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Abstract

Boron (B) toxicity frequently affects plant performances and productivity, especially in arid and semi-arid environments. In this experiment, loquat seedlings were subjected to 25 μM (control) or 400 μM B (B excess) to test the hypothesis that (i) B alter sugar/polyol metabolism in polyol-producing tree species as loquat and (ii) changes of leaf and stem anatomy assist young tissues against toxic effect of B. Gas exchange parameters were monitored until 147 days from the beginning of the experiment (FBE), one week later the first visible symptoms of B toxicity appeared in the upper part of stems. At 147 FBE, plant biometric parameters and pattern of B accumulation, leaf and stem anatomy, chlorophyll a fluorescence kinetics as well as biochemical measurements were assessed in top (asymptomatic) leaves and upper stem bark. Boron accumulated principally (in the row) in top leaves > top bark > top wood in B-stressed plants, but no changes in allocation pattern were found between controls and B-stressed plants. Excess B promoted the increase in the spongy layer of top leaves and caused the development of cork and numerous collenchyma cells with increased cell wall thickness. This mechanism, which has never been described before, can be considered an attempt to store excessive B in tissues where B ions are less harmful. The accumulation of sorbitol (B-complexing polyol) in top leaves and stem bark can be considered as a further attempt to detoxify B excess. However, B toxicity drastically affects the photosynthetic rate of top leaves, mainly due to non-stomatal limitations, i.e., reduction of ambient CO_2 use efficiency and of photosystem II (PSII) efficiency, modification of the partitioning excess energy dissipation in PSII, thus leading to an increased level of lipid peroxidation. Our results suggest that changes in sugar metabolism associated with leaf and stem bark thickening partially assist (but not totally preserve) young tissues of loquat plants under B stress.

Keywords	Boron mobility; Chlorophyll fluorescence; Loquat tree; Polyalcohol-producing species; Photosynthesis; Sugars
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Athens, September 5th, 2018

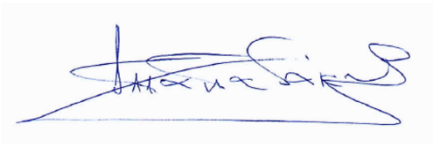
To the Editor-in-Chief of *Journal of Plant Physiology*

Dear editor,

please find enclosed a manuscript submitted for possible publication in *Journal of Plant Physiology*. The manuscript is titled "**Changes in sugar metabolism associated to stem bark thickening partially assist young tissues of *Eriobotrya japonica* seedlings under B stress**" and deals with new aspects of boron(B)-induced changes in loquat plants' metabolism. In particular, we found that excess B promoted the increase in the spongy layer of top leaves and caused the development of cork and numerous collenchyma cells with increased cell wall thickness in the upper part of the stems. This mechanism, which has never been described before, can be considered an attempt to store excessive B in tissues where B ions are less harmful. The accumulation of sorbitol (B-complexing polyol) in top leaves and stem bark can be considered as a further attempt to detoxify B excess.

The present manuscript consists of unpublished work, which is also not under consideration for publication elsewhere. The authors have also carefully read and are fully aware of the *Journal of Plant Physiology*'s policies.

Best regards,



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Changes in sugar metabolism associated to stem bark thickening partially assist young tissues of *Eriobotrya japonica* seedlings under B stress

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ABSTRACT

Boron (B) toxicity frequently affects plant performances and productivity, especially in arid and semi-arid environments. In this experiment, loquat seedlings were subjected to 25 μM (control) or 400 μM B (B excess) to test the hypothesis that (i) B alter sugar/polyol metabolism in polyol-producing tree species as loquat and (ii) changes of leaf and stem anatomy assist young tissues against toxic effect of B. Gas exchange parameters were monitored until 147 days from the beginning of the experiment (FBE), one week later the first visible symptoms of B toxicity appeared in the upper part of stems. At 147 FBE, plant biometric parameters and pattern of B accumulation, leaf and stem anatomy, chlorophyll *a* fluorescence kinetics as well as biochemical measurements were assessed in top (asymptomatic) leaves and upper stem bark. Boron

accumulated principally (in the row) in top leaves > top bark > top wood in B-stressed plants, but no changes in allocation pattern were found between controls and B-stressed plants. Excess B promoted the increase in the spongy layer of top leaves and caused the development of cork and numerous collenchyma cells with increased cell wall thickness. This mechanism, which has never been described before, can be considered an attempt to store excessive B in tissues where B ions are less harmful. The accumulation of sorbitol (B-complexing polyol) in top leaves and stem bark can be considered as a further attempt to detoxify B excess. However, B toxicity drastically affects the photosynthetic rate of top leaves, mainly due to non-stomatal limitations, i.e., reduction of ambient CO_2 use efficiency and of photosystem II (PSII) efficiency, modification of the partitioning excess energy dissipation in PSII, thus leading to an increased level of lipid peroxidation. Our results suggest that changes in sugar metabolism associated with leaf and stem bark thickening partially assist (but not totally preserve) young tissues of loquat plants under B stress.

Keywords: Boron mobility, Chlorophyll fluorescence, Loquat tree, Polyalcohol-producing species, Photosynthesis, Sugars

Abbreviations: A_{400}, net photosynthesis at ambient CO_2; Car, carotenoids; Chl, chlorophyll; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Chl *a*+*b*, chlorophyll *a* + chlorophyll *b*; Chl *a*/*b*, chlorophyll *a* / chlorophyll *b*; C_i, intercellular CO_2 concentration; DW, dry weight; E, evapotranspiration rate; ETR, electron transport rate; FAA, (formaldehyde – acetic acid – ethanol) solution; FBE, from the beginning of experiment; F_0, minimal fluorescence of photosystem II in dark-adapted conditions; F_m, maximal fluorescence of photosystem II in dark-adapted conditions; F_m', maximal fluorescence of photosystem II in light-adapted conditions; F_s, steady-state fluorescence; F_v/F_m, maximal efficiency of photosystem II in dark-adapted conditions; FW, fresh weight; g_s, stomatal conductance; LHCII, light harvesting complexes of photosystem II; MDA, malondialdehyde; PSII, photosystem II; q_P, proportion of open reaction centers; RCII, reaction centers of PSII; TCA, trichloroacetic acid; WUE, water use efficiency; Φ_{NPQ}, quantum yield of regulated energy loss in PSII; Φ_{NO}, quantum yield of regulated energy loss in PSII; Φ_{PSII}, operational efficiency of PSII

1. Introduction

Since the 1920s, when the essential role of boron (B) in *Vicia faba* was established (Warington, 1923), B has been considered an essential micronutrient for plant development. It has been hypothesized that B plays a key role in several metabolic processes, though is primarily involved in cell wall structure, where B cross-links two rhamnogalacturonan II (RGII) monomers by a borate bridge, providing stability to the cell-wall matrix (O'Neill et al., 2004). Boron is unique as a microelement given that it is characterized by an extremely narrow window between essential and toxic concentrations (Nable et al., 1997). Besides geothermal stems, the presence of B in the environment arises primarily from the weathering of B-containing minerals, which significantly contributes to the natural enrichment of B in the soil and water (Butterwick et al., 1989). However, the most impactful source of B is certainly the seawater (on average 3-4.5 mg L^{-1} B dissolved as boric acid) which can contaminate fresh water in coastal areas following tidal cycles. Seawater volatilization also originates $B(OH)_3$-containing aerosol and produces a gradient of B sedimentation in soil from the cost to the inland. Different to other elements, human activities only play a minor role as compared to the amplitude of the environmental B enrichment arising from natural sources, but in some cases agricultural lands in arid/semiarid environments or in proximity to industrial areas can cause B toxicity in plants (Rámila et al., 2016). In general, edaphic concentrations of B in the soil solution ranging from 5 to 100 mg L^{-1} are toxic for many plant species, even though a large degree of inter- (Ferreyra et al., 1997) and even intra-specific tolerance (Papadakis et al., 2003, 2004; Cervilla et al., 2007; Landi et al., 2013a, 2013b) has been observed.

In the soil solution, B is primarily present as boric acid $B(OH)_3$, which can either diffuse passively or be taken up by the root hair cells via anionic channels. However, active transport only occurs when the availability of B is below the sub-optimal plant's demand (Dannel et al., 2000). Under physiological conditions, B exists as $B(OH)_3$ or tetra hydroxyl borate anion $B(OH)_4^-$ in plants, though 98% (or even higher) of the total B exists in free form as $B(OH)_3$ at lower pH values (e.g., 5.5 in the apoplast spaces) (reviewed by Woods, 1996). Boric acid is a weak acid at cytoplasm pH (7.0-7.5) and, under physiological conditions, $B(OH)_3$ can easily bind to molecules with mono, di- and poly-hydroxyl groups such as ribose, apiose, sorbitol, mannitol and other polyalcohols (Raltson and Hunt, 2001).

Under a condition of excessive B supply, B is principally absorbed passively by the roots (Brown et al., 2002) due to a high permeability of $B(OH)_3$ to lipid bilayers (Brown and Shelp, 1997). Once absorbed, B is translocated to leaves via non-living xylem cells following the transpiration stream. Xylem translocation is therefore directed principally to the mature leaves because they represent the sites with the highest transpiration rate and usually older leaves show more evident symptoms of B toxicity than younger tissues (Princi et al., 2016). Thus, for a long time it has generally been accepted that B is an immobile nutrient in the phloem tissues and therefore it tends to accumulate in highly-transpiring mature leaves. More recently, in some plant species which use sugar alcohols (i.e., mannitol and sorbitol) for the phloem translocation of photosynthates in place of sucrose (e.g., *Pyrus*, *Malus*, *Prunus*, *Allium* and *Brassica*), B has been found to be uniformly distributed within plants, or even at a higher concentration in young tissues than in mature leaves (for a review see Camacho-Cristobal et al., 2008). These results demonstrate B's ability to move along the phloem flux due to its capability of binding with *cis*-hydroxyl groups of sorbitol and mannitol, which originates diol-B complexes (Reid et al., 2004). This bond is likely to allow B to be transported through the phloem where it is present as a stable polyol-B complex, with mannitol and sorbitol as ligands (Hu and Brown, 1997). Phloem translocation does not follow the transpiration stream and it supplies the major proportion of nutrient requirements for actively-growing organs which do not readily transpire (e.g., young leaves, stems and fruits) (Brown and Shelp, 1997) and in which B symptoms occur in sugar-alcohol translocating species, including loquat.

Loquat is considered to be very sensitive to B toxicity (López-Gómez et al., 2007), but the available knowledge on how B surplus affects loquat metabolism is still limited. In particular, no studies have investigated in depth the changes occurring in B partitioning in loquat organs and the consequences of B allocation on stem/leaf sugar and polyol pattern when loquat plants are grown under condition of B excess. In this experiment, hydroponically-grown control (25 μM B) *versus* high-B-treated (400 μM B) loquat seedlings were monitored for 147 days in terms of gas exchange parameters, until the first visible symptoms appeared in the upper part of the plant stem. At 147 days from the beginning of experiment (FBE), plant biometric parameters and pattern of B accumulation, leaf and stem anatomy, chlorophyll *a* fluorescence kinetics as well as biochemical parameters (carbohydrates, pigments, proline, lipid peroxidation by-products, and H_2O_2) were

measured in the top (asymptomatic) leaves and upper stem bark in order to increase the available knowledge of the anatomical/physiological/biochemical B effects in loquat plants.

2. Material and methods

2.1. Plant material and experimental design

One-and-a-half-year-old seedlings of loquat (*Eriobotrya japonica* Lindl.) uniform in stem diameter, leaf area and height, were grown in black plastic pots containing a mixture of peat:perlite (2:1, v/v) in a glasshouse located at the arboretum of the Agricultural University of Athens (Latitude 37.981907, Longitude 23.705639). Plants were irrigated, according to their needs, with a full strength Hoagland's nutrient solution containing 25 μM B (controls) or 400 μM B (B excess). Each watering (fertigation) supplied enough solution to fill the pores (field capacity), allowing some drainage for salt leaching from the planting medium. Furthermore, with this procedure the B concentrations in the planting media were identical to the original solution (Papadakis et al., 2003). Climatic parameters were monitored by weather station located inside the glasshouse. The minimum, maximum, and averaged air temperature were 18, 28, and 24.8 °C respectively. Maximum temperature reached up to 30-32 °C in sunny hours. Daily global radiation averaged 11.5 MJ m^{-2}. First visible symptoms of B toxicity appeared about 140 days FBE. Symptoms appeared exclusively in the upper part of stems (in the form of browning areas and bark cracking). This is why the upper plant tissues, i.e. leaves and bark of upper part of the stem, were sampled at 147 days FBE (when the experiment was stopped) and analyzed for the biochemical parameters detailed below.

To evaluate as to whether basal leaves were also suffering for physiological impairments due to B-excess, gas exchanges were measured in both basal and top fully-expanded leaves. Chlorophyll *a* fluorescence parameters were only measured in top leaves as the basal leaves did not show differences in gas exchange parameters when compared to controls.

Leaf and stem bark (outmost layer of the stem) samples were collected from the middle of the upper half portion of the stem of each experimental plant for the determination of chlorophyll (Chl) and carotenoid (Car) content in leaves and carbohydrates, H_2O_2, proline and malondialdehyde (MDA) by-product content in leaves and bark. Finally, the above-ground portion (shoot) of each

plant was divided in two parts: top and basal. In both top and basal shoot portion, the leaves were firstly collected and then the stem was further separated into bark and wood. All collected plant materials (top leaves, top bark, top wood (interior part of the stem), basal leaves, basal bark, basal wood, root) were weighted (fresh weight; FW), washed initially with tap water and then twice with deionized water, oven-dried at 70 °C until constant weight (dry weight; DW).

2.2. Gas exchange and chlorophyll a *fluorescence analyses*

Gas exchange parameters [photosynthetic rate (A_{400}; μmol CO_2 m^{-2} s^{-1}), transpiration rate (E; mmol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (C_i; μmol CO_2 mol air^{-1}), stomatal conductance (g_s; mmol H_2O m^{-2} s^{-1}), instantaneous water use efficiency (WUE = A_{400}/E; [μmol CO_2 mmol H_2O^{-1})], and CO_2 use efficiency (A_{400}/C_i; [μmol CO_2 m^{-2} s^{-1} (μmol CO_2 mol air^{-1})] were measured in both basal and top fully-expanded leaves (one leaf per plant; six plants per treatment) using a an Portable Infrared Gas Analyzer (mod. Li-6400; LI-COR Inc., Lincoln, NE, USA) at 27, 50, 66, 118, 129 and 147 days FBE. Measurements were carried out in the morning (10.00–11.00 a.m.; solar time), in light-saturated light conditions (1200 μmol photons m^{-2} s^{-1}) and ambient CO_2 concentration (400 ± 5 μmol CO_2 mol air^{-1}), while leaf temperature ranged between 23 and 26 °C.

Chlorophyll *a* fluorescence parameters were measured with a MINI-PAM fluorometer (Walz, Effeltrich, Germany) at 147 FBE (only time-point when gas exchange parameters changed in top mature leaves). Chlorophyll fluorescence parameters were measured in the same top leaves used for gas exchange determinations after 40 min of dark adaptation. Values of F_0 and F_m were measured in dark-adapted leaves before and after a saturating pulse (8,000 μmol m^{-2} s^{-1} for 1 s), whilst the maximal photosystem II (PSII) photochemical efficiency [$F_v/F_m = (F_m - F_0)/F_m$] and the operating PSII efficiency [$\Phi_{PSII} = (F_m' - F_s)/ F_m'$] were calculated according to Genty et al. (1989). The proportion of open reaction center, q_P, was calculated according to Schreiber et al. (1986). The quantum yield of regulated (Φ_{NPQ}) or nonregulated (Φ_{NO}) photochemical energy loss in PSII and operational PSII efficiency (Φ_{PSII}) at increasing photon flux densities were determined as reported by Kramer et al. (2004) based on the lake model. Electron transport rate (ETR) was calculated as reported in Guidi et al. (2016). Light-response curves were performed using a light ramp of nine steps from 0 to 2900 μmol m^{-2} s^{-1} using the light source supplied by the MINI-PAM.

2.3. Boron concentration, content, and distribution in loquat organs

An aliquot (0.25-1.00 g) of dry plant parts (top leaves, top bark, top wood, basal leaves, basal bark, basal wood, root) was dry ashed for 5 h at 500 °C. Leaf powder were digested as described in Landi et al. (2013). B concentration was determined colorimetrically (420 nm) by using the Azomethine-H method (Wolf 1971). Afterward, by multiplying the concentration of B in each plant part by the corresponding dry weight, the B contents in leaves, stem and root as well as the total per plant B content were calculated. Finally, B partitioning to leaves, stem, and root, per each experiment plant and B treatment, was further computed as a percentage to the respective total B content found in the whole plant.

2.4. Chlorophyll and carotenoid determinations

Leaf samples (0.75 g FW) were grinded in a pre-chilled mortar with 10 ml of cold aqueous acetone (80%; v/v), transferred in falcon tubes, kept in the dark for 1 h at 4 °C, and vortexed at 15 min intervals. A centrifugation at 4,400 *g* for 5 min at 4 °C was followed. Chlorophyll and carotenoid concentrations were determined spectrophotometrically by collecting extract absorbance at 470 nm, 647 nm, and 663 nm, using the equations described by Lichtenthaler and Buschmann (2001).

2.5. Proline content

Leaf (0.1 g FW) and bark (0.25 g FW) samples were cut into small pieces and grinded in a pre-chilled mortar with 10 ml of cold aqueous ethanol (80%; v/v). The homogenate was placed in falcon tubes and then centrifuged at 4,400 *g* for 5 min at 4 °C. One milliliter of the supernatant was transferred into test tubes containing 2 ml of acid-ninhydrin. After vortexing, test tubes were maintained at 95 °C for 25 min in a water bath and then transferred to an ice bath to allow samples to cool down at room temperature. After centrifugation (4,400 *g* for 5 min at 4 °C), the absorbance of the supernatant was recorded at 520 nm and proline was quantified as described by Bates et al. (1983).

2.6. Lipid peroxidation and hydrogen peroxide assay

Leaf (0.5 g FW) and bark (1.0 g FW) tissues were homogenized in 10 ml 0.1% (v/w) trichloroacetic acid (TCA) at 4 °C. After centrifugation at 4,400 *g* for 15 min at 4 °C, the supernatant was used for the determination of both lipid peroxidation level and H_2O_2 concentration. Lipid peroxidation was measured as MDA by-products content determined by reaction with 2-thiobarbituric acid and the concentration of MDA was calculated from the difference of the absorbance at 532 and 600 nm using the extinction coefficient of 155 $mmol^{-1}$ cm^{-1}which takes into account the possible influence of interfering compounds in the assay (Heath and Packer 1968).

Hydrogen peroxide accumulation was measured spectrophotometrically as described by Junglee et al (2014) with few modifications. The reaction mixture consisted of 0.5 mL 0.1% TCA, tissue extract supernatant, 0.5 ml of 0.1 M potassium-phosphate buffer (pH 7.0), and 1 ml of 1 M KI (w/v). The reaction color was developed for 45 min in darkness and absorbance was measured at 390 nm. Hydrogen peroxide levels were calculated using a calibration curve prepared with eight known concentrations of H_2O_2.

2.7. Soluble carbohydrate and starch content

An aliquot of 30 mg of freeze-dried leaf or bark tissues were added to 2 ml of HPLC-grade water (Carlo Erba Reagents S.A.S, Val-de-Reuil, France) and vortexed for 20 sec. Then, extraction of the water-soluble carbohydrates was performed in a microwave oven for 2 min at 400 watt. After centrifugation (4,400 *g* for 10 min at 4 °C), the supernatant was removed and the process was repeated twice. The two supernatants were pooled together and filtered by using syringe filters (0.2 µm pore size). HPLC analyses were conducted using a HPLC pump (model 510 Waters, Milford, MA, USA) equipped with an HP refractive index-RI (HP 1047A, HP, Palo Alto, CA, USA). The mobile phase consisted of HPLC grade water. An aliquot (20 µl) of extract was injected into an Agilent HI-PLEX Ca^{2+} column (7.7 × 300 mm, 8 µm/ Mobile phase 100% DI H_2O, flow rate 0.6 ml/min, temperature 80 °C, detector: Refractive Index) (Agilent, Santa Clara, CA, USA). The processing of the chromatograms was done by means of Peak Simple Chromatograpy Data

System consisted of a hardware (Model 302, SRI Instruments, Bad Honnef, Germany) and the Peak Simple 4.51 chromatography acquisition and integration software for Windows (SRI Instruments, Bad Honnef, Germany). Sucrose, glucose, fructose, sorbitol and mannitol content were determined by fitting values to reference curves done with HPLC pure grade standards. The concentration of starch in loquat leaves was measured according to the procedure described by Vemmos (1999)**.**

2.8. Leaf and stem anatomy

Samples of fresh leaves and stems obtained from the upper-top part of loquat stems, were fixed and preserved in a FAA solution (70% aqueous ethanol: 40% formaldehyde in H_2O: pure glacial acetic acid, 90:5:5, v/v). Afterward, tissue sections were made using a cryotome (mod. Leica CM 1850; Leica, Wetzlar, Germania). Transverse sections of loquat stems and leaves were examined with an Olympus BX40 polarized light microscope and the micrographs were photographed with an Olympus DP71 digital camera (Olympus Optical Co., Hamburg, Germany). Measurements of morphological parameters, e.g., thickness of leaf abaxial and adaxial epidermis as well as palisade and spongy parenchyma thickness were determined with Cell^A imaging software (Olympus, Tokyo, Japan).

2.9. Statistical analysis

Experiment was set up following a completely-randomized experimental design with six plants for treatment. All data were subjected to Bartlett's test using the SPSS-17 software (SPSS INC., Chicago, IL, USA) to assess homoscedasticity of data across populations. For each parameter, means of controls and B-stressed plants were compared using the Student's *t*-test ($P<0.05$). For B allocation pattern, data were subjected to one-way ANOVA with plant organ as the variability factor and then the means were separated with Duncan's post-hoc test ($P<0.05$). Percentage values were angularly transformed prior analyses.

3. Results

3.1. Effects of B excess on plant growth and symptom development

At the end of the experiment, visible symptoms of the B toxicity only appeared in the stem of seedlings treated with 400 μM B (Fig. S1B, S1D), contrary to their leaves (both top and basal) that were symptomless. Extensive unframed lesions of purple and/or black colour with brown periphery were observed on stem surface which caused a decline of the naturally-occurring green color (see control plants in Fig. S1C). Furthermore, the bark cracking was observed in those areas, with particular emphasis around the stem nodes (Fig. S1D). It is also interesting to note that the wilting of apical stem's part was apparent in plants treated with 400 μM B some days prior to the development of the above-mentioned visible symptoms of B toxicity. Besides the visible injuries, assessment of allometric traits of loquat plants revealed no differences between control and B-excess treated plants in relation to any plant organ (Table 1).

3.2. Boron allocation patterns

Boron concentrations in all plant organs were notably greater in B-excess treated plants than in controls (Table 2); B concentrations in various plant parts were from 5.3-fold (e.g., basal leaves) to 11.3-fold (e.g., top leaves) higher in plants treated with 400 μM B than in those grown with 25 μM B. In the control plants, B concentrations increased in the row: basal wood (9.10 μg g^{-1} DW) < root, top wood, basal leaves < top leaves, top bark, basal bark (34.33 μg g^{-1} DW). The corresponding order was found under B-excess conditions: basal wood (51.97 μg g^{-1} DW) < basal leaves < root, basal bark, top wood < top bark < top leaves (379.74 μg g^{-1} DW). In terms of absolute values (not as concentrations) B content accumulated at whole plant level was ~9.2-fold higher in plants treated with 400 μM B than in controls (Table 2). In terms of B distribution, B partitioning to the leaves was proportionally higher in plants irrigated with 400 μM B than in control ones, while the opposite was found for stems (B partitioning to stems was proportionally higher in controls) (Table 2).

3.3. Gas exchange, chlorophyll fluorescence parameters and photosynthetic pigments

As reported in Fig. 1, only at 147 FBE were most leaf photosynthetic parameters significantly affected by B excess. The effect was evident in younger (top) leaves where values of A_{400} (Fig. 1A), E (Fig. 1B) and WUE (Fig. 1C) as well as the CO_2 use efficiency (Fig. 1D) were significantly impaired by B excess, whilst g_s was not affected (*data not shown*). Conversely, in the older (basal) leaves, only values of E were significantly lower in 400 μM B-treated plants *versus* controls (Fig. S2B).

Chlorophyll fluorescence highlighted a reduction of F_v/F_m (Fig. 1A) which was dependent on a more severe decline of F_m than F_0 values (*data not shown*). Only small reductions, especially at low light irradiances, were observed in Φ_{PSII} at increasing light (Fig. 2B), whereas B-treated plants had dramatically higher Φ_{NO} (Fig. 2C) and severely lower Φ_{NPQ} (Fig. 2D) values when compared to those of the controls. ETR also declined strongly in B-treated plants (Fig. 2E). Changes of Φ_{NPQ} occurred in a light-dependent manner, whereas Φ_{NO} and ETR declined constantly from 650 μmol PAR and 900 μmol PAR onward, respectively. The proportion of open reaction centres (q_P; Fig. 1F) was not influenced by B treatment, independent to the light intensity.

Regarding the leaf photosynthetic pigments, no significant differences were observed in terms of chlorophyll concentration and proportion (Chl *a*, Chl *b*, Chl *a*+*b*, Chl *a*/*b*) and total carotenoids in the top leaves of loquat plants treated either with 25 or 400 μM B (Table S1).

3.4. Soluble sugar, starch, proline, hydrogen peroxide, and lipid peroxidation by-products content in leaves and stem barks

As shown in Table 3, B excess resulted in a significant increase in total sugar levels in both stem barks and leaves. A deep analysis unveils the plants treated with B excess accumulated more non-translocated sugars (fructose + glucose) than the controls and the increment was attributable to the raise of both monosaccharides. Conversely, non-significant differences were recorded concerning the total levels of translocated sugars (sucrose + sorbitol + mannitol), even though some changes occurred in translocated sugar profile, similarly in both leaves and barks of B-excess treated plants. Notably, the leaves and stem barks of the plants treated with 400 μM B accumulated higher levels of sorbitol and reduced levels of sucrose, whereas mannitol content remained unchanged. Under high B, starch content decreased only in the stem bark. Finally, proline and

H_2O_2 levels were essentially unaffected by the B treatments, contrary to MDA levels that were about 2-fold higher in B-stressed than control plants.

3.5. Leaf and stem anatomy

Light micrograph of leaf and stem transverse sections are reported in Fig. 3. At the end of the experiment, B excess resulted in an increased thickness of the leaf blade, which is mainly due to the increased thickness of the spongy parenchyma layer. An increase in the number of oil droplets was also observed within the palisade cells adjacent to the adaxial epidermis of B-excess treated plants (Fig. 3B). As reported in Fig. 3D, excess of B also provoked morpho-anatomical changes in loquat stems which are supportive of an affected secondary growth of treated plants. Specifically, an increased number of cork parenchyma cells, a thicker epidermis and a higher number of collenchyma cells with thicker cell walls were indeed evident in the stems of B-excess treated plants.

4. Discussion

In most plant species B is essentially immobile into the phloem and it moves solely following the transpiration stream, whereas in plants that biosynthesize high levels of polyols, including sorbitol and mannitol as in loquat (Table 3), B is readily remobilized to sink organs by the phloem flux in the form of B-polyol complexes (Brown and Shelp 1997). In the current investigation, a high phloem mobility of B was identified by its allocation pattern in loquat organs (Table 2) along with the appearance of visible symptoms of B toxicity exclusively in younger (upper) part of the shoots. El-Motaium et al. (1994) observed that in polyol-producing species grown under B excess, B principally accumulated in stems rather than in leaf margins of older leaves, where B typically accumulates in sucrose-translocating species. Accordingly, a completely different pattern of B accumulation, and consequently in the development of toxicity symptoms, was observed in citrus trees where B is phloem immobile (Papadakis et al., 2003).

Since loquat trees produce fruits from apical flower buds, the basipetal allocation of B excess, and thus the development of necrotic areas in younger-apical parts of stems, can severely reduce the loquat fruit production. While deleterious effect of B excess on plant biomass were not

observed, the study of the leaf and stem anatomy/physiology revealed that the B excess had triggered multiple alterations that are probably associated with internal mechanisms adopted by plants (in the attempt) to cope with B toxicity. Boron excess promoted the development of a thicker spongy mesophyll layer and increased the occurrence of lipid droplets. Similar effects of B toxicity on the mesophyll structure was observed in citrus trees (Huang et al., 2014; Papadakis et al., 2004). Chapman et al. (2012) associated the occurrence of an elevated number of lipid droplets in non-seed tissues with biotic and abiotic stress responses. However, the precise role of the lipid droplets when promoted by abiotic stresses, such as in B toxicity, remains to be elucidated.

In stems, where the symptoms of B toxicity were also macroscopically evident, B excess caused considerable morphological alterations. The B-treated plants exhibited notable acceleration of the stem secondary growth, producing a layer of cork cells and several layers of collenchyma cells. Collenchyma cells are characterized by large and thick pectin-containing cell wall and a small number of chloroplasts. In plant cells, pectins participate to control the cell wall porosity, thus are critical for its functionality (Leroux 2012). Moreover, it is well-documented that the B participates in the structure of the pectin network, functioning as a crosslink between rhamnogalacturonan II (RG-II) sidechains which are required for the development of cell wall, and it is estimated that in most plant species more than 90% of the total B is localized in the cell wall (Woods, 1996). Given that the phloem is the site of higher concentration of B of the entire plant, we consider that there is, possibly, a mechanism of plant adaptation to B surplus through binding and immobilizing the element within the cell walls of the newly formed non-photosynthesizing phloem tissues, which preserve other tissues, cell compartments and organelles less prone to B toxicity. To the best of our knowledge, such a mechanism is yet to be described as a plant response to B toxicity.

Although the physiological basis for B toxicity is not clear, three main causes have been proposed, taking into account our knowledge of B chemistry (i.e., the ability of B to bind compounds with two hydroxyl groups in the *cis*-configuration): (i) alteration of cell wall structure; (ii) metabolic disruption by binding to the ribose moieties of molecules such as ATP, NADH and NADPH; and (iii) disruption of cell division and development by binding to ribose, either as the free sugar or within RNA (Reid et al. 2004). It is therefore conceivable that plants submitted to B stress exhibit altered sugar metabolism and, in particular, we propose that the strong enhancement of sorbitol (which paralleled the steep reduction of sucrose content) observed in the present experiment represents a further attempt (besides the amount of B immobilized in larger/thicker

collenchyma cells) to "sequestrate" B ions into the phloem; this likely mitigates the harmful effect described above. In addition, the accumulation of sorbitol observed in young leaves may perhaps represent another way to complex B which preserves other key polyols and polyol-based compounds (i.e., ATP, NADH). In support of our hypothesis, the observation that sorbitol-B-sorbitol complexes have been already isolated in *Prunus* species and, notably, even fructose (another sugar that is incremented in loquat leaves and stem barks) can form fructose-B-sorbitol and fructose-B-fructose complexes (Hu et al., 1997). In addition, in a tobacco line transformed with sorbitol-6-phosphate dehydrogenase, the production of sorbitol was accompanied by an increase in the translocation and accumulation of B, even in young plant tissues, compared with a sorbitol-6-phosphate dehydrogenase anti-sense line (zero-sorbitol producer with no B accumulation in meristematic tissues) (Bellaloui et al., 1999). In turn, the reduction of sucrose content in young leaves and stems bark highlights a possible reduced supply of sucrose to non-photosynthesizing tissues.

Although the above-detailed mechanisms could have assisted the leaves in counteracting the effect of B toxicity, the physiological/biochemical performances of young leaves were severely altered, evidenced by the steep decline of photosynthesis, the loss of PSII functionality and the incurrence of oxidative stress (increment of MDA by-products in the leaves). The loss of carbon gain in young leaves was not attributable to the reduction of CO_2 diffusion by stomatal limitation (see unchanged level of g_s), even though a higher thickness of the leaves, as observed in leaves of B-stressed, may have reduced the internal diffusion of CO_2 to chloroplast (mesophyll conductance *sensu* Tomas et al., 2013). There is no consensus in the literature as to whether B is directly or indirectly responsible for stomata closures as in some cases stomatal limitations were reported (Landi et al., 2013c; Lovatt and Bates, 1984), whilst not in others (Sotiropoulos et al., 2002), therefore suggesting that it can be a specie-specific responsive trait and/or a feature dependent to the applied experimental conditions and leaf age (for a review see Landi et al., 2012). In addition, lack of increment in WUE, associated with unchanged level of proline, both usually observed under osmotic stress (Guidi et al., 2017) confirms that the main effect of B-toxicity does not directly participate in altering the osmotic cell balance (Reid et al., 2004). Conversely, multiple biochemical limitations were usually associated with B-stress and considered the main constrains of the photosynthetic process (Landi et al., 2013a; Papadakis et al., 2004), as below detailed for the present experiment. Firstly, even though speculative, the reduction of the carboxylation

efficiency may be attributable to the effect of B on ribulose, which is another *cis*-diol that can potentially bind to excessive B ions (Reid et al., 2004) or to the feedback regulation induced by glucose/fructose accumulation (Moreira Lobo et al., 2013). Secondly, the occurrence of photoinhibition and decline of both F_0 and F_m denote some damages to light harvesting complexes of PSII (LHCIIs) and PSII reaction centres (RCIIs). In particular, F_0 originates primarily from the LHCII, while F_m is associated with the reduction/oxidation state of the primary quinone electron acceptor of PSII (Q_A), thus in the rise from F_0 to F_m the RCII is involved as well (Lichtenthaler and Babani, 2004). Thus, the reduction of F_0 (associated to unchanged content/proportion of chlorophylls as found in the present study) is indicative of damages to the LHCII which have a reduced ability to capture photons and/or to tunnel photons to RCII. The decrease in F_m values may be related to denaturation of chlorophyll-binding proteins (Yamane et al., 1998), such as the antenna pigment-protein complex CP43-CP47 (Wang et al., 1999) and/or irreversible damages to the RCII (Klughammer and Schreiber, 2008). However, the fact that the proportion of open reaction centres (q_P) was not lower in B-excess treated plants is supportive for the incurrence of damages to LHCII rather than to RCII, which downstream the photon flux to RCII. The reduction of photon flux to RCII, accompanied by a decrease in the rate of consumption of ATP and NADPH for CO_2 assimilation by Calvin-Benson cycle (reduced CO_2 use efficiency), resulted in decreases in ETR values, especially when Mehler reaction (and operation of the water–water cycle *sensu lato*) and photorespiration does not compensate the lower rate of Calvin-Benson cycle (Flexas et al., 2002). In addition, complexation of B to ATP and NADPH changes the chemical structure of these coenzymes and reduces their affinity for enzyme catalytic sites (Reid et al., 2004).

Concerning the partitioning of absorbed light in photochemistry, controlled and non-controlled dissipative mechanisms (Φ_{PSII}, Φ_{NPQ}, and Φ_{NO}, respectively) the most remarkable result was the strong increase of Φ_{NO} and the decline of Φ_{NPQ} values in B-stressed plants. The Φ_{NO} usually reflects the fraction of energy that is passively dissipated in the form of heat and fluorescence, mainly due to the closed PSII, whereas Φ_{NPQ} corresponds to the fraction of energy dissipated in the form of heat *via* the regulated photoprotective NPQ mechanisms, namely Δ-pH- and xanthophyll-regulated thermal dissipation (Klughammer and Schreiber 2008, Pfündel et al. 2008). At saturating light intensities, a stronger enhancement of Φ_{NO} over Φ_{NPQ} reflects the suboptimal capacity of photoprotective reactions, which eventually leads to photodamage and photoinhibition, in most cases shifting from dynamic to chronic photoinhibition (Klughammer and Schreiber, 2008).

Indeed, the successful regulation in variable environmental stresses is generally aimed at maximal values of Φ_{PSII}, with the remaining loss 1-Φ_{PSII} aimed at a maximal ratio of Y(NPQ)/Y(NO), as occurred in B-stressed sweet basil plants, according to Landi et al. (2013b). In accordance with previous research that has focused on B toxicity, the oxidative stress observed in our experiments, in terms of both MDA by-products production, can be partially a direct consequence of an altered ability of light processing which leads to ROS generation and propagation of oxidative stress (Ardic et al., 2009; Cervilla et al., 2007; Gunes et al., 2006; Landi et al., 2013b; Landi et al., 2013c; Landi et al. 2014), even at the chloroplast membrane level.

5. Conclusions

The results of the present study indicate that B stress did not provoke serious alterations in plant biomass at the end of the experimental period, but it caused severe perturbation of several physiological and biochemical parameters in loquat seedlings. We observed a severe re-modulation of sugar metabolism with a shift from the production of sucrose to that of sorbitol and fructose. Both sorbitol and fructose have high affinity to form phloem mobile B-complexes which favors accumulation of B principally to young tissues, therefore sequestrating/immobilizing excess B in young leaves (usually more reactive and sensitive) and preserving them from B-triggered damages. On the other hand, prolonged B toxicity to young tissues (perhaps longer than the duration of the present experiment for which the biomass was unchanged between control and B-excess treated plants) can severely impair the plant growth. The observed increase in the number of collenchyma cells and the increase of the thickness of their cell wall in the stems of high B-treated plants represent a further attempt by the plant to “store” excessive B in tissues where B ions are less harmful. Besides these reactive mechanisms adopted by loquat seedlings, serious impairments of the photosynthetic process with severe alteration to PSII were observed in younger leaves, likely to be one of the causes of the incremented level of oxidative stress. In view of the new insights added by the present experiment, and remaining conscious that some aspects still need to be clarified, we believe that the exploration of plant responses to B excess still deserves further investigation.

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Conflicts of interest

The authors declare no conflicts of interest.

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Table 1
Effects of B concentration in the nutrient solution (25 or 400 μM) on various growth parameters of loquat seedlings.

Parameter	Plant part	25 μM B	400 μM B	*t-test*
Dry weight (g plant^{-1})	Leaves	62.96±7.72	58.22±3.99	n.s
	Stems	31.92±2.77	34.52±3.13	n.s
	Roots	14.78±1.33	12.70±0.59	n.s
	Whole plant	109.65±11.6	105.44±7.39	n.s

Mean ± S.E., n = 6; Each replicate (n) corresponds to a different plant per B treatment. n.s = P≥0.05 after Student's t-test between 25 and 400 μM B.

Table 2
Effects of B concentration in the nutrient solution (25 or 400 µM) on the B concentration (µg g^{-1} DW) and content (absolute quantity, µg) in various vegetative parts of loquat plants as well as on B distribution in different plant organs (% total plant content).

Parameter	Plant part	25 µM B	400 µM B	*t-test*
Boron concentration (µg g^{-1} DW)	Top leaves	33.63±0.83c	379.74±33.01e	***
	Top bark	33.81±2.15c	277.05±9.82d	***
	Top wood	22.91±1.58b	215.77±10.64c	***
	Basal leaves	25.93±1.48b	138.55±4.30b	***
	Basal bark	34.33±1.16c	214.37±7.20c	***
	Basal wood	9.10±0.32a	51.97±2.06a	***
	Root	22.34±0.95b	196.16±18.78c	***
Boron content (µg)	Leaves	1978.17±189.37	19295±2096.5	***
	Stems	657.5±56.89	5446.5±540.11	***
	Root	328.5±30.17	2525.83±296.32	***
	Entire plant	2964±271.9	27266.67±2612.16	***
Boron distribution (% total plant B content)	Leaves	66.64±0.55	70.31±1.87	*
	Stems	22.26±0.66	20.04±0.73	*
	Root	11.1±0.29	9.66±1.44	n.s

Mean ± S.E., n = 6; Each replicate (n) corresponds to a different plant per B treatment. n.s = $P>0.05$; * = $P<0.05$; *** = $P<0.001$ after Student's *t*-test between 25 and 400 µM B. For B concentration in plant organs, mean keyed with different letters are not statistically different in the column (Duncan's post-hoc; $P\leq0.05$).

Table 3
Effects of B concentration in the nutrient solution (25 or 400 μM) on carbohydrates, proline, malondialdehyde by-products and hydrogen peroxide measured in leaves and stem's bark of loquat plants.

Parameter		25 μM B	400 μM B	*t-test*
Carbohydrates in leaves (% DW)	Sucrose	5.48±0.1	4.62±0.08	***
	Glucose	0.89±0.07	1.31±0.06	***
	Fructose	0.59±0.03	0.76±0.05	*
	Mannitol	1.32±0.05	1.31±0.08	n.s
	Sorbitol	7.91±0.46	10.13±0.35	**
	Total sugars	16.18±0.6	18.12±0.53	*
	Translocated sugars	14.7±0.53	16.05±0.44	n.s
	Non-translocated sugars	1.48±0.09	2.06±0.11	**
	Starch	0.27±0.01	0.26±0.01	n.s
Carbohydrates in stem's bark (% DW)	Sucrose	5.87±0.32	4.66±0.15	**
	Glucose	0.31±0.01	0.62±0.08	**
	Fructose	0.33±0.02	0.79±0.06	***
	Mannitol	1.14±0.04	1.12±0.06	n.s
	Sorbitol	5.42±0.35	8.03±0.74	**
	Total sugars	13.06±0.48	15.21±0.8	*
	Translocated sugars	12.42±0.47	13.8±0.71	n.s
	Non-translocated sugars	0.64±0.03	1.41±0.12	***
	Starch	0.53±0.05	0.37±0.04	*
Proline (μmol g^{-1} FW)	Leaves	1.50±0.03	1.53±0.05	n.s
	Stem's bark	0.99±0.05	1.07±0.08	n.s
MDA (μmol g^{-1} FW)	Leaves	1.17±0.08	2.36±0.15	***
	Stem's bark	0.15±0.03	0.88±0.14	***
H_2O_2 (μmol g^{-1} FW)	Leaves	2.27±0.29	2.44 ±0.20	n.s
	Stem's bark	1.16±0.11	1.32±0.17	n.s

Mean ± S.E., $n = 6$; Each replicate (n) corresponds to a different plant per B treatment. n.s = $P>0.05$; * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$ after Student's *t*-test between 25 and 400 μM B.

Table 4
Morphometric assessments of the leaves and the bark of stems of loquat plants grown hydroponically under normal (25 μM B) or excessive B (400 μM B).

Parameter		25 μM B	400 μM B	*t*-test
Leaf histological component (μm)	Upper epidermis	23.89±0.67	25.94±1.18	n.s
	Palisade parenchyma	95.21±2.74	104.77±3.75	n.s
	Spongy parenchyma	81.13±2.29	113.66±6.32	***
	Lower epidermis	18.04±0.83	19.19±0.44	n.s
	Entire lamina	218.27±2.13	263.56±7.11	***
Stem's bark epidermis (μm)		13.53±0.4	31.89±1.42	***

Mean ± S.E., $n = 4$; Each replicate (n) corresponds to the assessment of five microphotographs per each experimental plant and sampled organ (leaf or stem). n.s = $P>0.05$; *** = $P<0.001$ after Student's *t*-test between 25 and 400 μM B.

Table S1
Effects of B concentration in the nutrient solution (25 or 400 μM) on photosynthetic pigments measured in leaves of loquat plants.

Parameter		25 μM B	400 μM B	*t-test*
Chl and Car content (mg g^{-1} FW)	Chl *a*	1.02±0.05	0.89±0.05	n.s
	Chl *b*	0.73±0.08	0.59±0.04	n.s
	Chl *a*+*b*	1.75±0.11	1.48±0.08	n.s
	Chl *a*/*b*	1.45±0.14	1.51±0.07	n.s
	Car	0.17±0.02	0.14±0.06	n.s

Mean ± S.E., $n = 6$; Each replicate (n) corresponds to a different plant per B treatment. n.s = $P>0.05$ after Student's *t*-test between 25 and 400 μM B.

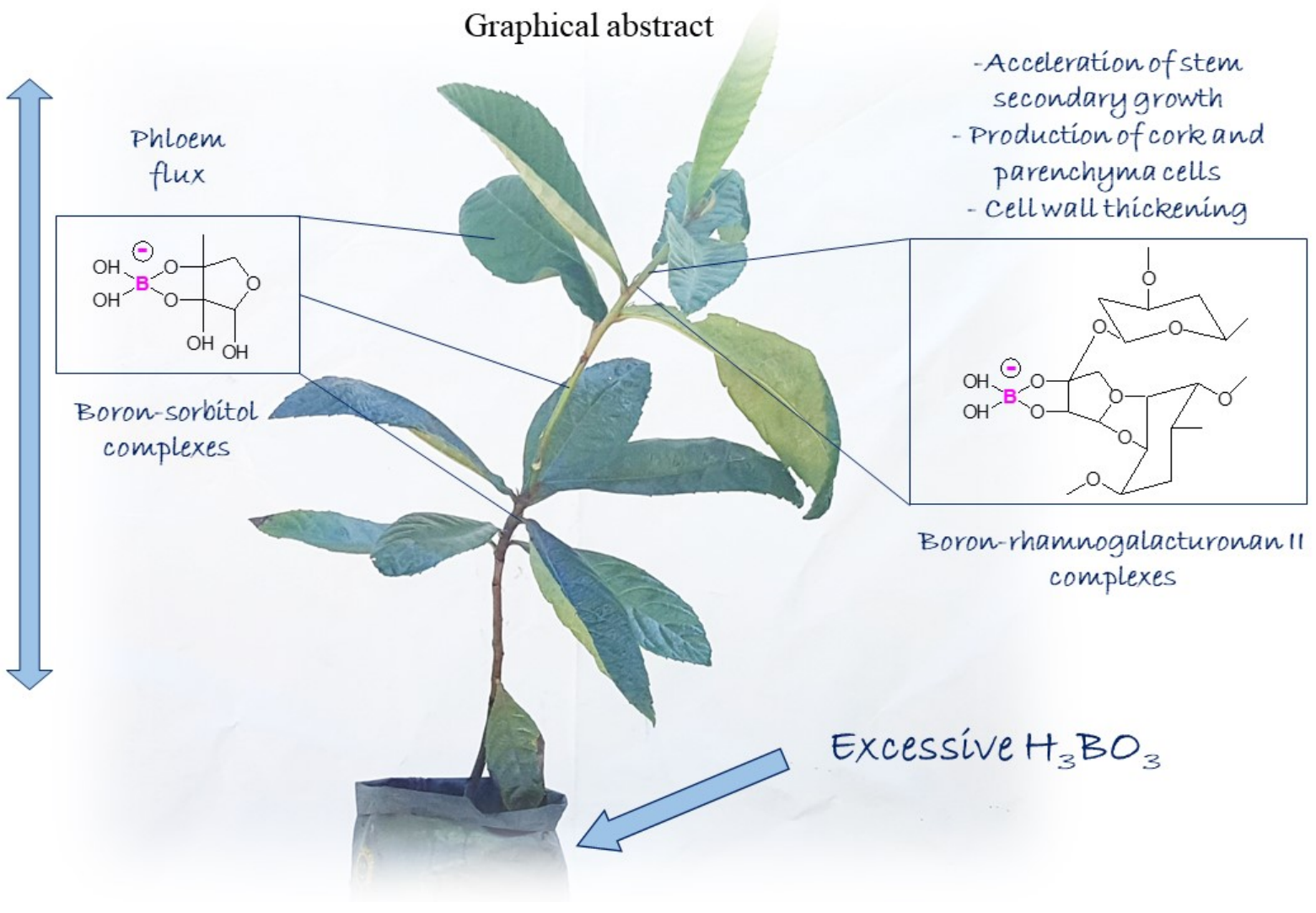
Figure legends

Fig. 1. (A) net photosynthesis at ambient CO_2, (B) evapotranspiration rate, (C) intrinsic water use efficiency, and (D) CO_2 use efficiency in top asymptomatic leaves of *Eriobotrya japonica* seedlings subjected to safe concentrations (25 µM; continuons line) or excess B (400 µM; dotted line). For each sampling time, asterisks denote significant differences of the means (n = 6 ± S.E) after Student's *t*-test (P≤0.05). n.s = P>0.05; * = P<0.05; *** = P<0.001.

Fig. 2. (A) maximal efficiency of PSII in dark-adapted conditions, (B) operational efficiency of PSII, (C) regulated and (D) non-regulated energy loss in PSII, (E) electron transport rate and (F) proportion of open reaction centres in PSII at increasing photon flux densities. Parameters were measured at 147 days after the beginning of experiment in top asymptomatic leaves (n = 6 ± S.E) of *Eriobotrya japonica* seedlings subjected to safe concentrations (25 µM) or excess B (400 µM). In panel A, asterisks denote significant differences of the means after Student's *t*-test (P<0.001).

Fig. 3. Micrograph transverse section of control (A) and B-excess treated (B) top leaves, and control (C) and B-excess treated (D) upper stems of *Eriobotrya japonica* seedlings.

Graphical abstract



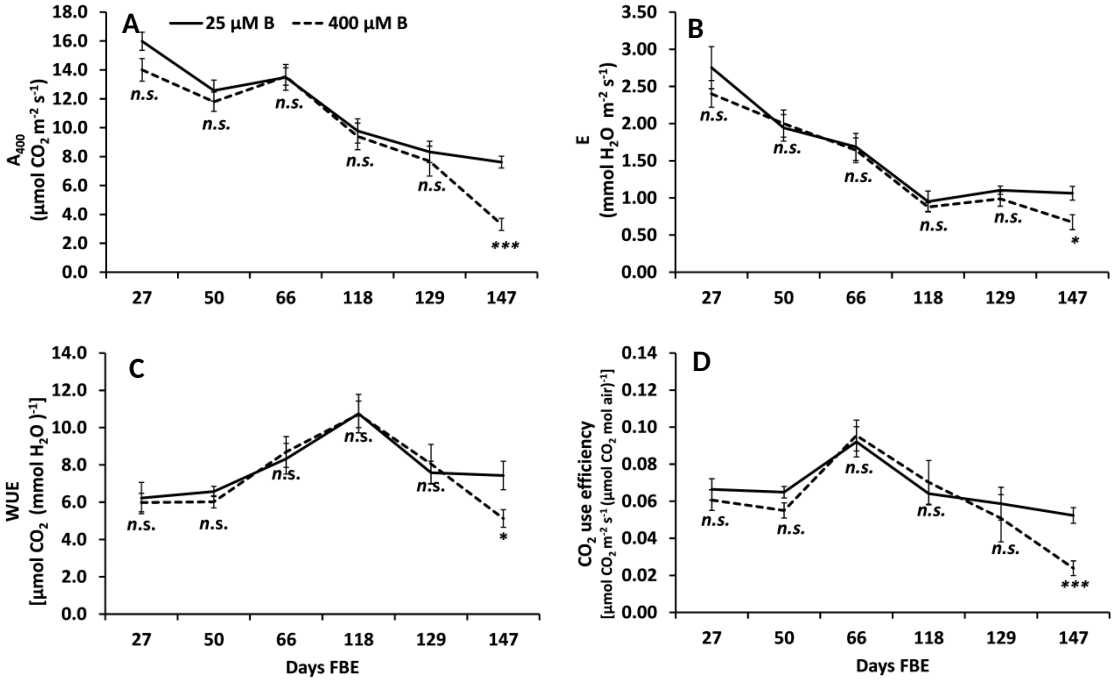

A
25 µM B
400 µM B
A_{400} (µmol CO_2 m^{-2} s^{-1})
B
E (mmol H_2O m^{-2} s^{-1})
C
WUE [µmol CO_2 (mmol H_2O)$^{-1}$]
D
CO_2 use efficiency [µmol CO_2 m^{-2} s^{-1} (µmol CO_2 mol air)$^{-1}$]
n.s.
*
***
27
50
66
118
129
147
Days FBE

Figure 1

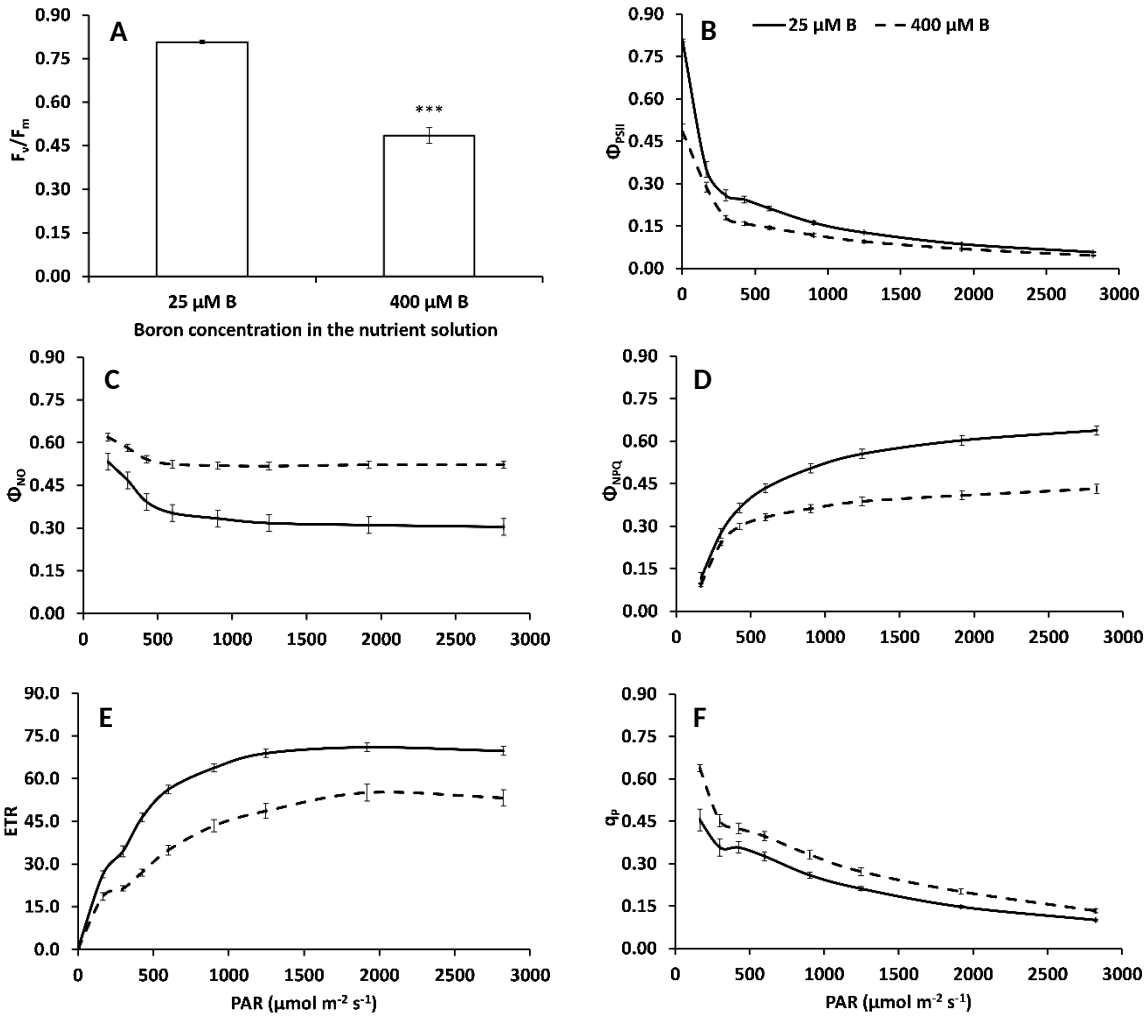

A
B
C
D
E
F
***
25 µM B
400 µM B
Boron concentration in the nutrient solution
PAR (µmol m-2 s-1)
PAR (µmol m-2 s-1)

Figure 2

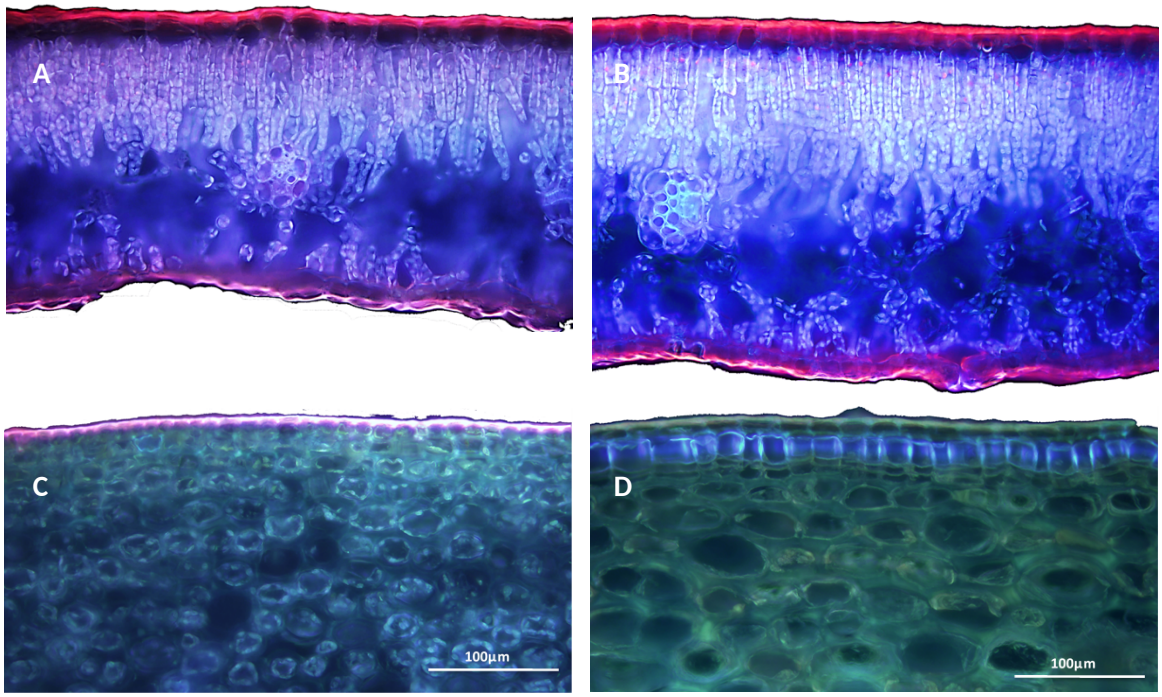


Figure 3

Fig. S1

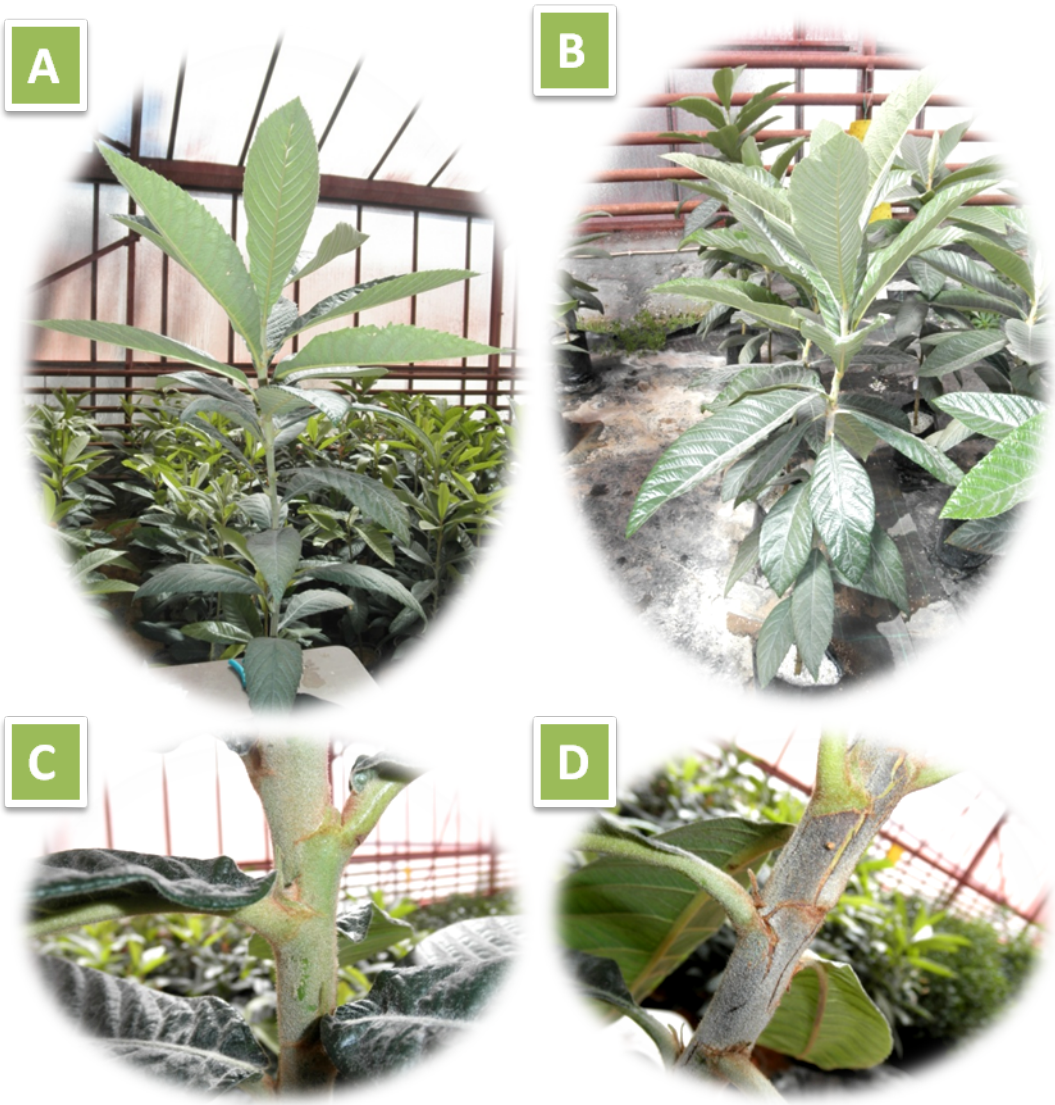


Fig. S1. Shoot (A, B) and upper stem (C, D) in *Eriobotrya japonica* seedlings subjected for 147 days to safe concentrations (25 µM; A and C) or excess B (400 µM; B and D) in the nutrient solution.

Fig. S2

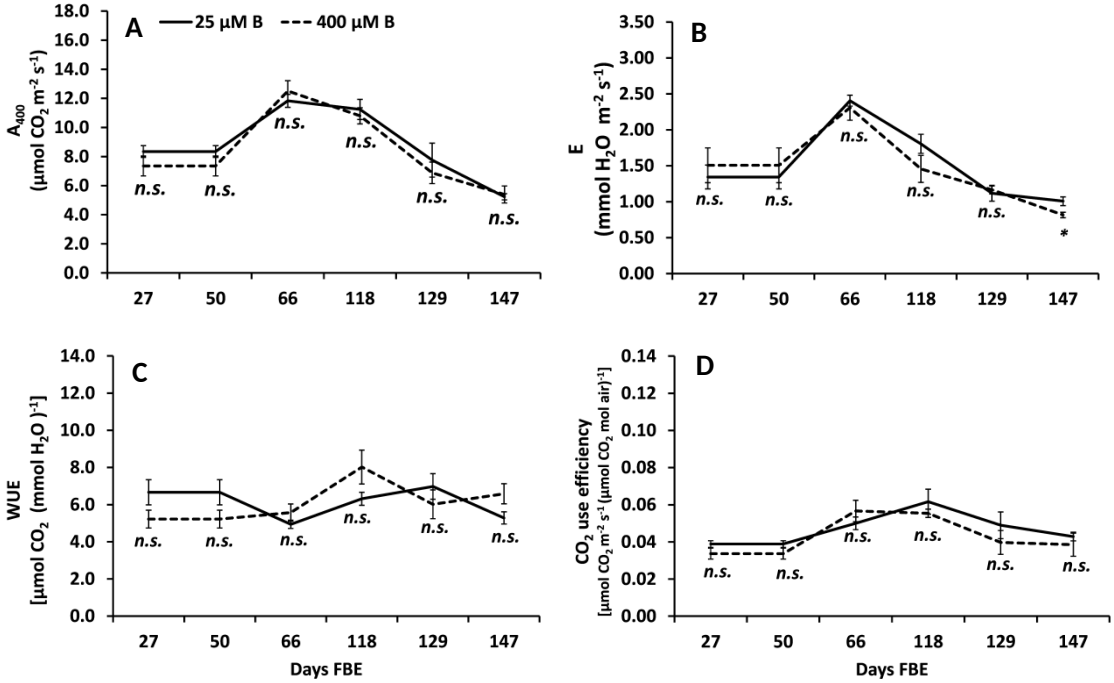


Fig. S2. (A) net photosynthesis at ambient CO_2, (B) evapotranspiration rate, (C) intrinsic water use efficiency, and (D) CO_2 use efficiency in basal leaves of *Eriobotrya japonica* seedlings subjected to safe concentrations (25 µM; continuons line) or excess B (400 µM; dotted line). For each sampling time from the beginning of experiment, asterisks denote significant differences of the means (n = 6 ± S.E) after Student's t-test (P≤0.05). n.s = P>0.05.