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Propagation

Boron on *in vitro* growth and enzymatic activity of Blueberry

Jessé Neves dos Santos[1], Ricardo Antonio Ayub[2], Isabela Letícia Pessenti[3], André Belmont Pereira[4]

Abstract - Boron (B) is essential for plants metabolism and most culture mediums use the same concentration, but in different quantities this nutrient may provoke growth alterations. Thus, the purpose of this study was to examine the effect of B on *in vitro* growth of blueberry in three experiments. The first experiment of multiplication (evaluated at 90 days) used 3 concentrations of 2-isopentenyladenine-2iP with 4 concentrations of boric acid-BA (factorial scheme 3x4). The second used 4 concentrations of BA and 1 concentration of 2iP and the third (rooting) used 4 concentrations of BA and 1 concentration of indolbutyric acid-IBA both evaluated at 180 days (unifactorial scheme). All the experiments had 4 replicates with explants. 6.2 mg L^{-1} of B and 5.0 mg L^{-1} of 2iP generated the highest shoot quantity (18.4, 25.5 respectively). From the interaction of these concentrations, there was the highest activity of POD and PPO enzymes. Under B deficit was seen a larger number of shoot-tip necrosis (9), red leaves (31) and high activity of the PAL, IAAO and POD enzymes. With IBA the correlation between IAAO and the roots' growth was positive, showing that blueberry rooting depends of both B and IAAO regulation.
Index terms: *Vaccinium ashei* Reade; Boric acid; Plant propagation.

Boro no crescimento *in vitro* e atividade enzimática de Mirtilo

Resumo - Boro (B) é essencial para o metabolismo das plantas, e a maior parte dos meios de cultura usa a mesma concentração, mas em diferentes quantidades esse nutriente pode provocar alterações de crescimento. Assim, o propósito deste estudo foi examinar o efeito do B no crescimento *in vitro* do mirtileiro de três experimentos. O primeiro experimento de multiplicação (avaliado aos 90 dias) usou 3 concentrações de 2-isopenteniladenina-2iP, com 4 concentrações de ácido bórico-BA (esquema fatorial 3x4). O segundo usou 4 concentrações de BA e 1 concentração de 2iP, e o terceiro (enraizamento) usou 4 concentrações de ácido bórico e 1 concentração de ácido indolbutírico-IBA, ambos avaliados aos 180 dias (esquema unifatorial). Todos os experimentos tiveram 4 repetições, com 5 explantes cada. 6.2 mg L^{-1} de B e 5.0 mg L^{-1} de 2iP geraram a maior quantidade de brotações (18.4 e 25.5, respectivamente). Da interação dessas concentrações, houve a maior atividade das enzimas POD e PPO. Sob deficiência de B, foi visto maior número de necrose do ápice (9), folhas vermelhas (31) e alta atividade das enzimas PAL, IAAO e POD. Com IBA, a correlação entre IAAO e o crescimento das raízes foi positiva, mostrando que o enraizamento do mirtileiro depende da regulação do B e da IAAO.
Termos para indexação: *Vaccinium ashei* Reade; ácido bórico; propagação de plantas.

Corresponding author: isabelaleticiapessenti@gmail.com.br

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[1]MSc. in Agronomy by the State University of Ponta Grossa (UEPG) Ponta Grossa – PR, Brazil. Email: jessenevessantos@hotmail.com (ORCID 0000-0002-4228-8298)

[2]Agronomist, PhD, Professor Researcher, State University of Ponta Grossa (UEPG), Ponta Grossa – PR, Brazil. Email: rayub@uepg.br (ORCID 0000-0003-3240-8417)

[3]PhD Student in Agronomy of State University of Ponta Grossa (UEPG) Ponta Grossa – PR, Brazil. Email: isabelaleticiapessenti@gmail.com.br (ORCID 0000- 0002-5176-3134)

[4]Agronomist, PhD, Professor Researcher, State University of Ponta Grossa (UEPG), Ponta Grossa – PR, Brazil. Email: abelmont@uepg.br (ORCID 0000-0002-1673-9841)

Introduction

Blueberries (*Vaccinium* spp.) are members of the Ericaceae family and belong to small fruit crops. Due to its high nutraceutical and pharmaceutical value, as its high concentrations of antioxidant compounds, a fact that has been encouraging its consumption at a commercial scale in order to diminish precocious aging, as well as to combat diabetes and other diseases. Blueberries are traditionally propagated by cuttings of the stem. The propagation of cutting may not be adequate for all cultivars due to low rooting percentages and may also not be an effective method to rapidly increase the number of starting materials or propagules for the commercial introduction of new cultivars (ANTUNES, 2006; MARTINEAU et al., 2006; AYUB et al., 2017; SOUZA et al., 2014; FAN et al., 2011).

Micropropagation has been considered the most effective method for rapid increase of propagules throughout the year. It has been reported that micropropagated blueberry plants produce more fruits (rabbit eye group), as well as generate cuttings with higher rooting capacity compared to plants propagated by traditional cutting (FAN et al., 2011). This technique may be divided into some steps such as multiplication and rooting. In the multiplication, the medium is supplemented with cytokinin, which plays a role in either aerial part or radicular system by means of the receptors CRE1, AHK2 and AHK3 (INOUE et al., 2001; LÓPES-BUCIO et al., 2007). Conversely, in the rooting it is necessary to enhance the levels of internal indoleacetic acid (IAA) (MENG et al. 2019). The main auxin present in plants turns out to be IAA, which is regulated by the enzyme indole acetic acid oxidase (IAAO). Some studies show that the greatest activity of this enzyme is coupled to the highest levels of IAA and to a low rooting (FU et al., 2011; ELMONGY et al., 2018; MENG et al., 2019), which in turn characterizes an ideal medium comprised of zeatin and 2Ip (2-isopentenyladenine) as source of cytokinin along with indolbutyric acid (IBA) as auxin (SCHUCH et al., 2008; CUCE et al., 2013; ABREU et al., 2014; CUCE; SOKMEN, 2017; FAN et al., 2017).

B stablishes crossing linkages of Rhamnogalacturonan II (RGII) molecules onto the cell wall (O'NEILL et al., 2004, CHORMOVA; FRY, 2016), acts in cell division and differentiation (ABREU et al., 2014; POZA-VIEJO et al., 2018), promotes translocation of sugars and synthesis of proteins (LANDI et al., 2012). Moreover, B participates in roots formation for conditioning hormonal metabolism, such as IAA regulation. The roots of blueberry are devoid of radicular hairs (BABA et al., 2018); therefore, nutrition must be thoroughly managed so physiological efficiency might be conducive to a better root growth and also to reclamation of mineral nutrients.

Higher or lower enzymatic activities might be reported when B deficiencies are detected with regards to polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), peroxidase (POD) and IAAO. PAL is an enzyme that starts the cycle of phenylpropanoids responsible for the formation of phenolic compounds such as lignin (Lin; Northcote 1990; Su et al., 2019). PPO is responsible for the degradation of phenolic compounds that will form quinones (CAKMAK; ROMHELD, 1997), which in turn might bring about phenolic oxidation of explants. POD regulates the levels of hydrogen peroxide, plays a role in lignin biosynthesis and in conjunction with IAAO participates in inactivation of IAA (ROUT, 2006; WANG et al., 2018).

For blueberry research demonstrated that B enhanced crop biomass concerning plantlets and was conducive to phenolic compounds production such as chlorogenic acid, ferulic acid, myricetin and quercetin (MERIÑO-GERGICHEVICH et al., 2017; MERIÑO-GERGICHEVICH et al., 2019). Nevertheless, the influences of B in the blueberry micropropagation and its physiological functions on enzymatic activity are not clear yet. Thus, the aim of the current research was to assess the effect of boron on the *in vitro* growth of blueberry and also to identify the impact of such a mineral element on the biochemical alterations of the plants of the species under scrutiny.

Material and methods

Plant material and culture medium

Plants of blueberry (*Vaccinium ashei*) originated from germination of seeds and cloned *in vitro* were segmented in microcuttings of 1.0 cm of size and placed in WPM (LLOYD; MCCOWN, 1980) at rates of B modified in accordance with the trial being supplemented with 20.0 g L^{-1} of sucrose, 0.1 g L^{-1} of myo-inositol and 6.0 g L^{-1} of agar with pH adjusted to 5.3. 25.0 mL of medium was added up in containers hermetically sealed with plastic lids and priorly autoclaved at 120°C for 20 min.

Experiment I – B and cytokinin on the shoot multiplication of blueberry

Experimental design adopted in this trial was completely randomized (CRD) in 3x4 factorial scheme in light of three concentrations of 2iP (0.0, 2.5 and 5.0 mg L^{-1}) and four concentrations of boric acid (0.0, 3.1, 6.2 and 9.3 mg L^{-1}), totaling twelve treatments with four replicates. Each replicate was depicted by a container with five explants. At 90-day cultivation *in vitro* response variables were assessed as follows: callus diameter (CD), number of leaves (NL), number of shoots (NS), length of shoots (LS), and fresh weight of plantles (FW). Four treatments were selected for enzymatic analyses as a function of the biggest number of shoots, being such treatments allotted

in containers in view of CRD in 2x2 factorial scheme with two concentrations of 2iP (0.0 and 5.0 mg L^{-1}) and two concentrations of boric acid (0.0 and 6.2 mg L^{-1}).

Experiment II – B applied for blueberry shoot multiplication at 180-day cultivation with cytokinin

Experimental design adopted in this trial was completely randomized (CRD), taking into account four concentrations of boric acid (0.0, 3.1, 6.2 and 9.3 mg L^{-1}) with four replicates comprised by five explants each. WPM was supplemented with 5.0 mg L^{-1} of 2iP priorly established. At 180-day *in vitro* cultivation CD, NS, NL, LS, FW, number of red leaves (NRL) and number of shoot-tips necrosis (STN), density, length and classification of roots, and chemical quantification of B present in the aerial part of the plants. Roots were classified as first and second order for every single root belonging to the third order (FITTER, 1982; BABA *et al.*, 2019).

Experiment III – B applied for blueberry rooting at 180-day *in vitro* cultivation with auxin

Experimental design adopted in this trial was completely randomized (CRD), considering four concentrations of boric acid (0.0, 3.1, 6.2 and 9.3 mg L^{-1}), totaling four treatments with four replicates five explants for each replicate. The WPM was supplemented with 2.5 mg L^{-1} of IBA priorly established. At 180-day *in vitro* cultivation the following response variables were evaluated: CD, NS, NL, LS, FW, number of roots and length of the biggest root, and rooting percentage (RP).

Growth chamber

Shortly after setting of the containers belonging to experiment I, II and III in a laminar flux, such containers were placed in a growth chamber at a temperature range of 25 ± 2°C, photoperiod of 16 h, and photons flux density for light period equivalent to 27 μmol m^{-2} s^{-1}.

Biochemical and chemical analyses

At all of the experiments after the morphological analysis blueberry plants were stored in the refrigerator at -20 °C temperature for both chemical and biochemical analysis procedures. For B determination 0.2 g of dry matter of aerial part of the plants was used in compliance with what preconizes the Azomethine Colorimetric method (MALAVOLTA *et al.*, 1997).

In order to prepare the enzymatic extract, 0.03 g of the leaf tissue or external green portion of the callus was weighed and macerated in mortar, making use of liquid nitrogen and homogenized in 3.0 mL-buffer solution with potassium phosphate at 50.0 mM (PH 7.0) supplemented with 0.1 mM of ethylenediamine tetra-acetic acid - EDTA. Such a solution was transferred to an eppendorf tube containing 0.0003 g of polyvinylpyrrolidone (PVP) and centrifuged at 13000.0 g for 30 min at 4 °C. The floating enzymatic extract was stored in refrigerator at -20 °C until analyses of enzyme activity regarding POD (E.C. 1.11.1.7), PPO (E.C 1.10.3.1), PAL (E.C. 4.3.1.5) and IAAO (EC. 1.2.3.7) were performed.

POD activity was determined by means of a modified approach proposed by Urbanek *et al*. (1991) and Fu *et al.* (2011). For such, 100.0 μL of enzymatic extract was added to 2.9 mL-buffer solution with sodium acetate (50mM) pH 5.2, hydrogen peroxide (60 mM) and guaiacol (20mM). Furthermore, the solution in question was incubated in a water bath for 10 min at 30 °C and then readings at 480 nm from a spectrophotometer model UV 1650 PC (Shimadzu, Kyoto, Japan) were taken. One single activity unit of POD is corresponding to a rise of 0.01-fold larger enzyme amount for 1 g of fresh weight per minute.

Aiming at determining PPO activity (GAUILLARD, 1993), 50.0 mL-buffer solution with potassium phosphate (0.1 M) pH 6.0 containing catechol (25.0 mM) was utilized as enzymatic extract. 200.0 μL of enzymatic extract was added to 2.8 mL of subtract in test tubes, which were maintained for 10 min in water bath at temperature of 30 °C. Readings at 410 nm wavelength from a spectrophotometer model UV 1650 PC (Shimadzu, Kyoto, Japan) were taken. One single activity unit of POD is corresponding to a rise of 0.01-fold larger enzyme amount for 1 g of fresh weight per minute as preconized by Fu *et al*. (2011).

In order to determine PAL (RODRIGUES *et al.*, 2006; FU *et al.*, 2011), 100.0 μL of enzymatic extract with 400.0 μL-buffer solution with *tris-hydroxymethyl aminomethane* (TRIS) and 500.0 μL of phenylalanine was taken into consideration herein. Shortly after incubation in water bath at 40 °C 60.0 μL of chloride acid (0.5 M) was added with readings at 290 nm wavelength from a spectrophotometer model UV 1650 PC (Shimadzu, Kyoto, Japan) being taken. One single activity unit of PAL is corresponding to a rise of 0.01-fold larger enzyme amount for 1 g of fresh weight per minute.

IAA oxidase was obtained by means of the modified method proposed by Fu *et al*. (2011), with 0.1 mL of enzymatic extract being added to a solution containing 0.2 mL of magnesium chlorite (1.0 mM), 0.1 mL of 2.4-dichlorophenol (1.0 mM), 0.2 mL of IAA (1.0 mM) and 0.5 mL-buffer solution with phosphate pH 6.0 immersed in water bath for 30 min at 30 °C. Furthermore, 0.2 mL of the mixture was added to 0.4 mL Salkowski reagent (1 mL of iron chlorite (0.5 M) with 50.0 mL of perchloric acid (35%)) and subjected to water bath for 30 min at 30 °C, with readings at 530-nm wavelength from a spectrophotometer model UV 1650 PC (Shimadzu, Kyoto, Japan) being taken. One single unit of IAAO was expressed in μg of degraded IAA per 1 g of fresh weight throughout one-hour period.

Statistical analysis

Experimental data collected from the three experiments under scrutiny were subjected to Shapiro Wilk test to verify normality of the data distribution, as well as to a regression study defining the critical point of the regression curve by means of the first derivate in view of coefficients of determination higher than 0.95. In order to assess the effect of qualitative factors on the response variables scrutinized at each one of the experiments, Tukey test at 5% reliability was applied to experimental data of the variables that didn't suited the regression study. In addition, coefficients of Pearson correlation for all variables assessed at the three experiments were also calculated and presented. For such statistical investigations, the R statistical program (R CORE TEAM, 2018) along with the ExpDes package were utilized in the current study.

Results

Experiment I

There was non-significant interaction between boric acid and 2iP treatments for all scrutinized variables other than for CD (Figure 1A and B), which evidenced the highest average of 6.8 mm at 5.0 mg L^{-1} of 2iP and 0.0 mg L^{-1} of boric acid, meanwhile under lack of 2iP and 3.1 mg L^{-1} of boric acid the highest average obtained was 3.5 mm. For NS (Figure 1C and D) a significant difference between boric acid and 2iP concentrations was detected in light of no interaction effect between such factors.

We noticed that 5.0 mg L^{-1} of 2iP was conducive to a higher NS (25.2) (Figure 1D), and the lowest LS (35.5 mm) (Figure 1G) as it can be seen in the Figure 2C. This concentration of 2iP also resulted in the highest NL (121.1) (Figure 1F) and FW (0.637 g) (Figure 1H). Conversely, among boric acid concentrations, we came up with the highest NS (18.4) at 6.2 mg L^{-1} (Figure 1C) and the highest NL (90.7) at 9.3 mg L^{-1} (Figure 1E).

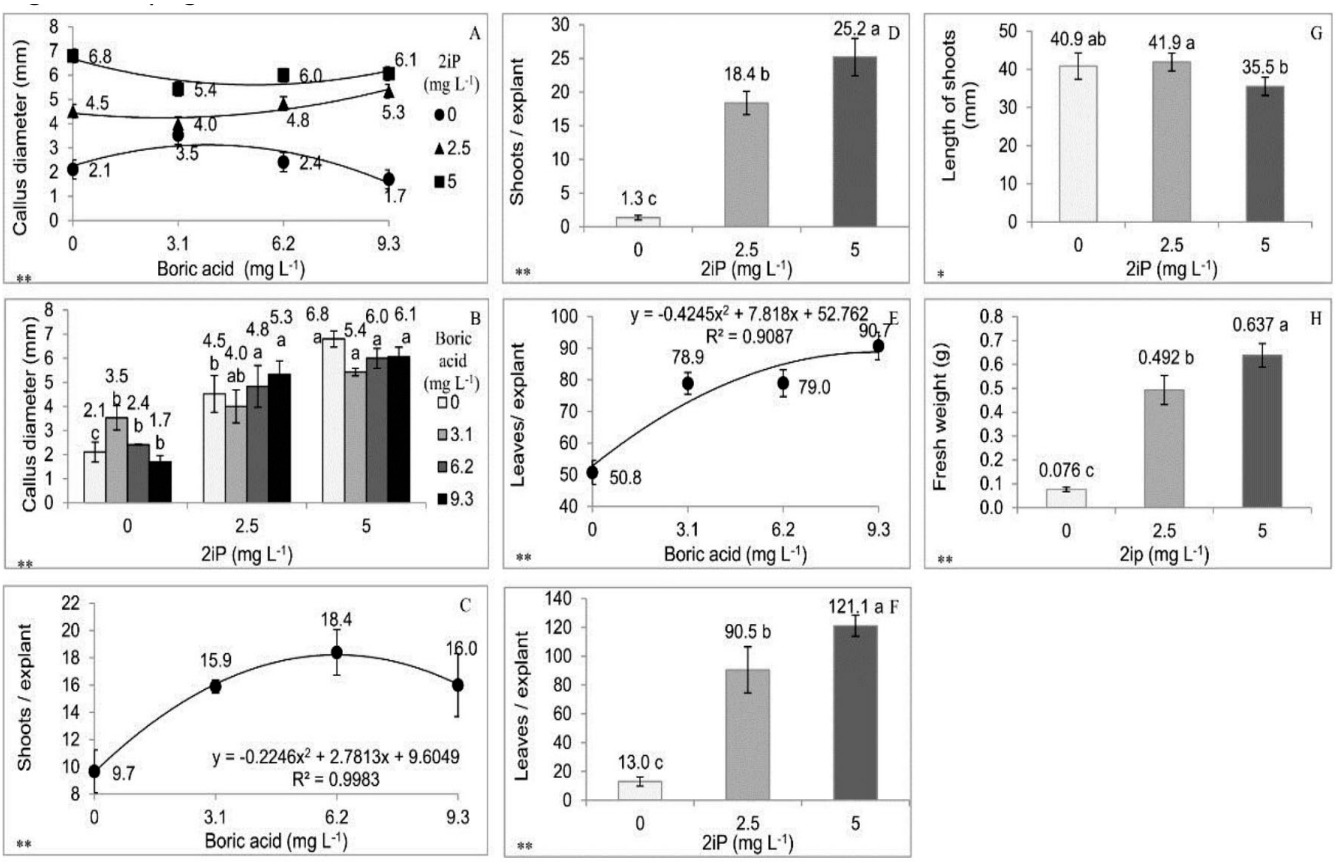

Figure 1. Influence of boric acid and 2iP on the development of aerial part of blueberry at 90-day cultivation. A and B – callus diameter, C and D – number of shoots, E and F – number of leaves, G – length of shoots, and H – fresh weight. Averages followed by the same letters among concentrations of 2iP did not statistically differ by means of the Tukey test at 5% reliability; *significant at 5% reliability; **significant at 1% reliability; ns – non-significant; A and B had data transformed by the formula $\sqrt{x+1}$ prior to statistical analysis; C, D, E and F had data transformed by the formula log (x+1) prior to statistical analysis.

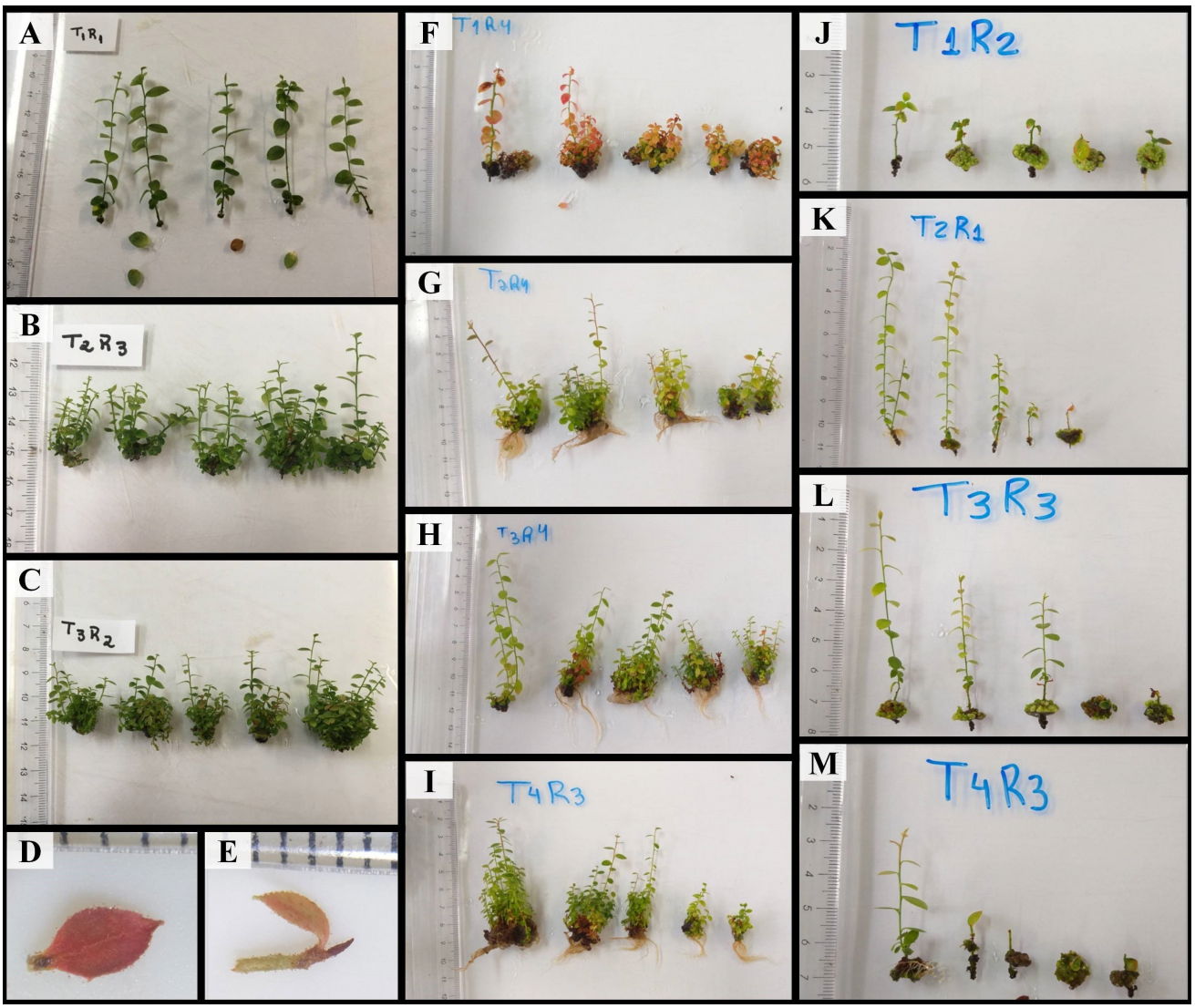

Figure 2. Plants of blueberry. A, B and C – Plants grown at 90-day cultivation in a medium containing 0, 2.5 and 5.0 mg L^{-1} of 2iP, respectively, without boric acid. D and E – symptoms evidencing deficiency of boron at 180-day *in vitro* cultivation. F, G, H and I – plants grown at 180-day cultivation in a medium containing 0, 3.1, 6.2, and 9.3 mg L^{-1} of boric acid, respectively, with 5.0 mg L^{-1} of 2iP concentration. J, K, L and M – plants grown for 180 days in a medium containing 0, 3.1, 6.2, and 9.3 mg L^{-1} of boric acid, respectively, with 2.5 mg L^{-1} of IBA concentration.

In view of a study of Pearson correlation (Table 1), a strong correlation was found for the variables NS, FW and CD among themselves along with the influence of 2iP treatment. For leaf enzymatic activity, there were interaction effects between 2iP and boric acid concentrations regarding leaf PPO and POD (Figure 3 A and B), with the highest averages reached at 6.2 mg L^{-1} of boric acid and 5.0 mg L^{-1} of 2iP. On the other hand, leaf PAL (Figure 3C) was impinged mainly upon 2iP treatment, pointing out a strong correlation with NL (Table 1). Moreover, under lack of boric acid and 2iP the lowest averages for leaf PPO were observed. For leaf POD the lowest average was found at 0.0 mg L^{-1} of 2iP along with 6.2 mg L^{-1} of boric acid. No treatments effects were detected on leaf IAAO.

Table 1. Pearson correlation study taking into account the following variables from experiment I related to *in vitro* multiplication: BA – boric acid; C – cytokinin; PPOL – leaf PPO; PALL – leaf PAL; PODL – leaf POD; IAAOL – leaf IAAO; LS – length of shoots; FW – fresh weight; CD – callus diameter; NL – number of leaves; NS – number of shoots.

	BA	C	PPOL	PALL	PODL	IAAOL	LS	FW	CD	NL	NS
BA	1.00	ns	0.60*	ns	ns	ns	ns	ns	ns	ns	ns
C	-	1.00	0.65**	0.90**	0.59*	0.64**	ns	0.95**	0.97**	0.84**	0.87**
PPOL	-	-	1.00	0.72**	0.71**	0.56*	ns	0.58*	0.54*	0.62*	0.80**
PALL	-	-	-	1.00	0.54*	0.56*	ns	0.76**	0.82**	0.87**	0.76**
PODL	-	-	-	-	1.00	0.66**	ns	0.57*	ns	0.60*	0.72**
IAAOL	-	-	-	-	-	1.00	ns	0.61*	0.57*	ns	0.72**
LS	-	-	-	-	-	-	1.00	ns	ns	ns	ns
FW	-	-	-	-	-	-	-	1.00	0.94*	0.67**	0.88**
CD	-	-	-	-	-	-	-	-	1.00	0.77**	0.85**
NL	-	-	-	-	-	-	-	-	-	1.00	0.68**
NS	-	-	-	-	-	-	-	-	-	-	1.00

*significant at 5% reliability; **significant at 1% reliability; ns – non-significant.

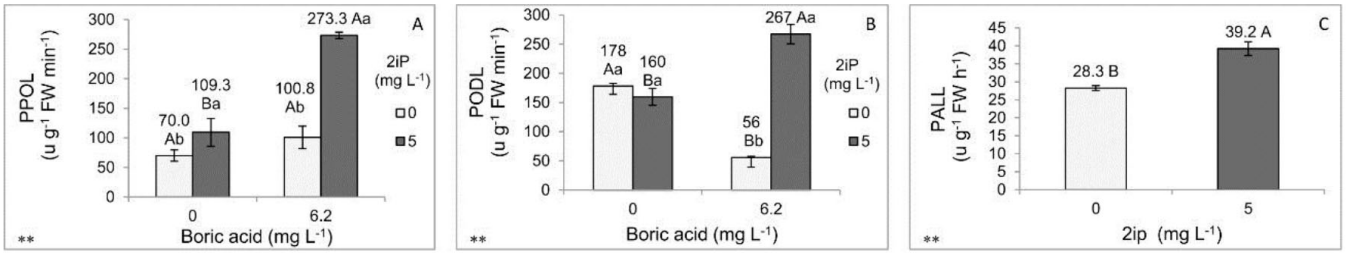

Figure 3. Influence of boric acid and 2iP on the leaf (L) enzymatic activity at 90-day *in vitro* cultivation. A – polyphenol oxidase; B – peroxidase; C – phenylalanine ammonia lyase. Averages followed by the same capital letters among BA concentrations along with small letters among 2iP concentrations did not statistically differ by means of the Tukey test at 5% reliability.

Experiment II

No effect of treatments on NS, CD, FW and NL was observed. The highest LS (Figure 4A) was obtained at 6.2 mg L^{-1} of boric acid coupled with low NRL and also STN (Figure 4B and C). Under B deficiency, there was a more pronounced incidence of symptoms under scrutiny (Figure 2D and E). There was a substantial increment of B present in aerial part of the plants as a function of boric acid application to the medium (Figure 4D). It was possible to verify a significant increase in the number of first and second order roots in conjunction with length of the largest root (Figure 4E and F) as a function of increments in boric acid concentration. Under conditions of absence of B there was no rooting of the plants (Figure 2F), whereas all treatment with boric acid demonstrated roots (Figure 2G, H, I). For enzymatic activity of callus, a high activity of PPO and POD was evidenced (Figure 5A and B) under lacks of B. At the same time, the activity of the IAAO enzyme in the callus triggered the highest value under 6.2 mg L^{-1} of boric acid, with drops in its activity at the highest concentration (Figure 5C).

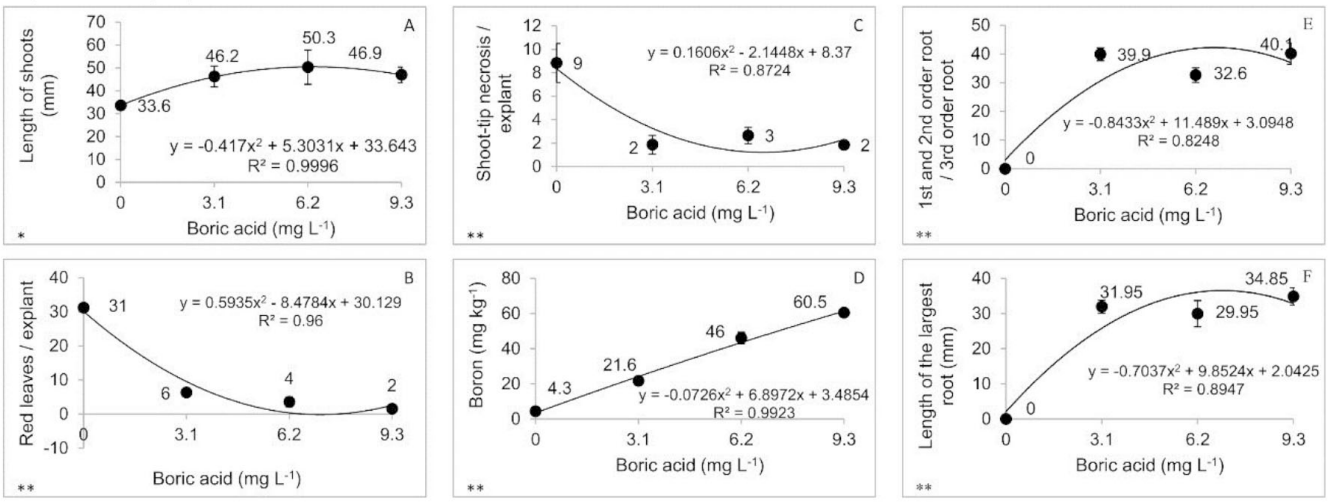

Figure 4. Influence of boric acid on the aerial part of blueberry grown for 180 days in a medium containing 5.0 mg L^{-1} of 2iP. A – Length of shoots; B – number of red leaves; C – number of shoot-tips necrosis; D – amount of boron in aerial part; *significant at 5% reliability; **significant at 1% reliability.

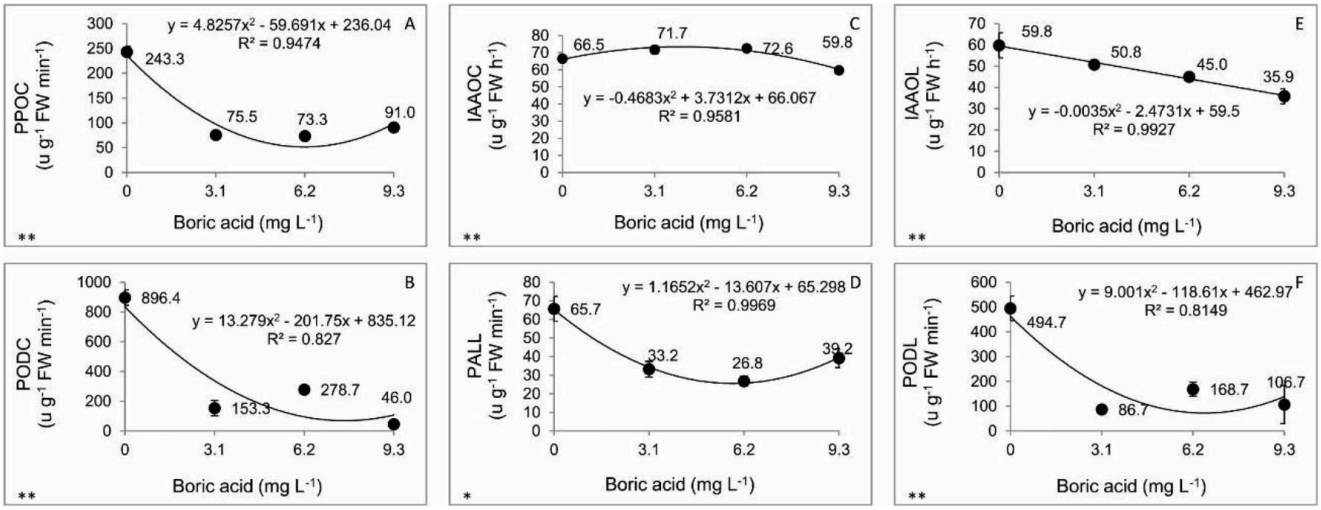

Figure 5. Influence of boric acid on enzymatic activity of blueberry grown in a medium containing 5.0 mg L^{-1} of 2iP. A, B and C – activity of PPO, POD and IAAO enzymes in the callus, respectively. D, E and F – activity of PAL, IAAO and POD enzymes in the leaf, respectively. *significant at 5% reliability; **significant at 1% reliability.

Leaf enzymatic activity (Figure 5D, E, F), a higher activity of all enzymes under lack of B was noticed. At a Pearson correlation study (Table 2), a high positive correlation was detected among boric acid concentration, amount of B in aerial part of the plants, number of first and second order roots, and length of the largest root. On the other hand, there was a strong negative correlation between boric acid and NRL as well as amount of B in aerial part of the plants and leaf IAAO. In the face of NRL and STN, such variables revealed a conspicuous positive correlation with POD and PPO in the callus and also a strong negative correlation with variables that depict root development.

Table 2. Pearson correlation study taking into account the following variables from experiment II related to *in vitro* multiplication: BA – boric acid; LS – length of shoots; CD – callus diameter; NL – number of leaves; NRL – number of red leaves; NNBT – number of necrosed bud-tips; NS – number of shoots; FW – fresh weight; NR12 – number of roots of first and second order; NR3 – number of roots of third order; LLR – Length of the longest root; ABAP – amount of boron in aerial part; PPOL – leaf PPO; PALL – leaf PAL; PODL – leaf POD; IAAOL – leaf IAAO; PPOC – callus PPO; PALC – callus PAL; PODC – callus POD; IAAOC – callus IAAO.

	BA	LS	CD	NL	NRL	NNBT	NS	FW	NR12	NR3	LLR	ABAP	PPOL	PALL	PODL	IAAOL	PPOC	PALC	PODC	IAAOC
BA	1	ns	ns	ns	-0.85**	-0.66**	ns	ns	0.74**	0.72**	0.78**	0.98**	ns	ns	ns	-0.69**	-0.72**	ns	-0.82**	ns
LS	-	1	ns	ns	-0.65**	-0.51*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.59*	ns	-0.55*	ns
CD	-	-	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
NL	-	-	-	1	ns	ns	0.76**	0.55*	0.53*	0.52*	0.53*	ns	-0.69**	ns	-0.52*	ns	ns	ns	-0.52*	ns
NRL	-	-	-	-	1	0.80**	ns	ns	-0.94**	-0.90**	-0.92**	-0.83**	ns	0.67**	0.89**	0.74**	0.96**	ns	0.95**	ns
NNBT	-	-	-	-	-	1	ns	ns	-0.77**	-0.78**	-0.81	-0.62	ns	0.50*	0.78**	0.70**	0.85**	ns	0.83**	ns
NS	-	-	-	-	-	-	1	0.67**	ns	0.51*	ns	ns	-0.66**	ns	ns	ns	ns	ns	ns	ns
FW	-	-	-	-	-	-	-	1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
NR12	-	-	-	-	-	-	-	-	1	0.97**	0.93**	0.71**	-0.62*	-0.61*	-0.87**	-0.65**	-0.93**	ns	-0.96**	ns
NR3	-	-	-	-	-	-	-	-	-	1	0.93**	0.70**	-0.70**	-0.54*	-0.89**	-0.68**	-0.90**	ns	-0.96**	ns
LLR	-	-	-	-	-	-	-	-	-	-	1	0.78**	-0.64**	-0.64**	-0.91**	-0.72**	-0.93**	ns	-0.93**	ns
ABAP	-	-	-	-	-	-	-	-	-	-	-	1	ns	ns	-0.69**	-0.88**	-0.71**	ns	-0.77**	ns
PPOL	-	-	-	-	-	-	-	-	-	-	-	-	1	ns	0.53*	ns	ns	-0,62*	-0.58*	ns
PALL	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.68**	ns	0.64**	ns	0.65**	ns
PODL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.64**	0.92**	ns	0.90**	ns
IAAOL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.67**	ns	0.78**	ns
PPOC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	ns	0.92**	ns
PALC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	ns	-0.74**
PODC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	ns
IAAOC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1

*significant at 5% reliability; **significant at 1% reliability; ns – non-significant.

Experiment III

Under deficiency of B, there was reduction in NS (15.2 mm), NL (4) and RP (20%) (Figure 6A, C, E). The lowest CD (5.7 mm) was ascribed to the concentration of 9.3 mg L^{-1} of boric acid; however, at all concentrations a substantial callus formation took place (Figure 2J, K, L, M). We envisioned an increasing proclivity for RP insofar as boric acid concentration rises (Figure 6E). For the B variables, either number of roots or length of the larger root did not evidence significant statistical differences. As to callus enzymatic activity, a high activity of PPO (Figure 7A) at the concentration of 6.2 mg L^{-1} was found. With regards to leaf enzymatic activity, deficiency of B was conducive to a high activity of both enzymes PAL and POD (Figure 7C and D), decreasing at other concentrations. Conversely, leaf IAAO (Figure 7E) revealed low activity under conditions of lack of B as opposed to the concentrations 3.1 and 6.2 mg L^{-1} of boric acid. Leaf PPO did not show any effect of treatments on all the variables in study. In light of a Pearson correlation approach (Table 3), boric acid negatively correlated with CD, and leaf POD and PAL and positively with RP, which in turn positively correlated with leaf IAAO and negatively with leaf POD and CD. Leaf IAAO presented a negative correlation with leaf POD, differently from observations reported in previous experiments.

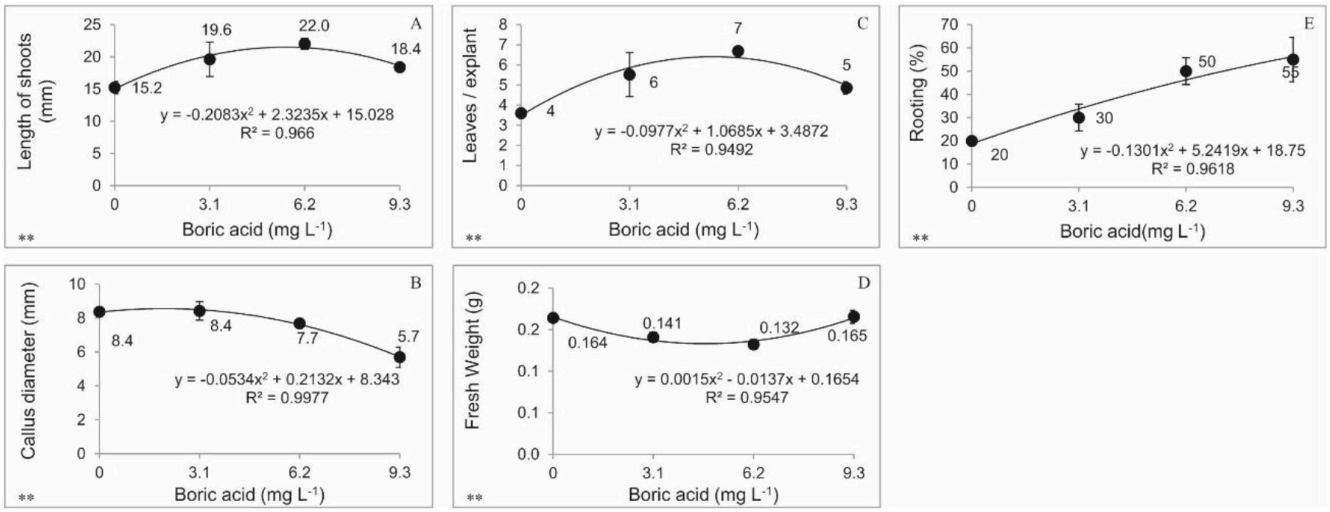

Figure 6. Influence of boric acid on the growth of blueberry grown for 180 days in a medium containing 2.5 mg L^{-1} of IBA. A – length of shoots; B – callus diameter; C – number of leaves; D – fresh weight; E – rooting percentage. **significant at 1% reliability.

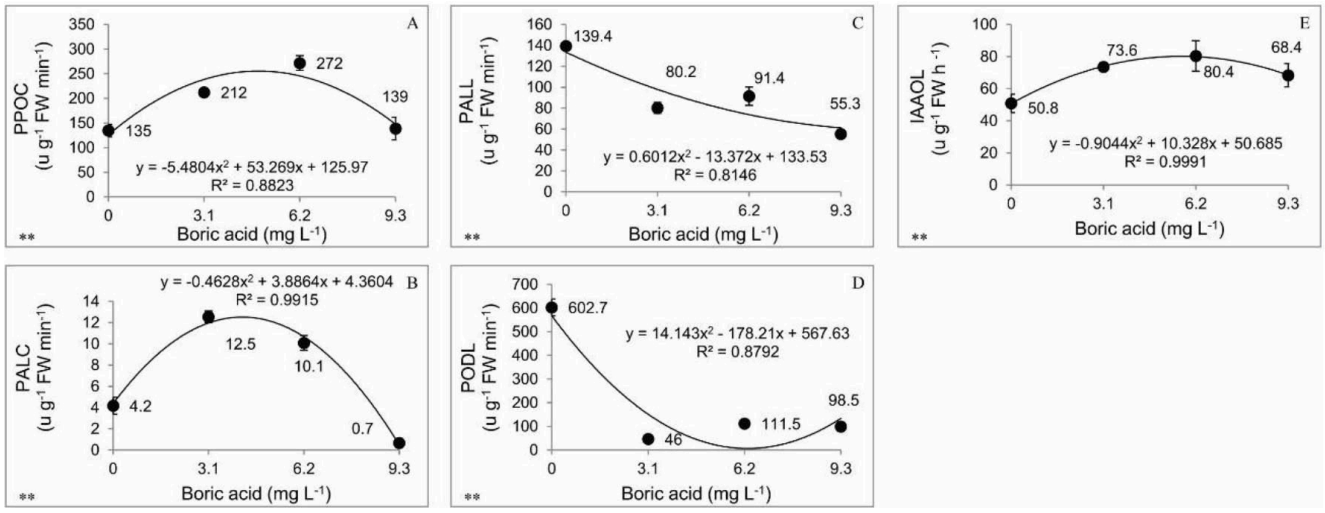

Figure 7. Influence of boric acid on the enzymatic activity of blueberry grown in a medium containing 2.5 mg L^{-1} of IBA. A and B – activity of PPO and PAL enzymes in the callus, respectively; C, D and E – activity of PAL, POD and IAAO enzymes in the leaf, respectively; ** significant at 1% reliability.

Table 3. Pearson correlation study taking into account the following variables from experiment III related to *in vitro* multiplication: BA – boric acid; LS – length of shoots; CD – callus diameter; NL – number of leaves; NS – number of shoots; FM – fresh mass; NR – number of roots; LLR – length of the longest root; RP – rooting percentage; PPOL – leaf PPO; PALL – leaf PAL; PODL – leaf POD; IAAOL – leaf IAAO; PPOC – callus PPO; PALC – callus PAL; PODC – callus POD; IAAOC – callus IAAO.

	BA	LS	CD	NL	NS	FW	NR	LLR	RP	PPOL	PALL	PODL	IAAOL	PPOC	PALC	PODC	IAAOC
BA	1	ns	-0.74**	ns	ns	ns	ns	ns	0.78	ns	-0.60*	-0.71**	ns	ns	ns	ns	ns
LS	-	1	ns	0.62*	0.74**	ns	ns	Ns	ns	-0.68**	ns	-0.56*	ns	0.66	ns	ns	ns
CD	-	-	1	ns	ns	ns	ns	Ns	-0.59*	ns	ns	ns	ns	ns	0.54*	ns	ns
NL	-	-	-	1	ns	-0.67**	ns	Ns	ns	ns	ns	-0.78**	0.65**	0.74**	0.72**	ns	ns
NS	-	-	-	-	1	ns	ns	Ns	ns	-0.51**	ns	ns	ns	ns	ns	ns	ns
FW	-	-	-	-	-	1	ns	Ns	ns	ns	ns	ns	-0.62**	-0.74	-0.64**	ns	ns
NR	-	-	-	-	-	-	1	0.60*	ns	ns	ns	ns	ns	ns	ns	ns	ns
LLR	-	-	-	-	-	-	-	1	ns	-0.59	ns	ns	ns	ns	ns	ns	ns
RP	-	-	-	-	-	-	-	-	1	ns	ns	-0.53*	0.50*	ns	ns	ns	ns
PPOL	-	-	-	-	-	-	-	-		1	ns	ns	ns	ns	ns	ns	ns
PALL	-	-	-	-	-	-	-	-	-	-	1	0.54*	ns	ns	ns	ns	ns
PODL	-	-	-	-	-	-	-	-	-	-	-	1	-0.73**	-0.51*	ns	ns	ns
IAAOL	-	-	-	-	-	-	-	-	-	-	-	-	1	0.72**	ns	ns	ns
PPOC	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.67**	ns	ns
PALC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	ns	ns
PODC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	ns
IAAOC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1

*significant at 5% reliability; **significant at 1% reliability; ns – non-significant.

Discussion

B and 2iP concomitantly acted on the formation of shoots. This is ascribed to the fact that B turns out to be directly involved with expression of the receptor of CRE1 cytokinin, which plays a pivotal role in cellular differentiation, whilst under B deficiency conditions promotes its repression (ABREU *et al*., 2014; POZA - VIEJO *et al*., 2018) and stimulates callus formation – a non-differentiated tissue (Figure 1A and B). On the other hand, the greatest shooting under the influence of B is a result of tissue differentiation (Figure 2A) (WANG *et al*., 2018). Therefore, the presence of B involved with the expression of receptors and in the view of cytokinin present in the medium, favors a better development along with a higher density population.

The treatment with 6.2 mg L^{-1} of boric acid and 5.0 mg L^{-1} of 2iP activated leaf POD (Figure 3B), which is associated with cellular differentiation process. Vatankhah *et al.* (2010) reported the expression of seven isoforms of peroxidases in both aerial part and root differentiation, demonstrating its participation in the synthesis of lignin that is crucial for the xylem differentiation and also formation of shoots (WANG et al., 2018). Rises in leaf PAL activity in light of cytokinin presence (Figure 3C) and its correlation with the larger SN, NL and FW of such physiological processes (Table 1). This takes place because of a demanding requirement of enzyme for the formation of compounds such as lignin present in the xylem, which in turn plays a more active role in young leaves (Hajiboland *et al*., 2015).

Second experiment, the largest SL attained with 6.2 mg L^{-1} of boric acid might be associated with its biological function on cellular wall (Figure 5A). This is because B stablishes crossing linkage of RGII molecules (O'NEILL *et al.*, 2004; ZHOU *et al*., 2015; CHORMOVA; FRY, 2016), strengthening plant tissues. Conversely, under deficiency of B, a lower development of SL was observed (Figure 5A), corroborating the outcomes obtained by Hajiboland *et al*. (2015). Moreover, in the face of lack of B a more intense absorption of Ca leading to thickening of cell wall owing to lignification takes place in such a way as to reduce its plasticity and consequently, cellular enlargement (WANG *et al*., 2015), which in turn induces expression of such a symptom.

Other symptoms related to deficiency of B often reported in the literature refer to necrosis of the aerial part-tip along with leaf yellowness (BAIRU *et al.* 2009; ZHOU *et al*., 2014; GÓMEZ-SOTO *et al.*, 2019). The STN was noticed under lack of B conditions (Figure 4C) in compliance with the findings of Thakur and Kanwar (2011), whose authors eliminated such a symptom by means of a supplementation of B in the medium. STN seems to be rather associated with failure in cellular division and also with malformation of structural compounds (ZHOU *et al*., 2015).

As to yellowness itself, there were no evidences of such a process other than reddish leaves (RL) in the plant community (Figure 4B). From the Pearson correlation study, we came up with a strong correlation between RL and activity of PPO and POD enzymes in the callus (Table 2) (Figure 5A and B), as well as a negative correlation between RL and boric acid. This occurs as a function of a severe B deficiency, which brings about accumulation of phenolic compounds (YANG *et al.*, 2013). The highest activity of PPO in the callus triggers degradation of such compounds forming quinones that are toxic molecules capable of promoting necroses in the tissues, reducing absorption of these compounds along with other nutrients, and conditioning expression of deficiency symptoms (Figure 2D and E) (CAKMAK; ROMHELD, 1997; HAJIBOLAND; FARHANGHI, 2010). PPO and POD are trigger-factors to rooting (MACEDO *et al*., 2013; ELMONGY *et al*., 2018; WANG *et al*., 2018). This particular experiment, the negative correlation between activity of such enzymes in the callus (Figure 5A and B) and radicular formation (Figure 4E and F) indicates that the enzymes at issue mainly play a role in metabolic regulation dependable on environmental stress, instead of induce root formation.

Under deficiency of B in the medium bereft of auxin, a more intense activity of leaf IAAO (Figure 5E) in conjunction with a negative correlation between leaf IAAO and variables expressing root system development was detected herein (Figure 4E and F), an observation that was to be in accordance with Fu *et al.*, (2011); Elmongy *et al., (*2018); Meng *et al*., (2019), whose studies revealed that an increase in IAAO reduces levels of free auxin and inhibits rooting. Nevertheless, lack of B might be conducive to accumulation of auxin, which intensifies IAAO expression as reported by Eggert and Wiren (2017). Such authors found a negative correlation between B and auxin amount in *Brassica naupus*. On the other hand, Su *et al*. (2019) identified a negative regulation of genes associated with auxin itself and its transport under B deficiency conditions.

However, plants grown in a medium with a high concentration of auxin were a target of a lower activity of IAAO (Figure 7E) under lack of B, whereas at further boric acid concentrations there was a considerable rise in IAAO activity. This reveals that B helps internal regulation of this particular enzyme directly and not only in a secondary manner as a function of increments in internal content of IAA in the plants, which in turn triggers negative regulation of genes and reduction in transportation of auxin. Fagan *et al.* (2015) suggested that IAAO is activated by B and Barker and Pilbeam (2007) verified that B protects the enzyme as it gets complexed with inhibitors.

The second experiment, there was a positive correlation between leaf IAAO and POD (Table 2), whereas at the third experiment a negative correlation between such variables was observed (Table 3). Some researchers preconize that POD might participate in degradation of internal IAA by keeping it at suitable levels. Sriskandarajah et al. (2006) evidenced an increase in IAAO and POD to control endogenic level of IAA. Rout (2006) verified that sticks treated with IBA suffered reductions in expression of IAAO throughout the radicular induction and proposed the participation of peroxidases in auxin metabolism. However, the negative correlation between IAAO and POD at the third experiment suggests that deficit of B in blueberry intensifies activity of leaf POD, which turns out to be related to synthesis of lignin (WANG et al., 2018) in conjunction with PAL.

The most intense activity of PAL is envisioned under deficiency of B conditions with drops up until the concentration 6.2 mg L^{-1} of boric acid, from which such an enzyme increases its activity (Figure 5D). Such a proclivity was also detected by Merino-Gergichevich et al. (2019), who scrutinized the enzyme activity in leaves of highbush blueberry plantlets subjected to different concentrations of B. The aforementioned authors at the concentration 3.0 mg L^{-1} of B obtained both the greatest leaf biomass and the lowest activity of PAL. Rises in PAL indicates the highest synthesis of lignin (LIN; NORTHCOTE, 1990) as verified by Su et al. (2019), who evidenced that deficiency of B positively regulates gens related to synthesis of the compound in question.

Since blueberry possesses roots deprived of radicular hairs, absorption of water and nutrients mainly occurs by means of the thinnest roots (of first and second order) (BABA et al., 2018). As a result, a well-balanced nutrition of blueberry is pivotal because the radicular system comes to being more sensitive to environmental stress. In light of the fact that plantlets production is expected to generate plants well-rooted, lack of B leads to lignification that impairs development of new roots (HAJIBOLAND et al., 2015). Baba et al. (2019) observed that in cuttings of blueberry diarch roots (i.e., the thin roots with two protoxylems) prevail. During the radicular initiation of the plants those roots are responsible for the absorption of nutrients, which will be compromised by B deficiency leading to accumulation of lignin. This, thus, causes roots to lose their ability to absorb water, energy and nutrients, being conducive as a consequence to a smaller growth of plants (ZHOU *et al*., 2015).

Conclusions

A desirable in vitro multiplication of blueberry was observed at 6.2 mg L^{-1} of boric acid and 5.0 mg L^{-1} of 2iP. A severe deficiency of B substantially intensifies activity of PPO, PAL, POD and IAAO enzymes in response to environmental stress and as a symptom was observed red leaves and higher callus formation. B is linked to blueberry rooting and directly regulates activity of IAAO enzyme.

Therefore, further specific-scientific investigations should be conducted in order to assess the impacts of B on both rooting and regulation of such a particular enzyme and its effects on the plants.

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