Plant and Soil

Boron accumulation and tolerance in sweet basil (Ocimum basilicum L.) with green or purple leaves

--Manuscript Draft--

Manuscript Number:	
Full Title:	Boron accumulation and tolerance in sweet basil (Ocimum basilicum L.) with green or purple leaves
Article Type:	Manuscript
Keywords:	anthocyanic variants; boron toxicity; mineral nutrition; rosmarinic acid; sweet basil; xylem sap.
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Abstract:	Background and aims There is a wide variability in plant tolerance to boron toxicity, which is often associated with the ability to limit boron accumulation. This study was conducted on two cultivars of sweet basil (Ocimum basilicum L.) with different boron tolerance: 'Tigullio', less tolerant and with green leaves; 'Red Rubin', more tolerant and with purple leaves. The main goal was to verify whether the greater boron tolerance of 'Red Rubin' is attributable to an exclusion mechanism. Methods In three greenhouse experiments, plants were grown hydroponically with solution boron concentration ranging from 0.25 (control) to 25 mg L-1. Results Tissue boron content increased with increasing boron supply. Boron concentrations in root and leaf tissues were comparable in 'Tigullio' and 'Red Rubin' or even higher in the purple cultivar. Boron supply did not affect the leaf content of total phenolic compounds and other nutrients. Leaf content of total phenols and rosmarinic acid were much higher in 'Red Rubin' than in 'Tigullio'. Conclusions The greater boron tolerance of 'Red Rubin' was associated with the ability to withstand higher concentrations of this element in plant tissues rather than to an exclusion mechanism. The high phenolic content was thought to contribute to the boron tolerance of 'Red Rubin'.

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Pisa, 9 February 2015

Dear Editor,

I submit the manuscript intitled "Boron accumulation and tolerance in sweet basil (*Ocimum basilicum* L.) with green or purple leaves" for publication in *Plant and Soil*.

The manuscript was revised by Dr. Beatrice Pezzarossa (Institute for Ecosystem Study, CNR, Pisa, Italy; Beatrice.pezzarossa@ise.cnr.it).

The graphs were prepared using GraphPad Prism 5.02 for Windows (GraphPad Software, 2008; version 5.02) and exported to EPS files.

Best regards

Alberto Pardossi

Boron accumulation and tolerance in sweet basil (*Ocimum basilicum* L.) with green or purple leaves

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ABSTRACT

Background and aims There is a wide variability in plant tolerance to boron toxicity, which is often associated with the ability to limit boron accumulation. This study was conducted on two cultivars of sweet basil (*Ocimum basilicum* L.) with different boron tolerance: 'Tigullio', less tolerant and with green leaves; 'Red Rubin', more tolerant and with purple leaves. The main goal was to verify whether the greater boron tolerance of 'Red Rubin' is attributable to an exclusion mechanism.

Methods In three greenhouse experiments, plants were grown hydroponically with solution boron concentration ranging from 0.25 (control) to 25 mg L^{-1}.

Results Tissue boron content increased with increasing boron supply. Boron concentrations in root and leaf tissues were comparable in 'Tigullio' and 'Red Rubin' or even higher in the purple cultivar. Boron supply did not affect the leaf content of total phenolic compounds and other nutrients. Leaf content of total phenols and rosmarinic acid were much higher in 'Red Rubin' than in 'Tigullio'.

Conclusions The greater boron tolerance of 'Red Rubin' was associated with the ability to withstand higher concentrations of this element in plant tissues rather than to an exclusion mechanism. The high phenolic content was thought to contribute to the boron tolerance of 'Red Rubin'.

Keywords anthocyanic variants; boron toxicity; mineral nutrition; rosmarinic acid; sweet basil; xylem sap.

Introduction

Boron (B) is an essential element for plants. However, in many regions in the world B concentration in soil and/or irrigation water often exceeds plant requirements, thus becoming a threat to crop productivity (Nable et al. 1997). Boron excess induces an oxidative stress and the occurrence of leaf chlorosis and necrosis, which reduce photosynthesis and dry matter accumulation (Landi et al. 2012). There is a wide intra- and inter-specific variability in plant tolerance to B toxicity, which is generally associated with reduced B accumulation in the shoot (Goldbach and Wimmer 2007; Reid, 2014).

This work was conducted on two cultivars of sweet basil (*Ocimum basilicum* L.) having different tolerance to B toxicity, as previously found in our laboratory (Landi et al. 2013a, 2014): 'Tigullio', less tolerant and with green leaves; 'Red Rubin', more tolerant and with purple leaves. Green-leafed sweet basil is widely cultivated and mostly used for food preparation (Makri and Kintzios 2007); for instance, it is the main ingredient of the famous Italian green sauce 'pesto'. Purple-leafed variants of sweet basil are commonly used for ornamental purposes (Makri and Kintzios 2007); in these plants, the purple color is attributable to high concentration of anthocyanins in the leaf epidermis (Landi et al. 2014). Previous works focused on the possible role of antioxidant systems and anthocyanins in B tolerance of 'Red Rubin' (Landi et al. 2013a, 2014). Recent research also indicates that antocyanins play a major role in the tolerance of this cultivar to high sunlight irradiance (Tattini et al. 2013).

The main goal of this study was to assess whether the greater B tolerance of 'Red Rubin' compared to 'Tigullio' is also attributable to the ability to restrict B uptake and accumulation. The second aim was to assess the B tolerance of the two basil cultivars using the piecewise linear model proposed by Maas and Hoffman (1977) for crop response to salinity. This model was also tested for B toxicity in a wide range of crop species, such as: broccoli, cauliflowers and radish (Francois 1986); garlic and onion (Francois 1991); squash, melon, cucumber and corn (El-Sheikh et al. 1971); sugar beet (Vlamis and Ulrich 1973); tomato (Francois 1984); wheat, barley and sorghum (Bingham et al. 1985). However, to the best of our knowledge, the Maas-Hoffman equation was never used to interpret the response of sweet basil to B supply. Another aim of this work was to ascertain whether the growth inhibition of sweet basil due to excessive B supply was associated with reduced uptake of other nutrients. Finally, we examined the effects of B supply on leaf content of total phenols and some caffeic acid derivatives, including rosmarinic acid, which is largely contained in sweet basil (Kiferle et al. 2011).

Materials and methods

Plant material and growing conditions

Three series of experiments were conducted on sweet basil seedlings grown in hydroponic culture in the spring (Experiments 1 and 2) or in the autumn (Experiment 3) of 2013 in a glasshouse in Pisa (central-western Italy).

Seedlings were transferred into separate hydroponic systems 21 (Experiment 1 and 2) or 42 (Experiment 3) days after sowing. Each hydroponic system consisted of a polystyrene tray floating in: a 50-L plastic tank (each tank hosted 16 plants) in Experiments 1 and 3; a 2-L pot (5 plants per pot) in Experiment 2. Planting density was approximately 96 plants m^{-2} of ground area.

Inside the glasshouse, plants were grown under natural light; the minimum and ventilation air temperature were 14 °C and 27 °C, respectively. During the experimental period, air temperature and daily global radiation averaged 23.5°C and 12.1 MJ m^{-2} day^{-1} in spring, and 21.4°C and 4.2 MJ m^{-2} day^{-1} in autumn.

In all the experiments, the nutrient solutions were prepared using tap water and contained the following concentrations of nutrients: 10.0 mM N-NO_3; 1.0 mM P-H_2PO_4; 8.0 mM K; 4.5 mM Ca; 2.0 mM Mg; 3.5 mM S-SO_4; 40 µM Fe; 1.9 µM B; 3.6 µM Cu; 9.2 µM Zn; 10.9 µM Mn; 1 µM Mo. Different B (boric acid) concentrations were tested in each experiment. The nutrient solution also contained small amounts of Na (2.0 mM) and Cl (4.5 mM), which were contained in the tap water.

The nutrient solution in each hydroponic system was checked every 1-2 days for pH, EC and ion concentrations, and replaced completely every week in Experiments 1 and 3, or every 2-3 days in Experiment 2. Large volume and/or frequent renewal of the nutrient solution minimized the variations of pH, EC and ion concentrations, which in all treatments remained within 3-5% of the desired values throughout the experiment (data not shown).

Experimental design

The treatments were defined by a combination of two factors: the concentration of B in the nutrient solution and the cultivar (‘Tigullio’ and ‘Red Rubin’). Treatments were arranged in a totally randomized design with four replicates, each consisting of a tank or a pot.

The following B concentrations were tested: 0.25, 5, 10, 15, 20 and 25 mg L^{-1} (corresponding to 0.023, 0.462, 0.924, 1386, 1.848 and 2.312 mM, respectively) in Experiment 1; 0.25, 5 and 20 mg L^{-1} in Experiment 2; 0.25 and 20 mg L^{-1} in Experiment 3. Control plants were grown at 0.25 mg B L^{-1}, which is an optimal concentration for most species grown in hydroponic culture (Sonneveld

and Voogt 2009). Boron concentrations were differentiated two weeks after planting. The treatments lasted 14, 21 or 35 days in Experiment 1, 2 and 3, respectively. **Table 1** shows the basic information on each experiment.

Determinations

At the end of each experiment, plants were sampled from each experimental unit for growth analysis and chemical analysis. Each sample consisted of two (Experiment 2) or four (Experiment 1 and 3) individual plants. Plants were separated in roots, stem and leaves to determine their biomass (dry weight, DW). Plant samples were dried in a ventilated oven at 80 °C until reaching a constant weight. Leaf area was determined with a digital planimeter.

Sub-samples of dry samples were analyzed for mineral content, as follows. Reduced N was quantified with Kjeldhal system while $N\text{-}NO_3$ was determined on aqueous extracts by nitration of salicylic acid. Sub-samples were also digested with a mixture of nitric and perchloric acid at 230 °C for 1 h and elements were determined as follows: B by the Azomethine-H colorimetric method; K, Ca, Mg, Cu, Fe and Mn by atomic absorption spectroscopy; P by Olsen's method. The same methods were used to analyze the nutrient solutions.

In Experiment 1, leaf content of total phenolic compounds and some selected caffeic acid derivatives (caffeic acid, caftaric acid, chlorogenic acid, cicoric acid, cynarin, ferulic acid, *t*-cinnamicacid, *p*-coumaric acid, rosmarinic acid) was determined in fresh leaf samples. Each sample consisted of all the leaves collected from four plants. Samples were rapidly washed in deionised water, gently dried with a towel, frozen in liquid nitrogen and stored at -80 °C before analyses, which were performed within a few weeks after sampling. Samples were not dried before extraction as desiccation was found to markedly reduce the content of rosmarinic acid in sweet basil tissues (Kiferle et al, 2011). The concentration of caffeic acid derivatives in ethanol-HCl (ethanol 80%, HCl 1%, H_2O 19%) extracts was determined by means of HPLC (Kiferle et al. (2011) and expressed per gram of dry weight. Peak identification was accomplished by LC-MS and LC-MS-MS; the detection limit of the analytical method was 0.05 g kg^{-1} DW. Leaf extracts (ethanol 80%, H_2O 20%) were also analyzed for the total phenol content using the Folin–Ciocalteau reagent (Kang and Saltveit 2002); values were expressed as mg gallic acid g^{-1} DW.

At the end of Experiment 1, plants were detopped above the collar to permit free exudation of the xylem sap for 20-30 min after the cut surface had been blotted with damp tissue and enclosed in an inverted test tube lined with wet filter paper. First droplets of the exudate were discarded to prevent contamination with phloem sap and fluids from damaged cells. Each sample (approximately 2 mL) consisted of the xylem sap collected from four to six different plants.

In Experiment 2, each pot was weighted every 2-3 days with and without the plants using an electronic balance (accuracy of 0.1 g) to determine the rate of leaf transpiration and plant water uptake. After measurements, the residual nutrient solution was discharged and pots were refilled with fresh nutrient solution.

Modeling growth response to B level

The relationship between B level and plant growth was examined using the Maas and Hoffman model for crop response to salinity (Maas and Hoffman 1977). According to this model, there is no real change in relative growth (Y^*; i.e. growth expressed as a percentage of maximum growth, Y^{max}) as B level (X) remains below a critical threshold (t); thereafter, Y^* decreases at a constant rate (s) per unit increase in B concentration:

$$Y^* = 100 - s \cdot (X - t) \qquad \textit{Eq. 1}$$

Data of root and leaf DW measured at the end of the experiment were analyzed using the procedure described by Magán et al. (2008) to identify values of Y^{max}, t and s. The analysis of variance (ANOVA) was conducted to determine Y^{max}, which was represented by the average of the treatments that did not differ statistically from the maximum value of leaf or root DW; Y^{max} was used to calculate Y^* at each B level. To determine the value of s, a series of linear regressions was performed to fit the complete pool of data at B levels below possible t values, with the highest coefficient of determination (R^2) value; the value of t was the intersection of this regression line with the horizontal line representing Y^{max}.

Statistics

Data were subjected to a two-way ANOVA with cultivar and boron treatment as variability factors and mean values were separated by the least significant difference test ($P<0.05$). Statistical analysis was performed using Statgraphics Plus 5.1 (Manugistic, Rockville, MD). Each experiment was repeated two or three times with similar findings; for the sake of brevity, the paper reports the results from a representative run of each experiment.

Results

Experiment 1

In this experiment, plants were grown at six different B concentrations ranging from 0.25 to 25 mg L^{-1}. Leaf chlorosis and necrosis, were much more evident in green-leafed cultivar ‘Tigullio’ than in

the purple-leafed cultivar 'Red Rubin'. In 'Tigullio', the symptoms appeared during the first week of treatment at B concentrations exceeding 5.0 mg L^{-1} and their severity increased with B level (data not shown). Towards the end of the experiment, few bottom leaves also dropped from the plants of 'Tigullio' grown at 20 and 25 mg B L^{-1}. In contrast, in 'Red Rubin' leaf symptoms were less severe and appeared only on plants grown at 20 and 25 mg B L^{-1}; no leaves dropped off in this cultivar.

Dry matter accumulation was greater in 'Tigullio' than in 'Red Rubin' (**Table 2**). Root, stem, leaf and total DW of 'Tigullio' were significantly reduced at external B concentrations above 5 mg L^{-1} compared to the control (**Table 2**). On the contrary, in 'Red Rubin' a significant reduction of both root, stem and leaf DW was observed only at 20 and 25 mg B L^{-1} (**Table 2**). At 20 mg B L^{-1} (the concentration tested in all three experiments), root and leaf DW were reduced by 53% and 36%, respectively, in 'Tigullio' and by 39% and 20% in 'Red Rubin'.

There were little differences between 'Tigullio' and 'Red Rubin' in terms of B concentration in leaf tissues for all B treatments (**Fig. 1a**). Leaf B content significantly increased with increasing B concentration of the nutrient solution (**Fig. 1a**). For instance, at 20 mg B L^{-1} leaf B content was nearly 15-fold higher than in the control (1188 ± 153.2 mg kg^{-1} DW vs. 80 ± 20 mg kg^{-1} DW). Similar trend was observed for root B concentration, which however was significantly higher in 'Red Rubin' than in 'Tigullio' at 0.25, 20 and 25 mg B L^{-1} (**Fig. 1b**). The concentration of B in the xylem sap did not differ in 'Tigullio' and 'Red Rubin' and was linearly related to external B concentration (**Fig. 2**). In the control, xylem sap B concentration was higher than the external concentration, while in other B treatments it was approximately half that of the surrounding solution (**Fig. 2**).

The Mass-Hoffman equations for relative growth of roots, leaves and the whole plant against the concentration of B in the nutrient solution or in plant tissues are shown in **Table 3**. The coefficients *t* and *s* were very similar when considering leaf and total DW for each sweet basil cultivar. The threshold B concentration was much higher in 'Red Rubin' than in 'Tigullio' (2.4 vs 14.3 mg L^{-1}). Minor differences between the two cultivars were noted in *s* values, which were approximately -1.8 and -2.2 % per mg B L^{-1} in 'Tigullio' and 'Red Rubin', respectively. When relative leaf growth was expressed as a function of leaf B content, *t* and *s* were, respectively, 206 mg kg^{-1} DW and -0.033 % per mg kg^{-1} DW in 'Tigullio', and 1102 mg kg^{-1} DW and -0.085 % per mg kg^{-1} DW in 'Red Rubin'. With regard to root growth 'Tigullio', the coefficients *t* and *s* were, respectively, 1.59 mg L^{-1} and -2.52 % per mg L^{-1} for external B concentration, and 66 mg kg^{-1} DW and -0.256 % per mg kg^{-1} DW for tissue B content. The parameters of equation 1 were greater for 'Red Rubin' roots; for instance, critical threshold of the concentration of B in the nutrient solution or in root tissues was 14.24 mg L^{-1} and 228 mg kg^{-1} DW, respectively.

Among selected caffeic acid derivatives, only rosmarinic acid was found in all leaf samples at concentrations above the detection limit. In both basil genotypes, B supply did not affect the leaf content of total phenols and rosmarinic acid, which however were much higher in 'Tigullio' than in 'Red Rubin' (data not shown); these quantities averaged, respectively, 27.17 ± 0.65 and 10.34 ± 0.27 g kg^{-1} DW in 'Tigullio', and 42.84 ± 0.98 and 25.73 ± 0.69 g kg^{-1} DW in 'Red Rubin'.

Experiment 2

In this experiment, both sub-toxic (5 mg L^{-1}) and toxic (20 mg L^{-1}) B concentrations, according to the findings of Experiment 1, were tested against the control (0.25 mg L^{-1}).

Leaf symptoms of B toxicity were observed only on the plants grown at 20 mg B L^{-1} and were more severe in 'Tigullio' than in 'Red Rubin'. In both the cultivars, no differences were found between 0.25 and 5 mg B L^{-1} in terms of leaf area and dry biomass (**Table 4**). In 'Tigullio' plants treated with a solution B concentration of 20 mg L^{-1}, root, stem and leaf DW were, respectively, 36%, 32% and 24% lower than in the control (**Table 4**); in contrast, in 'Red Rubin' root and leaf DW were no or little influenced by B supply. In the red-leafed cultivar, we noted a significant decrease of stem and total DW in plants grown at 20 mg B L^{-1} compared to the controls (**Table 4**).

Increasing B supply resulted in a significant increase of the B content of root, stem and leaf tissues (**Table 5**). Leaf B content was significantly higher in 'Red Rubin' than in 'Tigullio' at external B concentrations of 5 (+49%) and 20 (+57%) mg L^{-1} (**Table 5**). Compared to the control, the B content of root and stem tissues increased significantly at 20 mg B L^{-1} in both cultivars (**Table 5**); root B content was 33% greater in 'Red Rubin' than in 'Tigullio' at 20 mg B L^{-1}.

At each B level in the nutrient solution, there were little or no differences between 'Tigullio' and 'Red Rubin' as regards the total uptake of B (**Fig. 3a**), which was calculated based on dry matter accumulation and B content in root, stem and leaf tissues. In both cultivars, B uptake increased significantly with increasing B level in the nutrient solution; on average, it was 3.7- and 9.3-fold higher at 5 and 20 mg B L^{-1}, respectively, than in the control (**Fig. 3a**).

With optimal B supply, B accumulated in the leaves accounted for 60% and 73% of total B uptake in 'Tigullio' and 'Red Rubin', respectively; this proportion rose to 80%, on average, at 5 and 20 mg B L^{-1}, with little differences between the two cultivars (data not shown).

Total water uptake (**Fig. 3b**) and transpiration (data not shown) were similar in the plants grown at 0.25 and 5 mg B L^{-1}, while these parameters were significantly reduced (-15%, on average) at 20 mg B L^{-1}. Water uptake was lower in 'Red Rubin' than in 'Tigullio' (0.467 ± 0.011 vs. 0.540 ± 0.017 L $plant^{-1}$). In both genotypes, leaf transpiration accounted for 94-96% of total plant water uptake regardless of B supply (data not shown).

The uptake concentration of B, which was computed as the ratio between the total uptake of B and water, markedly increased with increasing B concentration in the nutrient solution, with slight differences between 'Tigullio' and 'Red Rubin' and no significant interaction between genotype and B level (**Fig. 3c**). The average B uptake concentrations determined at 0.25 (0.24 ± 0.02 mg L^{-1}), 5 (0.88 ± 0.05 mg L^{-1}) and 20 mg B L^{-1} (2.59 mg L^{-1}) were significantly different. Similar results were obtained when B uptake concentration was calculated using the volume of water and the amount of B that had been either transpired or accumulated by the leaves (data not shown).

Little or no effects of B level and genotype were observed on the leaf content of reduced N, which averaged 43.53 ± 3.13 g kg^{-1} DW. In contrast, N-NO_3^- accumulated much more at 20 mg B L^{-1} than at lower B levels, in particular in 'Red Rubin' (**Fig. 4**). Leaf N-NO_3 content was more than 2-fold higher in 'Red Rubin' than in 'Tigullio' (**Fig. 4**). Neither plant genotype nor B supply affected leaf concentrations of other nutrients (data not shown), which averaged: 5.98 ± 0.40, 68.17 ± 4.91, 17.00 ± 0.41, 4.27 ± 0.18 g kg^{-1} DW for P, K, Ca and Mg, respectively; 290.1 ± 61.2, 210.2 ± 19.1, 20.5 ± 1.9 and 143.2 ± 21.7 mg kg^{-1} DW for Fe, Zn, Cu and Mn, respectively.

Experiment 3

The effects of B supply on the leaf content of this element and other nutrients was also assessed in the autumn. Due to lower sunlight irradiance and shorter photoperiod compared to the spring, the autumn season was supposed to reduce leaf transpiration, thus limiting the transport of B to the shoot.

Excess B reduced shoot DW to a similar extent in 'Tigullio' (-28%) and in 'Red Rubin' (-24%), but it inhibited root growth only in the green-leafed cultivar (-25%; **Table 6**).

The two cultivars accumulated similar amounts of B in the roots at both 0.25 and 20 mg B L^{-1} while leaf B content was significantly greater in 'Red Rubin' than in 'Tigullio' at both B levels (**Table 6**).

Plant genotype and B supply did not affect the leaf content of reduced N (data not shown), which averaged 57.5 ± 1.7 g kg^{-1}. Leaf N-NO_3 concentration did not differ at 0.25 and 20 mg B L^{-1} (data not shown) and was significantly lower in 'Tigullio' (14.6 ± 0.5 g kg^{-1} DW) than in 'Red Rubin' (18.4 ± 1.0 g kg^{-1} DW). On average, leaf N-NO_3 was more than 5-fold higher in autumn than in spring (16.50 ± 2.72 vs. 4.57 ± 1.27 mg kg^{-1}).

There were little or no differences between genotypes and B levels regarding leaf concentrations of other nutrients (data not shown), which averaged: 4.09 ± 0.08, 48.22 ± 1.62, 13.52 ± 0.45, 2.82 ± 0.11 g kg^{-1} DW for P, K, Ca and Mg, respectively; 186.2 ± 13.2, 194.7 ± 8.7, 24.8 ± 1.1 and 122.5 ± 3.1 mg kg^{-1} DW for Fe, Zn, Cu and Mn, respectively.

Discussion

Growth analysis

As expected, 'Red Rubin' was more tolerant to excess B than 'Tigullio', as growth inhibition occurred at higher B supply concentration in the purple-leafed cultivar than in the green-leafed cultivar. Moreover, leaf chlorosis and necrosis, the typical symptoms of B toxicity in sweet basil (Landi et al., 2013a), were less severe and occurred at higher tissue B concentrations in 'Red Rubin' than in 'Tigullio'. In general, the severity of leaf injuries increased with increasing B concentration in the nutrient solution and was greater in the older leaves than in the younger leavesdue to the scarce phloem mobility of B in sweet basil and the difference in cumulative transpiration among leaves of different age. Similar results were found in other species in which B is phloem immobile, such as strawberry (Brown et al. 1999) and tomato (Guidi et al. 2011).

The Mass-Hoffman model was used to assess the response of both basil cultivars to B supply. Previous findings (Bingham et al. 1985; El-Sheikh et al. 1971; Francois 1984, 1986, 1991; Vlamis and Ulrich 1973) indicate that the coefficients of the Mass-Hoffman equation are fairly independent and that the relative tolerance to B toxicity is better described by the threshold concentration. Our findings are in agreement with this conclusion. In fact, the threshold for the concentration of B in the nutrient solution was 2.4 mg L^{-1} in 'Tigullio' and 14.3 mg L^{-1} in 'Red Rubin' when leaf growth was considered. In contrast, the slope was slightly higher (less negative) in 'Tigullio' than in 'Red Rubin' (-1.8 vs. -2.2% % per mg B L^{-1}). The tolerance to B of crop plants have been categorized by Maas (1984) according to the threshold B concentration in soil water as follows: very sensitive, <0.5 mg B L^{-1}; sensitive, 0.5 – 1.0 mg B L^{-1}; moderately sensitive, 1.0 – 2.0 mg B L^{-1}; moderately tolerant, 2.0 – 4.0 mg B L^{-1}; tolerant, 4.0 – 6.0 mg B L^{-1}; very tolerant, >6 mg B L^{-1}. Thus, 'Tigullio' and 'Red Rubin' fall into moderately tolerant and very tolerant categories, respectively. When considering root growth, 'Tigullio' would be classified as moderately tolerant as well.

Water and mineral relations

In experiment 2, cumulative water uptake and transpiration over the experimental period were, on average, slightly but significantly lower in 'Red Rubin' than in 'Tigullio'. In both cultivars, water uptake was significantly lower in plants exposed to 20 mg B L^{-1} than in the controls. This was a consequence of a reduction of leaf area and, most likely, of a lower stomatal conductance, as found in a previous work with 'Tigullio' and 'Red Rubin' plants (Landi et al. 2013a). Other authors reported that excess B reduced stomatal conductance, for instance in zucchini and cucumber (Landi et al. 2013b), and in jack pine (Apostol and Zwiazek 2004).

In our work, growth retardation under B-toxic conditions was not associated with any mineral deficiency, because leaf concentrations of macronutrients and other trace elements were invariably within the sufficiency ranges reported for sweet basil (Zheljazkov and Warman, 2003). Boron supply did not influence plant uptake and leaf content of other nutrients in broccoli (Smith *et al.*, 2010) and jack pine (Apostol and Zwiazek, 2004). On the contrary, large B application increased the content of N, P and K in tomato, and of N, P, Mg and S in pepper (Eraslan et al., 2007).

When compared to the control, leaf concentration of $N\text{-}NO_3$ significantly increased in both cultivars grown at 20 mg B L^{-1} in spring (Experiment 2); in contrast, no differences were found in autumn (Experiment 3; data not shown). Contrasting results have been reported on the influence of excess B on N uptake and assimilation. Toxic B concentrations increased leaf $N\text{-}NO_3$ content in onion (Inal and Tarakcioglu, 2001) while an opposite effect was observed in tomato (Cervilla et al. 2009). In plants of wheat and barley grown in a nutrient solution containing 10 mM B, the activities of glutamate dehydrogenase and nitrate reductase increased or decreased, respectively, compared to the control (Mahboobi et al. 2002). Either an increase (Eraslan et al. 2007) or a decrease (Cervilla et al. 2009) of nitrate reductase activity was found in tomato plants grown under B-toxic conditions.

Boron accumulation

The distribution of B within the plant is principally influenced by the rate of transpiration (Reid 2014) and a close relationship between leaf B accumulation and transpiration rate was found in different crop species such as tomato (Carmassi et al. 2013) and melon (Edelstein et al. 2005).

In our study, an increase of B supply concentration resulted in a correspondent increase in the B content of all plant organs in both ‘Tigullio’ and ‘Red Rubin’. Boron accumulated principally in the leaves, with little differences between the two cultivars. Thus, the distribution of B among different plant organs does not appear a B tolerance mechanism in the purple-leafed cultivar.

Under conditions of adequate or excessive concentrations in the growing medium, B is generally absorbed by the roots through a passive transport, moves within the plant in the xylem sap following the transpiration stream and accumulates in the shoot, especially in the leaves (Reid, 2014). In some sugar-alcohol producing species, B is remobilized through the phloem and excess B in the growing media can damage young tissues (Reid 2014). Energy-dependent mechanisms facilitate B uptake under deficiency conditions or limit its accumulation in the shoot in the presence of high B concentrations in the growing medium (Reid 2014). In a study with broccoli plants irrigated with 0.5 and 21 mg B L^{-1}, Smith et al. (2013) found that, at high B level, B uptake was much lower than a value determined as the product of plant transpiration times the B concentration

in the irrigation water, with the assumption of a simply passive B uptake. Smith et al. (2013) concluded that broccoli has a restrictive B uptake mechanism under high B conditions.

In our work, B uptake concentration increased with increasing B concentration in the nutrient solution in both basil cultivars; however, their ratio was close to 1.0 under control conditions and between 0.12 and 0.19 at 5 and 20 mg B L^{-1}, with minor differences between ‘Tigullio’ and ‘Red Rubin’ (**Fig. 3c**). Similarly, the concentration of B in the xylem sap was approximately half that of the nutrient solution when plants were grown under excess B conditions, while it was 2-3 times higher in the control plants. These findings suggest that a mechanism limiting B uptake was operating in sweet basil when grown under excess B conditions.

We noted that B concentrations measured in the xylem sap (Experiment 1; **Fig. 2**) were higher than calculated values of B uptake concentration (Experiment 3; **Fig. 3c**). In our experiments, xylem exudates were collected at the end of treatment while the uptake concentration was derived from the total amount of water and B taken up by the plants over the whole experimental period. Various reasons may account for the deviation in the solutes concentration of the xylem sap in isolated root systems and in intact plants (Schurr 1998). For instance, the concentration of xylem sap components changes when leaf transpiration rate oscillates and then it is influenced by the time of sampling (Schurr 1998). In castor bean plants, the concentration of nutrients and other solutes decreased in the xylem sap when flux rates increased from free root exudation (a process driven by root pressure) to values typical for transpiring plants (Schurr and Schulze 1995). In our work, the xylem sap was collected in the morning from detopped roots incubated at atmospheric pressure and most likely its concentration did not reflect that of intact plants.

B tolerance in ‘Red Rubin’

In plants, the tolerance to B toxicity is generally associated with the ability to maintain low B concentration in the shoot (Reid 2014). This exclusion mechanism involves the reduction of root uptake and/or xylem loading of B. Actual root uptake of B depends on the activity of either influx or efflux transporters (Hayes and Reid 2004). Reduced B accumulation in tolerant varieties of barley was associated with the active efflux of B from root cells (Hayes and Reid 2004). Genes encoding efflux transporters of B were identified in some B tolerant varieties of wheat and barley (Reid 2007).

At all B levels tested in our work, B concentrations in root and leaf tissues were comparable in ‘Tigullio’ and ‘Red Rubin’ or even higher in the purple-leafed cultivar (**Fig. 1b**; **Table 5** and **6**).

Landi et al. (2013a) found that leaves of ‘Red Rubin’ contained less B than ‘Tigullio’, in contrast with the findings reported in this work. These differences may be ascribed to the inter-experiment

variability that often occurs in studies on plant stress physiology. Such variability was also observed in the present work. We determined leaf B contents in seven separate runs: in four occasions, there were no significant differences between the two cultivars at all B concentrations tested, while in other runs leaf B content was significantly greater in 'Red Rubin' than in 'Tigullio'. Quite probably, the differences between these results and those reported by Landi et al. (2013a) are related to the different way in which leaf samples were collected. In this work, each sample comprised all the leaves of individual plants, while Landi et al. (2013) sampled fully expanded leaves from the 3rd and 4th older nodes. Leaf B content may vary noticeably depending on leaf position (Brown et al. 1999; Rees et al. 2011).

'Red Rubin' plants maintained their growth despite B content was higher than 1500 mg kg $^{-1}$ DW in the leaves and 700 mg kg $^{-1}$ DW in the roots, as found for instance in Experiment 2. Hence, the purple-leafed cultivar was less sensitive to excess B than 'Tigullio', not because it was more capable of restricting B uptake and accumulation in the shoot, but due a superior ability to tolerate high levels of B in root and leaf tissues.

Other B-tolerant plants species did not restrict B uptake and translocation to the shoot at high B supply and their tolerance was associated with the ability to withstand high leaf B content (>1000 mg kg^{-1} DW). Examples include: some *japonica* rice cultivars (Ochiai et al. 2008); hybrid poplar *Populus nigra x euramericana* (Rees et al. 2011); *Pucciniella distans* (Stiles et al. 2010); *Salix alba* and *Salix caprea* (Dellantonio et al. 2008). For instance, leaf B concentrations was as high as 1725 mg kg^{-1} DW in hybrid poplar plants grown with substrate B content of 8.6 mg kg^{-1} (93 mg Kg^{-1} of boric acid), which did not affect plant growth (Rees et al. 2011).

One possible mechanism of B tolerance in 'Red Rubin' is the detoxification of excess B through cellular exclusion and/or vacuolar compartmentalization. High intracellular concentration of soluble B is thought to disturb cellular metabolism by formation of complexes with some acceptor molecules such as NAD, RNA or ATP (Wimmer et al. 2003). There is some evidence that the redistribution of B from the symplast into the apoplast (Reid and Fitzpatrick 2009) or from the cytoplasm into the vacuole (Pang et al. 2010) may contribute to plant tolerance to B toxicity. Goldbach and Wimmer (2007) suggest that the formation of Ca-bound soluble B complexes in the apoplast could play an important role in plant tolerance to B toxicity.

Leaf phenols

Boron toxicity induces oxidative stress (Landi et al. 2012) and plants well equipped with powerful antioxidant machinery, either constitutive or induced, have been reported to have greater tolerance to oxidative damage (Gill and Tuteja 2010).

Phenols are secondary metabolites with multiple functions in plants; for example, they have a prominent antioxidant capacity and provide some degree of protection against different types of biotic and abiotic stress including mineral toxicity (Grace and Logan 2000). Contrasting results are reported on the effect of excess B on leaf total phenolic content, which remained either unchanged (e.g. in *Citrus*; Keles et al. 2004) or decreased (e.g. in broccoli; del Carmen Rodríguez-Hernández et al. 2013). In our study, leaf concentration of total phenolic compounds was much higher in 'Red Rubin' than in 'Tigullio' and it was not influenced by B supply.

Among the selected caffeic acid derivatives, rosmarinic acid was the sole compound found in all plant samples; it represented 32-38% of total phenolic content in 'Tigullio' and 58-59% in 'Red Rubin'. Rosmarinic acid is a caffeic acid derivative that is present in most species in the *Lamiaceae* family, in particular in sweet basil (Kiferle et al. 2011). This compound was found to accumulate to a large extent in sweet basil plants exposed to abiotic stress either *in vivo* (Nguyen and Niemeyer, 2008) or *in vitro* (Kiferle *et al.*, 2014).

Our findings on the effects of B on leaf phenolic content are in agreement with the conclusions of Landi *et al.* (2013a), who ascribed the greater B tolerance of 'Red Rubin' compared to 'Tigullio' to: i) an higher constitutive leaf content of antioxidants, such as ascorbate and glutathione; ii) an increased activities of antioxidant enzymes (ascorbate peroxidase, superoxide dismutase and catalase) in leaf tissues in response to high B supply. Landi et al. (2014) have also demonstrated that in 'Red Rubin' the anthocyanins in the leaf epidermis protect subjacent mesophyll cells from photo-oxidative stress when chloroplasts are damaged by excess B, thus contributing to B tolerance of this cultivar.

Concluding remarks

In both sweet basil cultivars 'Tigullio' and 'Red Rubin', growth inhibition due to excess boron was not dependent on reduced uptake of other nutrients. Calculated boron uptake concentrations and measured boron concentrations in the xylem sap suggest the presence in sweet basil of a mechanism that limits the uptake of this element at high external concentrations. The greater boron tolerance of 'Red Rubin' than 'Tigullio' was apparently due to the ability to tolerate high boron concentrations in both root and leaf tissues, and not to restrict boron uptake and accumulation into the shoot. Previous findings (Landi et al. 2013a) and the high leaf phenolic content found in this work suggest that an enhanced antioxidant system plays an important role in the boron tolerance of the purple-leafed cultivar. Further work is in progress to clarify whether the tolerance of 'Red Rubin' plants also results from intra- and/or inter-cellular compartmentation of boron.

Acknowledgments

This work was funded by the European Commission, Directorate General for Research (VII Framework RTD Programme; Project EUPHOROS), and by MIUR-PRIN 2009 (Ministero dell'Istruzione, dell'Università e della Ricerca, Italy, Project "Physiological response of vegetables crops to excess boron").

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Captions to Figures

Fig. 1 Influence of boron concentration in the nutrient solution on the concentration of this element in leaf (a) and root (b) tissues of two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 14 days (Experiment 1). The lowest B concentration tested was 0.25 mg L^{-1}. Each value is the mean (± S.E.) of four replicates; bars with the same letter indicate values that are not significantly different ($P<0.05$)

Fig. 2 Relationship between boron concentration in the xylem sap and in the nutrient solution in two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 14 days (Experiment 1). The lowest B concentration tested was 0.25 mg L^{-1}. The solid line represents a linear regression fit to the data ($Y = 0.456\ X + 0.532$; $R^2 = 0.902$; $P<0.001$; $n = 12$) while the dotted line represents the 1:1 relationship. Each value is the mean (± S.E.) of four replicates

Fig. 3 Influence of boron concentration in the nutrient solution on the total uptake of water (a) and boron (b) during the experimental period of two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 21 days (Experiment 2). The boron uptake concentration is also shown (a): in this graph, values above bars are the ratios between boron uptake concentration and external boron concentration. Each value is the mean (± S.E.) of four replicates

Fig. 4 Influence of boron concentration in the nutrient solution on nitrate accumulation in the leaves of two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 21 days (Experiment 2). Each value is the mean (± S.E.) of four replicates; bars with the same letter indicate values that are not significantly different ($P<0.05$)

Table 1. Basic information of the greenhouse experiments conducted in 2013 on sweet basil grown hydroponically with different concentrations of boron in the nutrient solution

	Experiment 1	Experiment 2	Experiment 3
Growing season	Spring	Spring	Autumn
Hydroponic system	50-L tank	2-L pot	50-L tank
Plants per tank or pot	16	5	16
Plant age at transplanting (days from sowing)	14	14	28
Plant age at the onset of boron treatment (days from sowing)	28	28	42
Treatment duration (days)	14	21	35
B concentrations under investigation (mg L^{-1})	0.25, 5, 10, 15, 20 and 25	0.25, 5 and 20	0.25 and 20

Table 2 Influence of boron concentration in the nutrient solution (B treatment) on root, stem, leaf and total biomass (dry weight, DW) of two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 14 days (Experiment 1).

Cultivar	B treatment ($mg\ L^{-1}$)	Root DW ($g\ plant^{-1}$)	Stem DW ($g\ plant^{-1}$)	Leaf DW ($g\ plant^{-1}$)	Total DW ($g\ plant^{-1}$)
'Tigullio'	0.25	0.353 ± 0.030 a*	0.694 ± 0.033 a	1.304 ± 0.072 a	2.351 ± 0.119 a
	5	0.319 ± 0.037 a	0.739 ± 0.043 a	1.225 ± 0.024 a	2.273 ± 0.098 a
	10	0.250 ± 0.021 b	0.559 ± 0.033 bc	1.062 ± 0.070 b	1.855 ± 0.097 b
	15	0.219 ± 0.024 b	0.589 ± 0.066 b	0.943 ± 0.069 c	1.752 ± 0.130 b
	20	0.167 ± 0.018 c	0.513 ± 0.023 cd	0.835 ± 0.070 d	1.515 ± 0.084 c
	25	0.158 ± 0.008 cd	0.402 ± 0.026 efg	0.835 ± 0.050 d	1.395 ± 0.077 cd
'Red Rubin'	0.25	0.128 ± 0.010 de	0.478 ± 0.017 d	0.760 ± 0.050 de	1.366 ± 0.060 cde
	5	0.129 ± 0.010 de	0.455 ± 0.017 de	0.703 ± 0.023 e	1.287 ± 0.047 de
	10	0.100 ± 0.010 ef	0.448 ± 0.026 def	0.705 ± 0.017 e	1.253 ± 0.035 de
	15	0.096 ± 0.010 ef	0.457 ± 0.028 d	0.672 ± 0.041 ef	1.225 ± 0.071 e
	20	0.078 ± 0.006 f	0.381 ± 0.058 fg	0.603 ± 0.028 fg	1.079 ± 0.080 f
	25	0.065 ± 0.005 f	0.375 ± 0.014 g	0.548 ± 0.018 g	0.988 ± 0.024 f
Analysis of variance (*P* values; ns = not significant)					
Cultivar		<0.001	<0.001	<0.001	<0.001
B		<0.001	<0.001	<0.001	<0.001
Cultivar x B		<0.01	<0.01	<0.01	<0.01

***** Each value is the mean (± S.E.) of four replicates; values followed by the same letter within a column are not significantly different ($P<0.05$)

Table 3. Maas-Hoffman equations for relative growth (Y*, %) of roots, leaves and the whole plant vs. the concentration of boron (B, mg L^{-1} or mg kg^{-1} DW) in the nutrient solution or in plant tissues of two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 14 days (Experiment 1).

Cultivar	Plant organ	Nutrient solution B concentration (mg B L^{-1})		Tissue B concentration (mg kg^{-1} DW)	
		Equation	R^2	Equation	R^2
'Tigullio'	Whole plant	Y* = 100 – 1.88 (X – 2.44)	0.932	-	-
	Leaves	Y* = 100 – 1.71 (X – 2.34)	0.904	Y* = 100 – 0.033 (X – 206)	0.958
	Roots	Y* = 100 – 2.52 (X – 1.59)	0.882	Y* = 100 – 0.256 (X – 66)	0.866
'Red Rubin'	Whole plant	Y* = 100 – 2.15 (X – 14.21)	0.929	-	-
	Leaves	Y* = 100 – 2.25 (X – 14.38)	0.955	Y* = 100 – 0.085 (X – 1102)	0.971
	Roots	Y* = 100 – 4.25 (X – 14.24)	0.934	Y* = 100 – 0.302 (X – 228)	0.923

Table 4 Influence of boron concentration in the nutrient solution (B treatment) on leaf area and biomass (dry weight, DW) in two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 21 days (Experiment 2).

Cultivar	B treatment ($mg\ L^{-1}$)	Leaf area ($cm^2\ plant^{-1}$)	Root DW ($g\ plant^{-1}$)	Stem DW ($g\ plant^{-1}$)	Leaf DW ($g\ plant^{-1}$)	Total DW ($g\ plant^{-1}$)
'Tigullio'	0.25	403.7 ± 15.1*	0.519 ± 0.027 a	0.642 ± 0.020 a	1.275 ± 0.047 a	2.436 ± 0.063 a
	5	391.7 ± 29.2	0.525 ± 0.028 a	0.607 ± 0.030 a	1.286 ± 0.034 a	2.418 ± 0.065 a
	20	272.1 ± 6.7	0.332 ± 0.024 b	0.433 ± 0.019 b	0.967 ± 0.035 b	1.731 ± 0.063 b
'Red Rubin'	0.25	341.8 ± 35.9	0.354 ± 0.041 b	0.388 ± 0.011 c	0.655 ± 0.0120 cd	1.397 ± 0.050 c
	5	352.7 ± 21.8	0.298 ± 0.053 b	0.355 ± 0.011 c	0.667 ± 0.010 c	1.320 ± 0.067 cd
	20	249.4 ± 20.6	0.341 ± 0.034 b	0.274 ± 0.012 d	0.593 ± 0.022 d	1.208 ± 0.037 d
Analysis of variance (*P* values; ns = not significant)						
Cultivar		<0.05	<0.001	<0.001	<0.001	<0.001
B		<0.001	<0.05	<0.001	<0.001	<0.001
Cultivar x B		ns	<0.01	<0.05	<0.001	<0.001

***** Each value is the mean (± S.E.) of four replicates; values followed by the same letter within a column are not significantly different ($P<0.05$)

Table 5 Influence of boron concentration in the nutrient solution on the content of this element in different plant organs of two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 21 days (Experiment 2).

Cultivar	B treatment (mg L^{-1})	B content (mg kg^{-1})		
		Roots	Stem	Leaves
'Tigullio'	0.25	93.1 ± 6.2 c	27.5 ± 9.6	77.5 ± 6.7 e
	5	164.2 ± 21.4 c	41.0 ± 13.4	340.1 ± 19.6 d
	20	525.8 ± 62.6 b	98.2 ± 9.5	962.5 ± 110.5 b
'Red Rubin'	0.25	71.0 ± 10.9 c	11.9 ± 6.7	119.0 ± 5.1 e
	5	144.8 ± 12.5 c	61.7 ± 5.7	506.4 ± 24.9 c
	20	701.5 ± 53.1 a	94.5 ± 8.2	1513.8 ± 72.6 a
Analysis of variance (*P* values; ns = not significant)				
Cultivar		ns	ns	<0.001
B		<0.001	<0.001	<0.001
Cultivar x B		<0.05	ns	<0.01

***** Each value is the mean (± S.E.) of four replicates; values followed by the same letter within a column are not significantly different (*P*<0.05)

Table 6 Influence of boron concentration in the nutrient solution (B treatment) on plant biomass (dry weight, DW) in of two cultivars ('Tigullio' and 'Red Rubin') of sweet basil (*Ocimum basilicum* L.). Plants were grown hydroponically under greenhouse conditions for 35 days (Experiment 3).

Cultivar	B treatment ($mg\ L^{-1}$)	Root DW ($g\ plant^{-1}$)	Shoot DW ($g\ plant^{-1}$)	Total DW ($g\ plant^{-1}$)	Root B content ($mg\ kg^{-1}$)	Leaf B content ($mg\ kg^{-1}$)
Tigullio	0.25	0.321 ± 0.013 a*	2.971 ± 0.198	3.292 ± 0.209	59.3 ± 9.6	17.2 ± 5.9 d
	20	0.241 ± 0.020 b	2.138 ± 0.225	2.379 ± 0.243	339.9 ± 24.2	907.7 ± 16.9 b
Red Rubin	0.25	0.149 ± 0.016 c	1.577 ± 0.110	1.726 ± 0.116	75.8 ± 6.4	80.5 ± 3.3 c
	20	0.162 ± 0.022 c	1.196 ± 0.065	1.358 ± 0.082	330.1 ± 27.6	1050 ± 21.7 a
Analysis of variance (*P* values; ns = not significant)						
Cultivar		<0.001	<0.001	<0.001	ns	<0.001
B		ns	<0.01	<0.01	<0.001	<0.001
Cultivar x B		<0.05	ns	ns	ns	<0.05

***** Each value is the mean (± S.E.) of four replicates; values followed by the same letter within a column are not significantly different ($P<0.05$)

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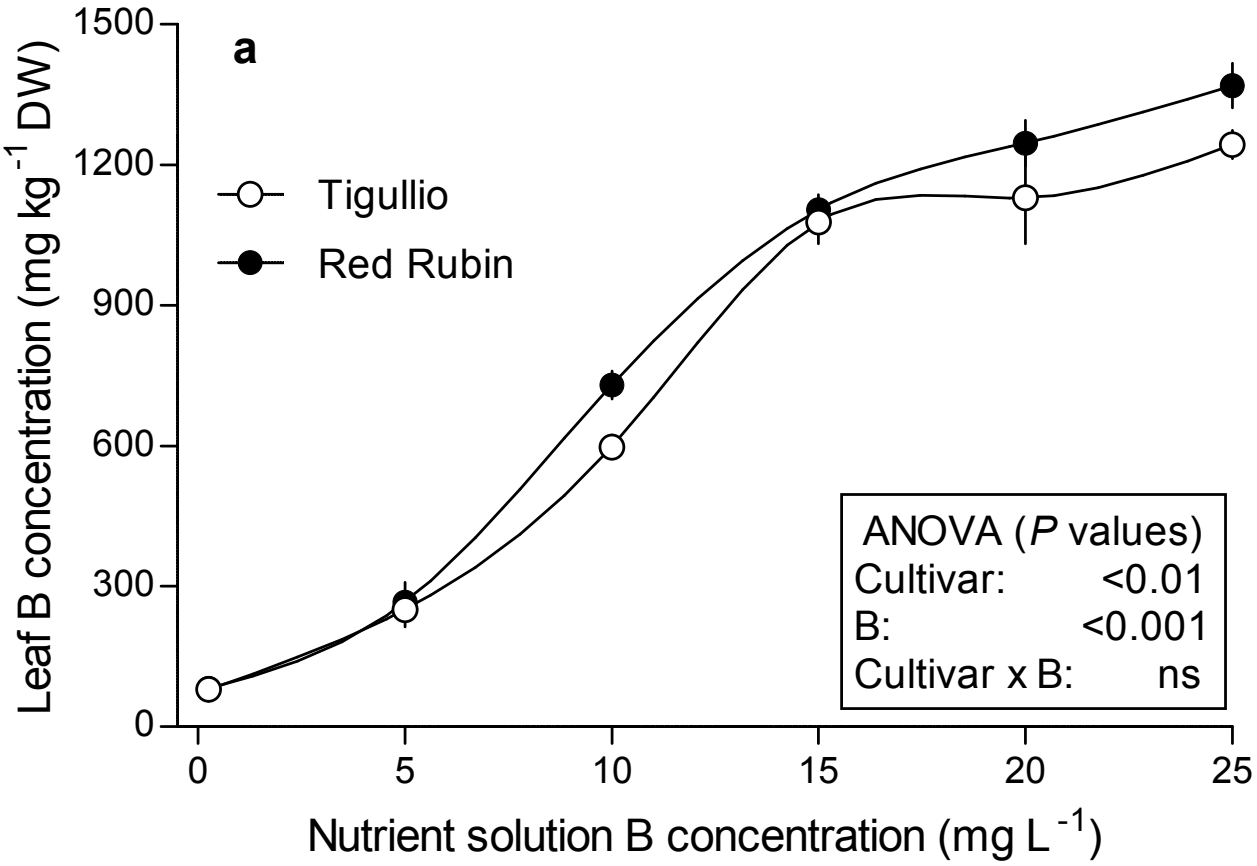

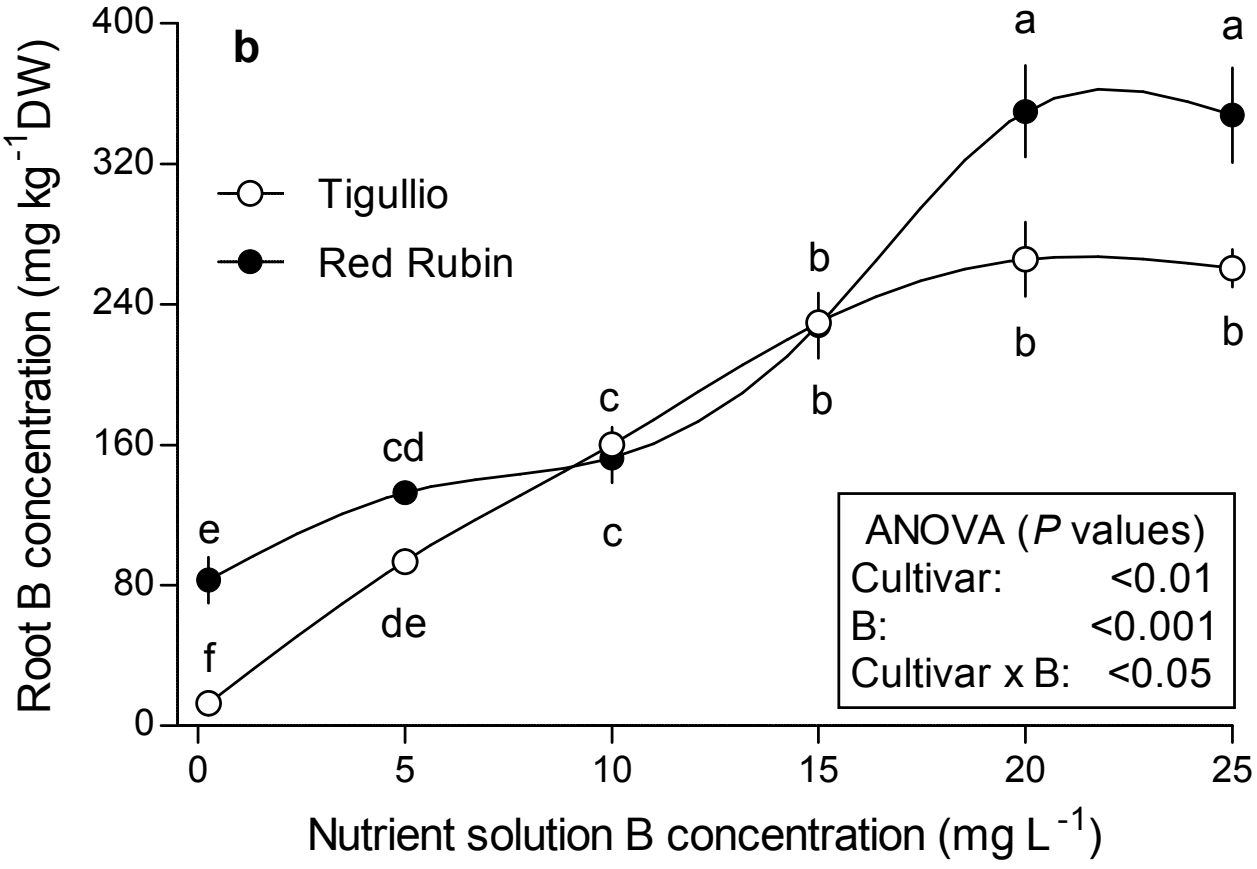

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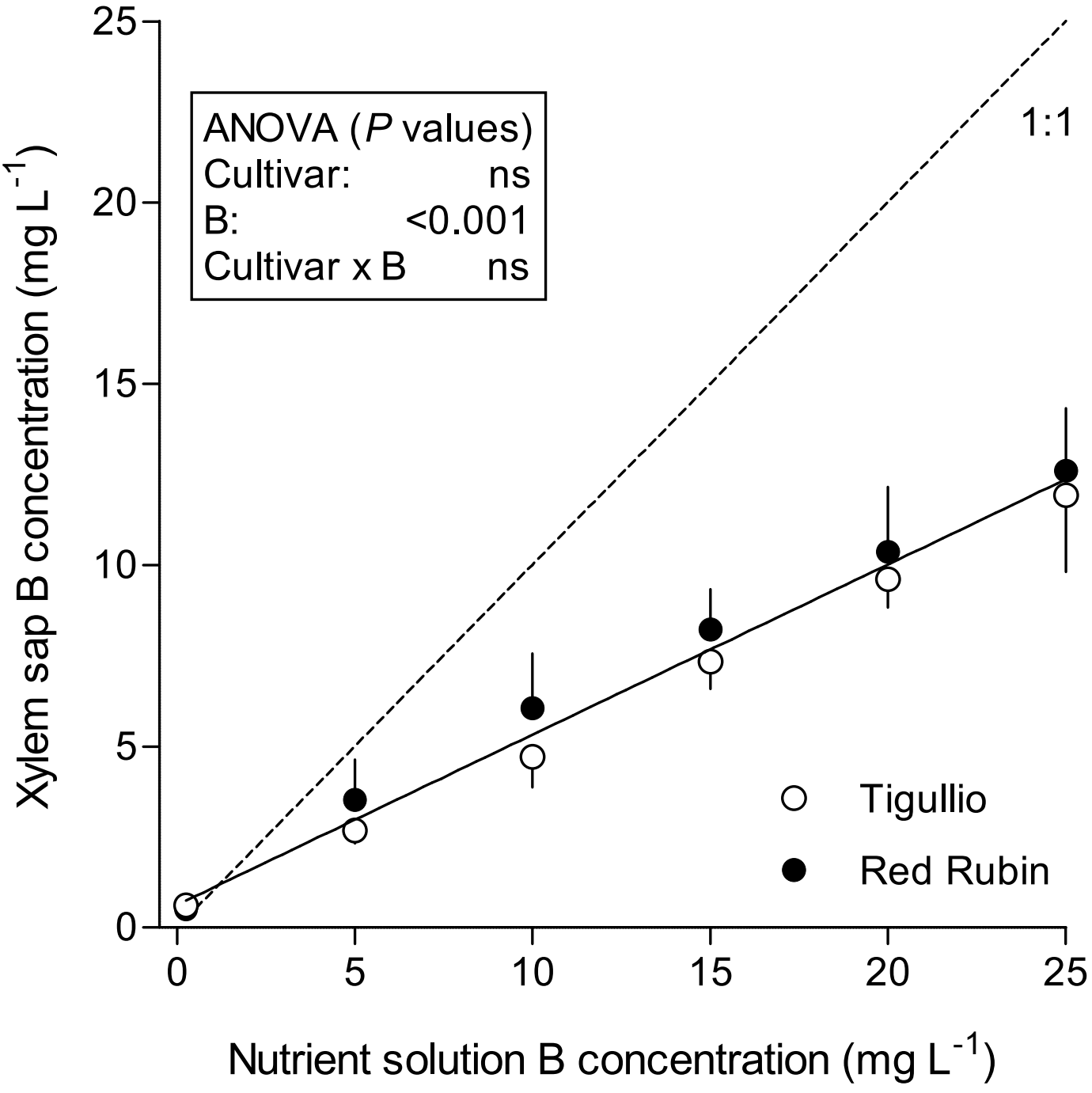

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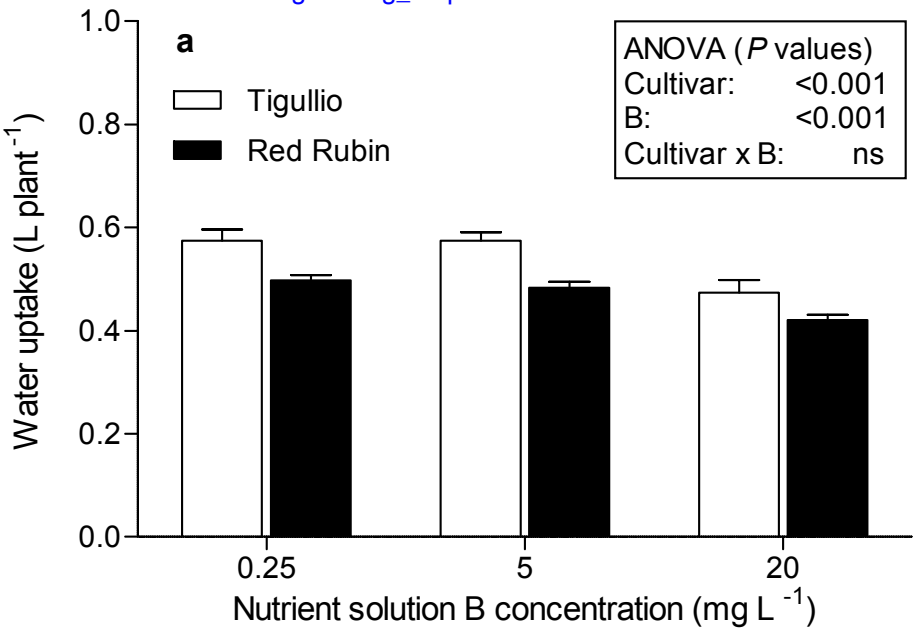

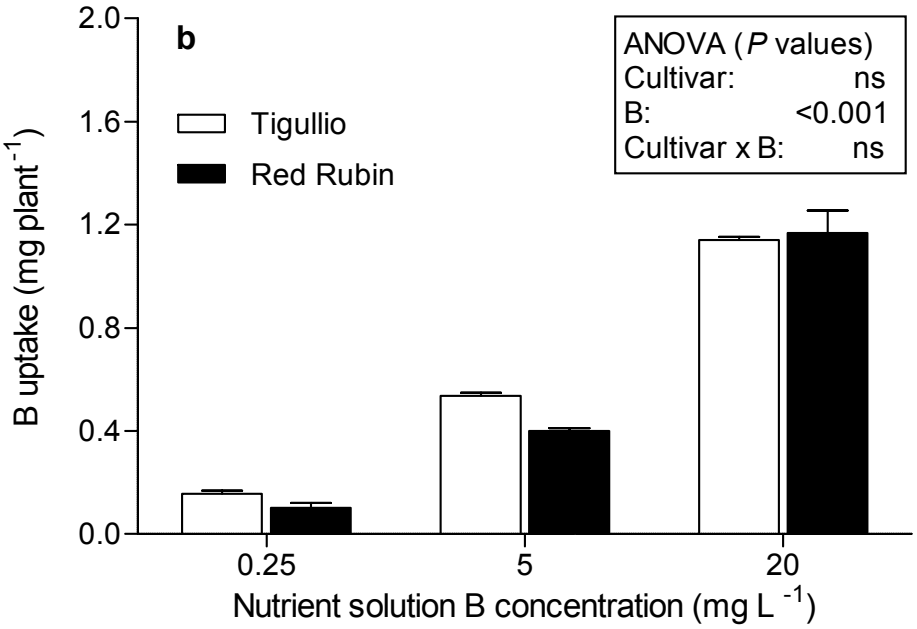

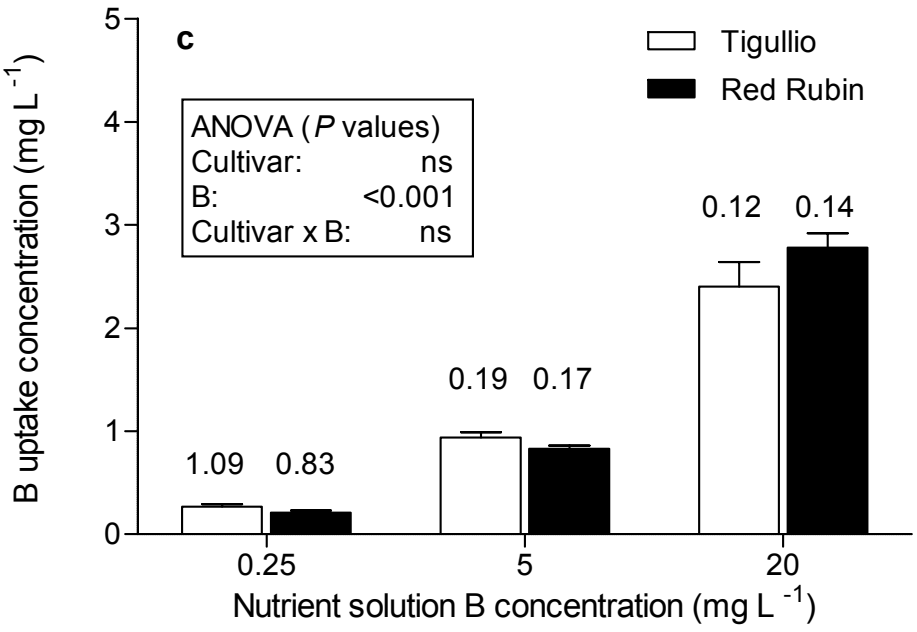

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