plants such as *Solanum lycopersicon* (Nable *et al.*, 1997). In general, B tolerant species can better survive to other salt stress (e.g., NaCl salinity) compared to sensitive plants. Moreover, within the same species more tolerant varieties have an endogenous B concentration lower than less tolerant varieties (Nable *et al.*, 1997; Cervilla *et al.*, 2007; 2012). This behavior appears to be related to the plant's ability to exclude B at root level: a reduced permeability of membrane lipids and/or the presence of carriers (BOR and NIP) essential for B extrusion from the cytoplasm may be responsible for reduced B accumulation (Miwa *et al*., 2007; Sutton *et al.*, 2007).

1.7. Boron: From Roots to Leaves

1.8. Uptake and Translocation Following Transpiration Stream

Boron uptake is considered to be a passive process: It is absorbed from the soil by roots mainly as not dissociated boric acid (H_3BO_3) and then transported to the leaves via the xylem. This is partly due to the high permeability of boric acid to lipid bilayers (Brown and Shelp, 1997; Dordas and Brown, 2001).

In plants, the long distance translocation of nutrient elements takes place in the vascular system consisting of the xylem and phloem. Upward movement from the roots to the shoots occurs in the nonliving cells of the xylem and this process is mainly driven by the gradient in water potential resulting from surface transpiration during the day. Thus, xylem translocation is mainly directed to the mature leaves because they represent the sites with the highest transpiration and, at the same time, they are sites with the lowest demand for nutrients (Pate, 1975).

In contrast, long distance translocation in the phloem, composed by living cells, occurs in both upward and downward directions. Phloem translocation does not follow transpiration stream and supplies the major proportion of nutrient requirements for actively growing areas, such as young leaves, fruits and seeds; all these organs do not readily transpire (Brown and Shelp, 1997). It has generally been accepted that B is an immobile nutrient in the phloem tissues and therefore tends to accumulate in highly-transpiring mature leaves. Kohl and Oertli (1961) demonstrated that B uptake followed the passive water flux from roots to leaves accumulated especially where termination of leaf veins terminate; these tissues show more evident symptoms of B toxicity such as chlorosis and necrosis. According to the previous hypothesis, higher B concentrations were found in leaf tissues than in phloem sap (Shelp, 1987; 1988). Moreover, since B is not efficiently remobilized, the

symptoms of B deficiency and toxicity occur in young and mature organs, respectively.

1.9. Evidence of Boron in Phloem Tissues

Recent findings suggest that the passive mechanism could not be the only mechanism involved in B translocation and tolerance to B toxicity (Chamacho-Cristobal *et al.*, 2008). Brown and Shelp (1997) showed that in some plant species B can be redistributed within the plant and the extent of B phloem mobility significantly varies among plant species. In many plants, including many important crop and tree species (e.g., *Pyrus*, *Malus*, *Prunus*, *Allium* and *Brassica*), B was found to be uniformly distributed under B deficiency (Brown and Hu, 1996); moreover, it was found that B concentration in young leaves was higher compared to mature leaves.

While in plants with low B mobility, the typical symptoms are chlorotic and/or necrotic patches (burn) of the older leaves where B tends to accumulate (Nable *et al.*, 1997), in plant species where B translocation occurs, the symptoms of B toxicity firstly appear in the meristemic regions and fruits and do not occur in mature leaves (Brown and Hu, 1996). Because this pattern of distribution cannot be explained by B distribution along transpiration stream, other possible mechanisms were suggested. These species commonly produce relatively high amounts of sugar alcohols (e.g., mannitol and sorbitol) which are used for the phloem translocation of photosynthates in place of sucrose (Brown *et al.*, 1999). Polyols, such as sorbitol and mannitol, contain *cis*-hydroxyl groups that can readily bind to boric acid originating diol-boron complexes; this binding is likely to allow B to be transported through phloem. Hu *et al.* (1997) showed that B was present as a stable polyol-B complex with mannitol and sorbitol as ligands. Furthermore, plants with high ability to produce sugar alcohols can better tolerate B deficiency than plants with a lower capacity. Transgenic tobacco plants transformed with the sorbitol synthesizing enzyme had higher growth and yield under B starvation compared with wild type (Brown *et al.*, 1999). It was argued that the transgenic lines had higher ability in B remobilization through phloem than control plants suggesting that B can move along the flow of B-binding sugar alcohol (Brown *et al.*, 1999).

Takano *et al.* (2001) found that B was transported via phloem in young leaves also in plants do not produce sugar alcohols, thus suggesting an alternative mechanism of translocation (Takano *et al.*, 2001). In these plants including *Arabidopsis thaliana* (Takano *et al.*, 2001) and *Brassica napus* (Stangoulis *et al.*, 2001), the translocation was reported under B limitation, but not in conditions of normal or luxury B supply. In fact, Tanaka and Fujiwara (2008) showed that an active B transport occurred in plants due to channels and transporters (BOR and NIR); this type of transport is probably enhanced under B deficiency. Since translocation occurs only under B limitation, this suggests that plants are capable of sensing B levels in the growing medium and within plant tissues. Consequently, they are able to regulate both root uptake and vascular movement of B. This regulation is essential to survive under conditions of limited B supply.

1.10. Proposed Mechanisms to Explain Boron Mobility

Several mechanisms are involved in B translocation. The passive diffusion across lipid membranes is mainly due to the high permeability coefficient of boric acid for lipid bilayer and, in the presence of excess B, it represents the main pathway for B transport (Tanaka and Fujiwara, 2008).

On the other hand, under conditions of limited B supply, other mechanisms are necessary to transport B against its concentration (Dannel *et al.*, 2000). BOR1 was the first B transporter found in *Arabidopsis* and it was shown essential under low B supply (Takano *et al.*, 2002). BOR1 is regulated at post-transcriptional level and the protein is degraded under adequate or luxury B supply suggesting a sophisticated regulation of this pathway in order to maintain a constant B uptake under variable growing conditions (Noguchi *et al.*, 2003; Takano *et al.*, 2002; 2005). However, these results suggest that BOR1 is not involved in B tolerance.

Nevertheless, an independent transgenic *A. thaliana* line has been generated showing that BOR4, another efflux-type borate transporter, is not degraded at the post-transcriptional level as occurs with BOR1. Accumulation of BOR4-GFP (Green Fluorescence Protein) and tolerance of B were positively correlated and the overproduction of BOR4-GFP improved plants growth under high B levels through boron efflux.

Furthermore, Takano *et al.* (2006) reported that also another channel, NIP5 was required to *Arabidopsis* to grown under low B supply. NIP5 belongs to Major Intrinsic Protein (MIPs) including aquaporine and it was supposed that this channel was a B importer at root level after the evidence that NIP5-deficient plants had a lower B concentration in roots and a reduced shoots elongation only under low B supply (Takano *et al.*, 2006). At present, nine NIPs have been reported in *Arabidopsis* (Takano *et al.*, 2006) and other members of this family are probably involved in this sophisticate pathway.

1.11. Can Antioxidants Increase Boron Tolerance in Plants?

When plants are subjected to environmental stress conditions such as high light intensity, extreme temperatures, drought, high salinity, herbicide treatment, or mineral disorders, the balance between the production of ROS and the quenching activity of the antioxidants is upset, often resulting in oxidative damage (Wise and

Naylor, 1987; Mittler, 2002). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have a great resistance to this oxidative damage (Dhindsa and Matowe, 1981; Wise and Naylor, 1987; Foyer and Shigeoka, 2011).

Glutathione (GSH) and ascorbate (AsA) are well known antioxidant compounds with recognized radicals scavenging activity even though an increasing number of evidences confirms that other molecules, such as phenols, anthocyanins and flavonoids, can increase antioxidant ability in many plant species especially herbaceous (Zheng and Wang, 2001; Javanmardi *et al.*, 2002; Shan *et al.*, 2005; Surveswaran *et al.*, 2007; Lee and Scagel, 2009). In plants, polyphenolic compounds are typically produced to protect plants against pests and diseases (Herms and Mattson, 1992; Crozier *et al.*, 2006; Mullen *et al.*, 2007), nutrient stress (Herms and Mattson, 1992) and exposure to UV radiation (Rozema *et al.*, 1997). Moreover, anthocyanins, which are responsible for the primary red and blue pigments in plants, have been recognized as contributing to plant growth, protection and development (Holton and Cornish, 1995) and as powerful antioxidant (Tsuda *et al.*, 1996; Gould *et al.*, 2002).

Non-enzymatic antioxidants are represented by enzymes such as, Catalase (CAT), Superoxide Dismutase (SOD), guaiacol Peroxidase (POD) and enzymes directly involved in Halliwell-Asada cycle: Ascorbate Peroxidase (APX) Glutathione Reductase (GR), Monodehydroascorbate Reductase (MDHAR), Dehydroascorbate Reductase (DHAR) (Melgar *et al.*, 2009).

It is really important for plants survival under stress conditions that these antioxidants act synergistically and cooperatively, thus providing better defense and regeneration of the active reduced forms. The most studied example of the antioxidant network is the ascorbate-glutathione (Halliwell-Asada) pathway in the chloroplasts, where it provides photoprotection by removing H_2O_2 (Noctor and Foyer, 1998) (**Fig. 1**). Components of this cycle have been detected in other cell compartments (Jimenez *et al.*, 1998).

Ascorbate works in co-operation not only with glutathione, but also takes part in the regeneration of α-tocopherol, providing synergetic protection of the membranes (Asensi-Fabado and Munné-Bosch, 2011). Tocopherol has been reported to be in direct interaction also with reduced glutathione (Fryer, 1992) and reduced coenzyme Q (Buettner, 1993). Kagan *et al.* (2000) suggested that tocopherol and reduced coenzyme Q, when they are both present in a membrane, showed combined antioxidant activity. Recently, redox coupling of plant phenolics with ascorbate in the H_2O_2-peroxidase system has been shown (Takahama and Oniki, 1997; Yamasaki and Grace, 1998; Ferreres *et al.*, 2011). It takes place in the vacuole, where H_2O_2 diffuses and can be reduced by peroxidases using phenolics as primary electron donors. Both AsA and the Monodehydroascorbic Acid Radical (MDHA) can reduce phenoxyl radicals generated by this oxidation. If regeneration of AsA is performed in the cytosol and AsA is supplied back to the vacuole, a phenolics and/or AsA peroxidas system could function in vacuoles and scavenge H_2O_2. This mechanism is specific for plant tissues and can improve stress tolerance under oxidative stress (Yamasaki and Grace, 1998).

1.12. Ascorbic Acid

AsA is considered one of the strongest molecules among antioxidants (Noctor and Foyer, 1998; Smirnoff *et al.*, 2001). Under physiological conditions AsA mostly exists in the reduced form (90% of the ascorbate pool) in leaves and chloroplasts (Smirnoff, 2000) and its intracellular concentration can build-up to millimolar range (e.g., 20 mM in the cytosol and 20-300 mM in the chloroplast stroma; Foyer and Lelandais, 1996). Evidences showed that AsA can directly scavenge hydroxyl and superoxide radicals, singlet oxygen; moreover, it can reduce H_2O_2 to water via ascorbate peroxidase reaction (Noctor and Foyer, 1998). AsA also acts as a cofactor of violaxantin de-epoxidase thus sustaining dissipation of excess excitation energy (Smirnoff, 2000) and can provide to tocopherol regeneration from tocopheroxyl radical providing membrane protection (Thomas *et al.*, 1992). The role of AsA in the plant's response to B toxicity is not simple to draw due to the limited number of studies on this topic.

B can significantly influence the plant ability to produce many compounds (including AsA) (Gunes *et al.*, 2006; Eraslan *et al.*, 2008). Singh *et al.* (2012) found variable levels of AsA production in different carrot genotypes; the level were higher under B deficiency rather than under B toxicity. We similarly found different reactions concerning the AsA levels in different cultivars of sweet basil (*Ocimum basilicum*; cv. Tigullio, green-leaved and cv. Red Rubin, purple-leaved) grown with excess B in the growing medium. In fact, in leaves of Tigullio plants an increase in AsA content was recorded; on the other hand, Red Rubin cultivar showed a decrease of this compound (*unpublished results*). More uniform was the trend of AsA reported by Cervilla *et al.* (2007) in two tomato (*Solanum lycopersicon*) cultivars (Josefina and Kosaco) grown with B in the nutrient solution. Both cultivars showed a general stimulation of antioxidants, including AsA. However, it is remarkable that the cultivar Josefina showed, at the same time, the lower B uptake in leaves and the higher AsA content compared to the cultivar Kosaco. A similar increase in AsA concentration was found in potato eaves (Mondy and Munshi (1993) and in orange (*Citrus grandis*) leaves Keles *et al.* (2004) after high B foliar supply. On the other hand, a decrease in AsA amount was reported by

Eraslan *et al.* (2007a) in both leaves and roots of carrot grown with 5 μM of B in the nutrient solution. When the plants were simultaneously treated with B and and Na_2SO_4 the concentration of AsA significantly increased. Similar results were found in lettuce (Eraslan *et al.*, 2007b): in this experiment the AsA concentration recorded in plants grown under B or NaCl stress was not significantly different from control plants while AsA increased as result of combined NaCl and B supply.

It is notable that AsA amount decreased in species and/or cultivar with high APX activity and then large AsA consumption compared to plant genotypes where APX plays a minor role in ROS scavenging. Eraslan *et al.* (2007a) in carrot and Han *et al.* (2009) in citrus recorded that high B concentration reduced AsA concentration while increasing APX activity. In contrast, in lettuce B increased APX activity compared to control plants while not significant differences were recorded in AsA concentration (Eraslan *et al.*, 2007b).

1.13. Gluthatione

A tripeptide glutathione (γ-glutamylcysteinylglycine) is an abundant compound in plant tissues. It has virtually been found in all cell compartments: cytosol, endoplasmic reticulum, vacuole and mitochondria where GSH executes multiple functions (Jimenez *et al.*, 1998). GSH is the main storage form of sulfur acts as a potent detoxifier of xenobiotics through GSH-conjugation and can represent as a precursor of phytochelatins (reviewed by Noctor *et al.*, 1998; May *et al.*, 1998). Together with its oxidized form (GSSG), glutathione maintains a redox balance in the cellular compartments. This property is of great biological importance, since it allows fine-tuning of the cellular redox environment under both optimal and stress conditions and provides the basis for GSH stress signaling. Indeed, the role for GSH in redox regulation of gene expression has been described in many papers (e.g., Wingate *et al.*, 1988; Alscher, 1989). Due to redox properties of the GSH/GSSG pair and reduced SH-group of GSH, it can participate in the regulation of the cell cycle (Sanchez-Fernandez *et al.*, 1997).

Functioning of GSH as antioxidant under oxidative stress has received much attention during the last decades. A central nucleophilic cysteine residue is responsible for high reductive potential of GSH. It scavenges cytotoxic H_2O_2 and reacts non-enzymatically with other ROS: superoxide radical, singlet oxygen and hydroxyl radical (Larson, 1988; Shao *et al.*, 2008). The central role of GSH in the antioxidative defence is due to its ability to regenerate ascorbic acid via the ascorbate–glutathione cycle (Foyer and Noctor, 2011) how mentioned above. Thus, the evaluation of its content and its redox status can be useful to understand the non-enzymatic antioxidant system reaction under several stresses (Ryang *et al.*, 2009).

In sunflower Ruiz *et al.* (2003) reported that B excess inhibited glutathione synthesis where as an external application of this antioxidant reduced damages induced by B toxicity. Moreover, LeNoble *et al.* (1996) and Ruiz *et al.* (2006) showed that B reduces Al phytotoxicity by stimulating GSH biosynthesis in the leaves and the increase in its concentration in the roots, where it could prevent injuries due to ROS accumulation. This study indicates the crucial role of GSH metabolism for Al detoxification. Notable was a simultaneous increase of total GSH and AsA recorded under B-deficiency in citrus plants while both these molecules decreased under B-excess condition (Han *et al.*, 2009). These results are partially in accord with Wang *et al.* (2011) who also reported an increase in total GSH content in pear leaves under high boron supply.

1.14. Phenols

Phenolics represent a large class of secondary metabolites (flavonoids, anthocyanins, tannins, hydroxycinnamate esters and lignin) abundant in plant tissues (reviewed by Grace and Logan, 2000). Antioxidative properties of polyphenols arise from: (i) high reactivity as hydrogen or electron donors; (ii) the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron due to their chemistry structural (chain-breaking function); (iii) the ability to chelate transition metal ions (termination of the Fenton reaction) (iv) the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora *et al.*, 2000). These changes could sterically hinder diffusion of free radicals and restrict peroxidative reactions. Moreover, it has recently been shown that phenolic compounds can be involved in the hydrogen peroxide scavenging cascade in plant cells (Takahama and Oniki, 1997; Dangles, 2012). An antioxidant role of flavonoids compounds has been reported due to their molecular structure (Pourcel *et al.*, 2006; Hernandez *et al.*, 2009; Agati and Tattini, 2010).

There is evidence that B is one of the nutrients responsible for the changes in concentration and metabolism of phenolic compounds in vascular plants, since B deficiency causes an accumulation of phenolics (Cakmak and Romheld, 1997). However, little information is available about phenols reaction under B stress. Ruiz *et al.* (1998) and Chamacho-Cristobal *et al.* (2002) reported an increase in phenolic content in the leaves of tobacco plants grown both under B deficiency or toxicity. Boron forms complexes (>90% of the foliar content) mainly with pectins and phenols in the cell wall and plasma membrane, respectively, resulting in higher stability of these structures. When B supply is adequate more than 60% of this element is in free form in leaves tissues (Brown and Hu, 1996). In view of above, in conditions of B excess phenols may play a role in B compartmentalization rather than in antioxidant activity.

During the last years anthocyanins, a particular group of phenols, have received great attention due to the

evidence of plant antioxidant capacity is related to the content of these compounds (Tsuda *et al.*, 1996; Gould *et al.*, 2002). The antioxidant activity of anthocyanins might be mainly due to their strong redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. It has also been suggested that the increase in anthocyanins level under salt stress is mainly due to their photoprotection of chlorophylls (Gould *et al.*, 2000). Moreover, anthocyanins are thought to be involved in osmotic regulation (Chalker-Scott, 2002). Many studies reported that plant tissues rich in anthocyanins are generally relatively resistant to drought and salt stresses (Chalker-Scott, 1999). A purple-leaved of pepper tolerated salt stress better than a green-leaved cultivar (Bahler *et al.*, 1991). The highly drought and salt tolerant resurrection plant *Craterostigma plantagineum* contained higher amount of anthocyanins during dehydration as compared to the hydrated stage (Sherwin and Farrant, 1998). Eraslan *et al.* (2008) showed an increase of anthocyanins concentration in *Spinacia oleraceae* grown under conditions of B excess and salt stress.

1.15. Antioxidant Enzymes

Boron stress causes an oxidative stress because of the formation of ROS such as superoxides and hydroxy and peroxy radicals as occur in many other ionic stress. The ROS that are by-products of hyperosmotic and ionic stresses cause membrane disfunction and cell death (Bohnert and Jensen, 1996). Generally, the plants defend against these ROS by induction antioxidative enzymes such as catalase, peroxidase, glutathione reductase and superoxide dismutase, which scavenge ROS (**Table 2**).

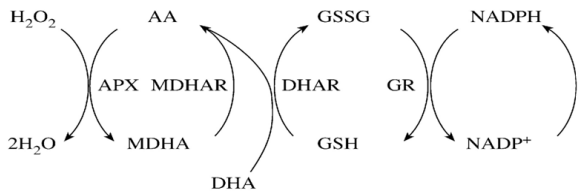

Fig. 1. Ascorbate-glutathione cycle (Halliwell–Asada). APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase

Table 2. The major ROS scavenger and antioxidant enzymes

Enzyme	EC	Reaction Catalysed
SOD	1.15.1.1	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \Leftrightarrow 2H_2O_2 + O_2$
CAT	1.11.1.6	$2H_2O_2 \Leftrightarrow O_2 + 2H_2O$
APX	1.11.1.11	$AA + H_2O_2 \Leftrightarrow DHA + 2H_2O$
MDHAR	1.6.5.4	$NADH + 2MDHA \Leftrightarrow NAD^+ + 2AA$
DHAR	1.8.5.1	$2GSH + DHA \Leftrightarrow GSSG + AA$
GR	1.6.4.2	$NADPH + GSSG \Leftrightarrow NADP^+ + 2GSH$

There are several reports of increasing activity of antioxidative enzymes under high B supply (Gunes *et al.*, 2006; Cervilla *et al.*, 2007; Eraslan *et al.*, 2007a; Ardic *et al.*, 2009; Wang *et al.*, 2011). SOD is the major $O_2^{\bullet-}$ scavenger and its enzymatic action results in H_2O_2 and O_2 formation. The H_2O_2 produced is then scavenged by CAT and several classes of peroxidases. Catalase, which is found in peroxisomes, cytosol and mitochondria, dismutates H_2O_2 into H_2O and O_2 (Mittler, 2002). Peroxidases (APX and GPX) are distributed throughout the cell and catalyze the reduction of H_2O_2 to H_2O. APX uses ascorbate as electron donor in the first step of the ascorbate–glutathione cycle and it is considered the most important plant peroxidase for H_2O_2 detoxification (Noctor and Foyer, 1998). GR, MDHAR and DHAR are involved in Halliwell-Asada cycle (**Fig. 1**) and their activities might influence the turn-over of reduced ascorbate regeneration. Moreover, recent researches have shown that over-expression of MDHAR can increase the amount of reduced ascorbate because MDHAR functions upstream of DHAR in the ascorbate recycling pathway (Ishikawa *et al.*, 2006).

SOD is responsible for the detoxification of O_2^- by forming H_2O_2, which also being toxic must be eliminated by conversion to H_2O in subsequent reactions. Superoxide radicals (O_2^-) are toxic byproducts of oxidative metabolism and can interact with H_2O_2 to form highly reactive hydroxyl radicals (OH^-), which are thought to be primarily responsible for oxygen toxicity in the cell (Mittler, 2002; Azevedo-Neto *et al.*, 2006). Generally, SOD activity increased during high B supply (Garcia *et al.*, 2001; Gunes *et al.*, 2006; Molassiotis *et al.*, 2006; Sotiropoulos *et al.*, 2006). For instance, Cervilla *et al.* (2007) observed an increase of SOD activity only in tomato cultivar Kosaco plants grown at 0.5 and 2.0 mM of B (compared to the control level). In contrast, Josefina cultivar plants grown in the same condition exhibited an upgrade of SOD activity only at 0.5 mM of B. At the highest B concentration SOD activity did not significantly change compared to the control. An increased in SOD activity was also reported in tomato grown with 2 mg L^{-1} of B in the nutrient solution by Kaya *et al.* (2009). Moreover, an increase in SOD activity was reported by Han *et al.* (2009) in citrus leaves both under B deficiency and excess and by Ardic *et al.* (2009) in two chickpea cultivars submitted to 1.6 and 6.4 mM of B in comparison to control plants (0.05 mM). Moreover, Ardic *et al.* (2009) reported that the increase in SOD activity was due to the induction of new isoforms of the enzyme rather than an increase of constitutive isoenzymes. Thus, the authors hypothesized that SOD is tightly controlled in response to B excess according to other researches (Garcia *et al.*, 2001; Karabal *et al.*, 2003).

The oxidative stress triggered by B toxicity increases O_2^- production and, consequently, SOD activity. For this reason the reaction catalyzed by this enzyme can play an important role in radicals scavenging but, at the same time, other enzymes are necessary to scavenge the hydrogen peroxide generate by SOD. In plants, a number of enzymes regulate H_2O_2 intracellular levels, but CAT and APX are considered the most important ones (Noctor and Foyer, 1998) and certain concentrations of H_2O_2 can promote CAT and APX activity (Bowler *et al.*, 1992). For instance, in pear Wang *et al.* (2011) reported an increase in the activity of these two antioxidant enzymes and a strongly H_2O_2 accumulation at 100 and 300 μM of B compared to control plants grown at 10 μM. On the other hand, in the same research, plants grown at 500 μM of B showed lower APX and CAT activity. The authors hypothesized that the accumulation of $O_2^{\bullet -}$ and H_2O_2 exceeded the scavenging ability and consequently ROS could be accumulates and causes lipid peroxidation and membrane damage. The increase in CAT was observed in many species exposed to B excess (Gunes *et al.*, 2006; Molassiotis *et al.*, 2006; Cervilla *et al.* 2007; Ardic *et al.*, 2009; Soylemezoglu *et al.*, 2009). Conversely, Keles *et al.* (2004) and Han *et al.* (2009) reported a decrease in CAT activity in citrus leaves due to B toxicity. Interestingly, Han *et al.* (2008; 2009) reported a concomitant buildup of APX activity under both conditions of limited and excessive B supply and suggested that in this species APX plays a major role in H_2O_2 scavenging than CAT. In agreement with these results (Eraslan *et al.*, 2007b), a strong CAT increase and a concomitant decrease in APX activity under B stress were observed in *Vitis* (Gunes *et al.*, 2006) and in lettuce under B stress. The increase of CAT activity was also recorded in tomato (Cervilla *et al.*, 2007), tobacco (Garcia *et al.*, 2001), hot pepper (Lee, 2006), pear (Wang *et al.*, 2011).

Furthermore, exposing plants to simultaneous B and other salt stress as Si (Gunes *et al.*, 2006) and NaCl (Eraslan *et al.*, 2008) a mitigation of the activity of APX and CAT was recorded as compared to the values found in plants treated only with B.

Since AsA is believed to play a great important role in the antioxidant response to many stresses, the regeneration of this molecule is also a relevant process. This pathway involves the enzymes GR, MDHAR, DHAR which are distributed in most cellular compartments (Ishikawa *et al.*, 2006). In general, their activity is enhanced by B toxicity (Cervilla *et al.*, 2007; Lopez-Gomez *et al.*, 2007; Wang *et al.*, 2011). On the contrary, under conditions of severe B toxicity, ROS production exceeds the ability of these enzymes to reduce DHA in to AsA (Wang *et al.*, 2011). Moreover, GR was stimulated by B toxicity in barley (Karabal *et al.*, 2003), citrus (Han *et al.*, 2009) and the cultivar Gökce of chickpea (Cicer arietinum) (Ardic *et al.*, 2009). On the contrary, a decrease of GR was recorded in another cultivar (Küsmen) of chickpea (Ardic *et al.*, 2009). In citrus leaves the activity of all of three enzymes was enhanced under B deficiency while excess B stimulated only the activity of GR (Han *et al.*, 2009). It is remarkable that B deficiency also increased the content of AsA and GSH while in plants subjected to B toxicity the amount of these two antioxidants decreased. This suggests that GR, MDHAR and DHAR are involved and necessary to promote an adequate turn-over of AsA and GSH regeneration.

Over-expression of MDHAR affected ascorbate accumulation and increased the redox status of this molecule toward reduction (Ishikawa *et al.*, 2006). Indeed, MDHAR functions upstream of DHAR in the ascorbate recycling pathway.

1.16. Effects of Boron on Photosynthesis

Information on the effects of B on photosynthetic process is still scarce even if it has known that boron excess inhibits photosynthesis (Han *et al.*, 2008; 2009; Ardic *et al.*, 2009; Guidi *et al.*, 2011; Chen *et al.*, 2012). Experimental data are sometimes contradictory. In condition of B toxicity, CO_2 assimilation decreased in many species such as summer squash (Lovatt and Bates, 1984), kiwifruit (Sotiropoulos *et al.*, 2002), Clementine mandarin (Papadakis *et al.*, 2004a), citrus (Papadakis *et al.*, 2004b; Han *et al.*, 2009; Sheng *et al.*, 2010) and pear (Wang *et al.*, 2011).

It has well known as the lower CO_2 assimilation in B deficient leaves is related to stomatal factors (Papadakis *et al.*, 2004a; Han *et al.*, 2009; Sheng *et al.*, 2009). Conversely, the reduction in CO_2 assimilation under B excess conditions seems to be related to a combination of different factors: oxidative load, decrease in photosynthetic enzymatic activities and an impaired electron transport rate (Han *et al.*, 2009). However, the mechanisms involved in the alteration of photosynthesis by B stress has not be elucidated yet.

Some authors found that the reduction in photosynthetic rate in plant subjected to B excess was accompanied by an increase in intercellular CO_2 concentration whereas stomatal conductance remained unaffected (Sotiropoulos *et al.*, 2002). In contrast, other authors observed a reduction in stomatal conductance (Lovatt and Bates, 1984; Papadakis *et al.*, 2004a). Pereira *et al.* (2000) hypothesized that one of the possible reasons for the reduction of photosynthesis by B excess was the structural damage of thylakoids; this, in turn, altered the rate of electron transport and influenced CO_2 photoassimilation which can also be limited by stomatal reduction.

The F_v/F_m ratio (maximum quantum yield of chlorophyll fluorescence) significantly decreased in many species following B toxicity (Larsson *et al.*, 1998; Papadakis *et al.*, 2004a; 2004b; Guidi *et al.*, 2011). The

reduction of F_v/F_m is sometimes related to an increase in F_0 (minimal chlorophyll fluorescence) which is closely related to the structural damage to the thylakoid membranes (Havaux and Lannoye, 1985). It is clear that the decrease in F_v/F_m ratio indicates that leaves are photoinhibited and it has well known as in these conditions molecular oxygen can represent an alternative electron acceptor for unused electrons and light (Velez-Ramirez *et al.*, 2011), which leads to the generation of ROS. This event can also explain the observed decrease in chlorophyll content leaves (Papadakis *et al.*, 2004b; Han *et al.*, 2009; Chen *et al.*, 2012) and chloroplast damage (Papadakis *et al.*, 2004; 2004b) in plants grown under conditions of B toxicity. Papadakis *et al.* (2004a) reported that leaves of Clementine orange treated with B excess showed a decrease in their thickness and a disorganization of mesophyll cells. Similar results were found by Sotiropoulos *et al.* (2002) in kiwifruit and by Kamali and Childers (1967) in peach. Thus, B excess can induce an alteration of leaf structure both in the species where B is phloem immobile (orange and kiwifruit) and in those where B mobility is high as peach (Brown and Shelp, 1997).

On the other hand, Guidi *et al.* (2011) found that a reduction of electron transport rate induced by boron excess attributable to an increase in J_{NPQ}, the quantum yield of excitation energy thermally dissipated. It has known that the loss of excitons through non-radiative decay in PSII is a down-regulated process and that leaves can alleviate the damage to photosynthetic processes by dissipating the excessive excitation energy into harmless heat (Kramer *et al.*, 2004). This hypothesis was substantiated by the higher values of J_{NPQ} found in central and asymptomatic leaf portions compared to the necrotic margins. However, boron toxicity also induced a decrease in the fraction of open PSII reaction centers and a slow re-oxidation of QA due to the inhibition of Calvin cycle activity.

In conclusions B can induce an alteration in the mesophyll cells that, in turn, reduces electron transport rate and light utilization. On the other hand, the reduced activity of some enzymes involved in CO_2 assimilation (ribulose-1,5-bisphosphate carboxylase/oxygenase and fructose-1,6-bisphosphate phosphatase; Han *et al.,* 2009) also determines a reduction in NADPH and ATP utilization, thus inhibiting in electron transport rate. Consequently, the reduction in electron transport rate determines an oxidative stress that generates ROS in the chloroplast. These ROS oxidized organic molecules as chlorophyll and lipid and, probably, induce cell death. The consequence of these events is the visible symptoms of damage which are typically located at the leaf margin where B is accumulated to a large extent (Guidi *et al.*, 2011).

2. CONCLUSION

Boron is a nutrient element that is involved in different plant processes such as cell division, cell wall synthesis, sugars translocation, protein synthesis and membrane functions. In general, B excess affects leaf tips and edges disorders. It is frequently contained at excessive concentration in both soil and irrigation water and it may induce several metabolic disorders (decreases in chlorophyll content, inhibition of photosynthesis, structural damage to thylakoids, deposition of lignin or suberin, peroxidation of lipids and altered activities of antioxidant pathways) and reduce plant growth.

Plants are frequently subjected to concomitant B toxicity and salinity because of plants are irrigated with water containing high level of B and salts or plants are grown in soils with high content of salts and B. It has been reported that the combination of high B levels and salinity induced less toxic effects on plant growth than would be expected probably because of reduced uptake of B in the presence of chloride and reduced uptake of chloride in the presence of B (Yermiyahu *et al.*, 2008). On the other hand, in the simultaneous presence of B and salts, boric acid influences the activity of membrane components which regulate the functions of aquaporin isoforms and ATPase increasing the tolerance to salts stress (Martinez-Ballesta *et al.*, 2008). Boron was assimilated from soils by root and then transported *via* xylem or phloem to the leaves with differences in relation to leaf age. Xylem translocation is mainly directed to the mature leaves while phloem translocation supplies the major proportion of nutrient requirements for actively growing areas, such as young leaves, fruits and seeds. Plant species show different sensitivity to B toxicity. Boron tolerance is likely associated with the ability to exclude B at root level: a reduced permeability of membrane lipids and/or the presence of carriers (BOR and NIP) essential for boron extrusion from the cytoplasm may be responsible for reduced B accumulation.

Although the physiological and biochemical mechanisms on the basis of B toxicity is not complete clear, certainly this element at high concentration induces: (i) alteration of cell wall structure; (ii) alterations of biochemical pathways binding to the ribose moieties of important molecules such as ATP and reduced form of NAD(P)H; (iii) alterations of cell division and development by binding to ribose within RNA (Reid *et al.*, 2004).

Plants species show different sensitivity to B toxicity being the tolerance related to a lower uptake (or a major efflux) of B into both roots and shoots. It has been reported as BOR1 is an efflux-type borate transporters essential to transport B from root to shoots, is degraded

in the presence of toxic levels of B. In addition to, another boron efflux-type transporter, BOR4 is related to the tolerance to B.

As in most ionic stresses, toxic levels of B cause the formation of ROS. In order to avoid the harmful effects of these reactive molecules, plants have evolved an effective scavenging system composed of antioxidant molecules and antioxidant enzymes such as SOD, CAT, APX and GR. In addition to, organic antioxidant compounds (e.g., phenols, AsA, GSH or anthocyanins) could enhance the reaction to these toxic reactive molecules. It has well known that B toxicity trigged ROS production and, in the same way, a general stimulation of enzymatic and non-enzymatic antioxidant systems was reported.

In conclusion, because of the relative little few information on different aspects of B toxicity, further researches are necessary in order to asses: (i) the biochemical or physiological target of the B toxicity; (ii) the identification of the carriers involved in B uptake and translocation and (iii) the contribute of antioxidants in scavenging of ROS induced by B and their involvement in B tolerance.

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