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Response to Boric Acid and Light in the Number and Biomass of Potato Microtubers Cv. "Floresta"

Efecto del ácido bórico y la luz en el número y biomasa de microtubérculos de papa cv. "Floresta"

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Abstract
In order to understand the performance of *Solanum tuberosum* L. "Floresta" in producing microtubers, the effect of four concentrations of boric acid (6.2 mg l^{-1}, 7.75 mg l^{-1}, 9.3 mg l^{-1}, and 10.85 mg l^{-1}) under two conditions—1) 8 hour dark and 2) 16 hour light photoperiod and 8 hour dark—was evaluated under *in vitro* culture conditions. Full-strength Murashige and Skoog medium was used as a control. The data analysis for the number and biomass of microtubers were significant for the condition ($p \leq 0.05$). Concerning the concentration of boric acid and the interaction between factors, a significant difference was obtained for biomass ($p \leq 0.05$). In dark conditions, a greater number of microtubers was obtained, although the biomass was lower compared to the light conditions where the biomass was higher, and the number of microtubers was lower. A concentration of 9.3 mg l^{-1} of boric acid in both types of conditions was the best treatment to produce a greater number and more biomass of microtubers. The differences identified in this work with regards to the number and biomass of microtubers were probably the combined result of genotype and specific culture conditions. Even considering this, the use of lighting conditions is proposed to induce larger and greener microtubers.

Keywords: 6-bencilaminopurine; microtuberization; *Solanum tuberosum*; tissue culture.

Resumen
Con el propósito de conocer la respuesta en el número y la biomasa de microtubérculos de papa (*Solanum tuberosum* L. "Floresta"), se evaluó, bajo condiciones de cultivo *in vitro*, el efecto de cuatro concentraciones de ácido bórico: 6.2 mg l^{-1}, 7.75 mg l^{-1}, 9.3 mg l^{-1} y 10.85 mg l^{-1}; en dos condiciones: oscuridad y fotoperiodo de 16 horas luz y 8 horas de oscuridad.

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El medio que se utilizó como testigo fue Murashige y Skoog. El análisis de los datos para el número y la biomasa de microtubérculos fue significativo para la condición de oscuridad ($p \leq 0,05$). En relación con la concentración de ácido bórico y la interacción entre los factores, se obtuvieron diferencias significativas para la biomasa ($p \leq 0,05$). De manera que, en la condición de oscuridad, se obtuvo un mayor número de microtubérculos; sin embargo, su biomasa fue menor. Para la condición con iluminación, la biomasa fue mayor y el número de microtubérculos fue menor con respecto a la condición con oscuridad. La concentración de 9,3 mg l^{-1} de ácido bórico en ambas condiciones fue el mejor tratamiento para producir un mayor número y biomasa de microtubérculos. Las diferencias indicadas en este trabajo en relación con el número y la biomasa de microtubérculos probablemente son el resultado conjunto del genotipo y las condiciones específicas de cultivo. Aun así, se propone la utilización de una condición de iluminación para inducir microtubérculos más grandes y verdes.

Palabras claves: 6-bencilaminopurina; cultivo de tejidos; microtuberización; *Solanum tuberosum*

The potato is the fourth most important food crop worldwide; this is due to its nutritional properties (Lakhotia *et al.*, 2014). However, for its production, the conventional propagation way is still used, which involves repeated tuber multiplication through clonal selection (Tadesse, Lommen, & Struik, 2001); these processes are inefficient, particularly in early generations, according to Gopal and Minocha (1998). Thus, the conventional asexual propagation, although maintaining the genetic stability of the cultivars, reduces seed quality over a few clonal generations, mainly because of the accumulation of phytosanitary problems (Dhital & Lim, 2012; Tadesse et al., 2001) and the low multiplication rates in the field (Dobránszki, Magyar, & Hudák, 2008). Therefore, *in vitro* propagation is the most viable alternative to alleviate the problems associated with conventional seed production systems (Tadesse et al., 2001), and to supply the quantity and quality of disease-free plant material that is required to establish large-scale plantations through the year (Dobránszki et al., 2008).

Microtubers, which are potato tubers produced by *in vitro* culture techniques, show many advantages that make them ideal propagules to produce high-quality seeds (Dhital & Lim, 2012; Motallebi, Kazemiani, & Yarmohamadi, 2013). First of all, disease-free, high-quality potato seeds can be obtained; and, compared to other propagation techniques, microtubers are more robust and easier to handle, which favors automatic planting processes (Dhital & Lim, 2012; Motallebi et al., 2013). Secondly, microtubers facilitate the maintenance and exchange of genetic material because small samples can be preserved, and subsequently sent in aseptic conditions (virus-free), even to countries with strong phytosanitary regulations (López, Sánchez, Mora, & Martínez, 2012). Thirdly, they have also been experimental research tools in many areas including plant metabolism, germplasm selection and evaluation, transformation, somatic hybridization, and *in vitro* selection of agronomically important characters such as maturity, abiotic stress tolerance, among others (Dobránszki et al., 2008).

In consequence, microtubers can be used in greenhouses to produce minitubers, which are an alternative way of producing potato seeds. However, current research addresses efforts to develop more specific and efficient protocols by using direct field planting of potato tubers produced *in vitro* because of the technical advantages in management compared to conventional seed (Park *et al.*, 2009).

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Many factors are affecting the induction and formation of microtubers. Growth regulators play a fundamental role in the process and have been extensively studied (Aksenova, Konstantinova, Lozhnikova, Golyanovskaya, & Sergeeva, 2009; Hoque, 2010). Nevertheless, potato microtuberization is influenced by many factors, including genotype, explant type, culture medium, and culture conditions (sucrose, photoperiod, light intensity, temperature) (Dhital & Lim, 2012; Li et al., 2005).

The tuber formation in some wild relatives and subspecies of potato is a process that is strictly dependent on short days or long periods of darkness (long nights), although the response of tuberization in many varieties of *Solanum tuberosum* showed an adverse effect on the presence of short days (Aksenova, Konstantinova, Golyanovskaya, Sergeeva, & Romanov, 2012; Aksenova et al., 2009). The above statements indicate that the investigation of the length of the photoperiodic response is essential to improve yields in the production of potatoes (Aksenova *et al.*, 2005).

In this research, we evaluated the effect of boric acid in two light conditions under *in vitro* culture conditions. Lots of research has been conducted on the physiological aspects of the nutrition of boric acid in plants, but there are no *in vitro* protocols evaluating the effect of this compound on the growth characteristics of potato. Boric acid is involved in the transport and control of sugars through the cell wall and membranes, the adenosine triphosphate (ATP) synthesis, the nucleic acids metabolism, the gibberellins synthesis, and the formation of starch, and affects the transpiration in leaves (Gutiérrez-Soto & Torres-Acuña, 2013). In greenhouse conditions, boric acid improved the role of auxin, the photophosphorylation rate, and the tuber growth (Puzina, 2004).

Particularly in recent years, technical and scientific advances have improved aspects such as conservation, production, genetics, and plant pathology. But the variability in the responses showed that, in the *in vitro* culture protocols, there appears to be a genotype-specific process (Sharma, Venkatasalam, & Singh, 2011); therefore, much research still needs to be done.

In Costa Rica, *S. tuberosum* "Floresta" and *S. tuberosum* "Granola" are the most cultivated cultivars. However, conventional seed tubers production has become a phytosanitary problem mainly due to PVX and PVY virus. The establishment of a virus-free *in vitro* stock for formally certified seed production systems is necessary to overcome these limitations (Barquero, Gómez, Brenes, & Valverde, 2001; Vásquez, Montero-Astúa, & Rivera, 2004).

The potential use of the *in vitro* culture as an alternative tool for potato microtuber production is essential to obtain asexual high genetic and phytosanitary quality seed. To confirm this assumption, we studied the response in yield of microtubers under two conditions: 1) dark and 2) 16 hours of photoperiod and 8 hours of dark; each one interacting with 6-Benzilaminopurine (BAP), boric acid (H_3BO_3), and 9% sucrose.

Methodology

Plant material

Single nodal sections (0.5-1.0 cm long) cut from greenhouse plants of *S. tuberosum* L. "Floresta" were used as plant material to initiate the investigation. The plants were disinfected and aseptically cultured according to the protocol described by Gopal, Minocha & Dhaliwal (1998). The research was carried out at the Plant Tissue Culture Laboratory, Escuela de Ciencias Agrarias, Universidad Nacional de Costa Rica.

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Microtuberization

Axillary buds of the second and third node below the apical bud from six-week-old *in vitro* axenic plantlets were aseptically subcultured and incubated under eight treatments. The purpose was to evaluate the effects of light conditions and H_3BO_3 concentration on a number and biomass of microtubers (Table 1). To guarantee the phytosanitary quality, several representative leaves were collected from each subcultured plant, and tested by enzyme-linked immunosorbent assays (ELISA) for Potato virus X (PVX), Potato virus Y (PVY), and Potato leafroll virus (PLRV).

Table 1
In vitro *treatments for microtuberization of Solanum tuberosum L. "Floresta"*

Treatment	Condition	Concentration of boric acid (mg l^{-1})
1	Light	6.2
2	Light	7.75
3	Light	9.3
4	Light	10.85
5	Dark	6.2
6	Dark	7.75
7	Dark	9.3
8	Dark	10.85

Source: own research.

The flasks were randomly transferred to a growth room under two different culture conditions. Cultures in the first condition were kept in darkness at 19°±2°C. In the second one, cultures were maintained under a 16h/day photoperiod with a light intensity of 2300 lux and a temperature of 23°±2°C.

Experimental design

The experiment was conducted in a complete 2 x 4 factorial design with eight treatments. Four flasks of 90x40 mm were used per treatment and four explants per flask corresponded to one experimental unit. Each treatment had 16 replicates, with a total of 64 explants per cultivar per treatment in each condition (Di Rienzo *et al.*, 2008).

Data recording and statistical analysis

The variables evaluated were biomass/plantlet (mg) and a number of microtubers/plantlet (NM). Data were recorded after four months of culture. To validate the model for each variable, a normal distribution and constant variance assumptions were confirmed. Significant differences among the treatments were found by using an analysis of variance, and followed by average comparison tests (Tukey Honest Significant Difference) at 5% level. Infostat software for the statistical analysis was used (Di Rienzo *et al.*, 2008).

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Results and discussion

The plants regenerated from single nodal explants were multiplied for the production of disease-free plants microtubers. ELISA showed that PVX, PVY and PLRV were absent in the *in vitro* plants produced.

In our study, the effect of the condition was statistically significant for the number (p=0.0019) and biomass of microtubers (p=0.0168). The value of the average number and biomass of microtubers was 2.83±0.28 and 0.24±0.03 g for the light condition, and 4.10±0.27 and 0.13±0.03 g for the dark condition, respectively.

The biomass and number of the microtubers changed in the different treatments at different light conditions (Figure 1). However, our data did not indicate a linear or polynomial regression for the number of microtubers in both conditions (p≥0.05; Figure 1A). On the contrary, a linear regression between the biomass and the light condition was obtained (p=0.0001; Figure 1B). For the dark condition, a nonlinear regression was determined (p=0.9513). Therefore, polynomial regressions were calculated in the model for more accurate prediction, but the differences were not significant (p=0.4819; Figure 1B).

Furthermore, the results showed that, under the light condition with a photoperiod of 16 hour light and 8 hour dark, the number of microtubers was lower, but their biomass was higher and greener. In this regard, Seabrook (2005) indicated that microtubers exposed to light during the induction phase and early development tend to have a well-developed periderm, are often green, and have improved resistance to disease and desiccation.

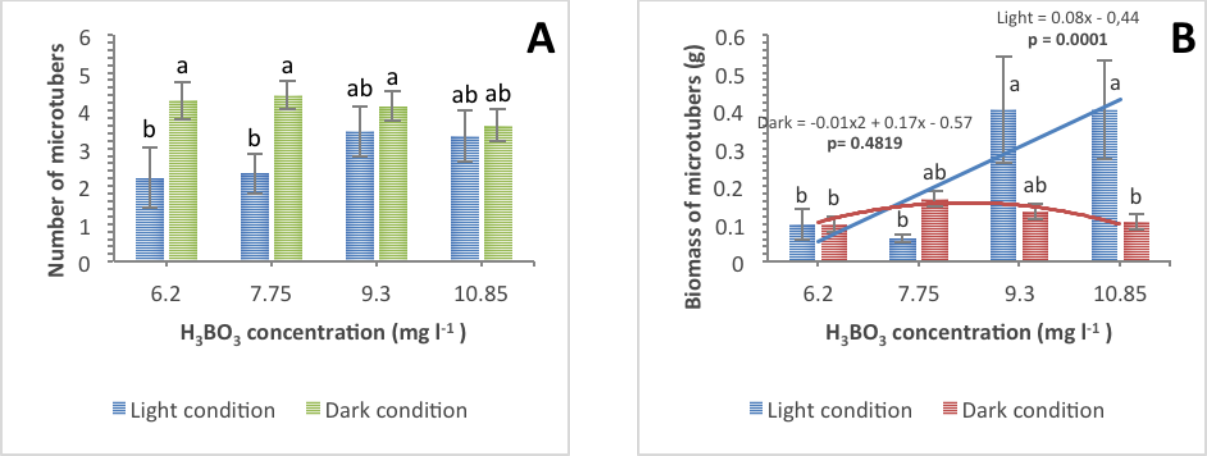

Figure 1. The effect of the condition on the number (A) and biomass (B) of microtubers *of Solanum tuberosum* L. "Floresta" under *in vitro* culture conditions. The means followed by different letters are significantly different at the 5% level of significance. The standard errors of the means are represented as bars. Source: own research.

In the late nineties, the importance of light (photoperiods of 10 and 14 hours) to obtain green microtubers was revealed. According to this finding, green microtubers performed better in cultivation at field level as compared to microtubers obtained in darkness, possibly through a greater number of buds, better survival rate, higher production and vigor, and, finally, a lower dormancy (Gopal *et al.*, 1998).

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This result could be related to photosynthesis. This is supported by the results obtained by Desjardins (1995), who argues that the light intensity increases the carbon dioxide levels, accompanied by increased levels of oxygen, thus promoting breathing of the emerging shoots and the entire process of photosynthesis.

The same author states that the photosynthetic activity of the *in vitro* shoots is low, which probably indicates that these shoots used more of the sugars from the medium than the photosynthetic products for the biomass production. It is important to point out that sugars are accumulated as starch in microtubers because they are reproductive structures that need to store energy. Furthermore, the results obtained in this survey indicate that most biomass was obtained in microtubers grown under light conditions.

On the other hand, in the dark condition, the number of microtubers was greater, with a lower biomass average and cream color (Figure 1A). Senescence was also found, and the few shoots were stunted. In this regard, Seabrook (2005) reported that explants obtained from shoots instead of tubers, and maintained in light, grew better than those grown in darkness. The results obtained in this investigation support the hypothesis that the darkness promotes premature senescence, and this reduces the potential of microtubers for thickening. In addition, the results of the present study are in agreement with Garner and Blacke (1989) who found that, in periods of total darkness, the microtuber weight percentage is reduced.

The results of the present study agree with Aksenova *et al.* (2009), who indicated that a photoperiod of 16 hours accumulated greater biomass compared to a photoperiod of 10 hours; while both light conditions still showed important differences in biomass accumulation when compared to the condition of darkness in *S. tuberosum* L., cv. "Desirée". Similarly, the fresh weight for the variety "Desirée" was higher in short days, both in media without growth regulators and those supplemented with Kinetin (1 mg l^{-1}) compared to the dark condition (Aksenova *et al.*, 2005).

Other research studies concluded that, regardless of cultivar and jasmonic acid treatments, the light condition of 8 hours benefited the *in vitro* tuberization, and increased the size and uniformity of microtubers, compared to *in vitro* tuberization under dark conditions (Pruski, Astatkie, & Nowak, 2002).

Thus, an appropriate combination of light and dark conditions with short days can synchronize and accelerate the initiation and development of microtubers, as well as increase their numbers (Dobránszki, Tábóri, & Ferenczy, 1999).

In this research, the use of different concentrations of boric acid was significant for the biomass ($p=0.0179$), and was not significant for the number of microtubers ($p=0.8012$). The mean weight value of the microtubers in the treatments one (0.1 ± 0.02g) and two (0.12 ± 0.02g) statistically differed from those values measured in the treatments three (0.27 ± 0.07g) and four (0.25 ± 0.07g). A linear regression between the biomass and the concentration of boric acid was calculated ($p=0.0042$; Figure 2). The difference among treatments, based on the results of the concentration effects of boric acid, does suggest a key role in the development of the biomass in *in vitro* plants.

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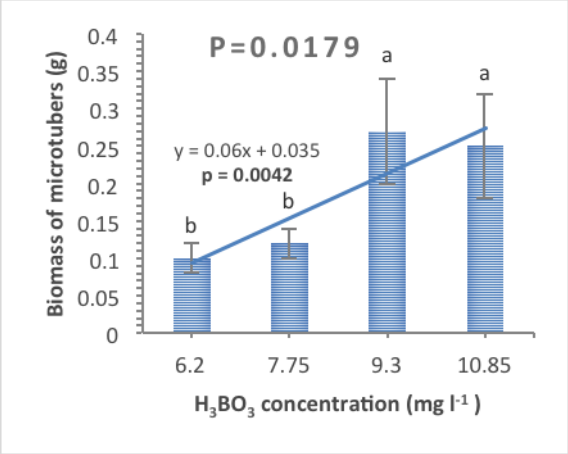

Figure 2. The effect of the concentration of boric acid on biomass of microtubers *of Solanum tuberosum* L. "Floresta" under *in vitro* culture conditions. The means followed by different letters are significantly different at the 5% level of significance. The standard errors of the means are represented as bars. Source: own research.

However, Puzina (2004) found that the treatment of potato seed tubers in greenhouse conditions with boric acid led to an increase in the role of auxin, the photophosphorylation rate, and the tuber growth. According to this author, the boric acid can produce direct changes in the biosynthesis of cytokinins enhancing auxin content and auxin/cytokinin ratio. Our findings indicate that BAP addition to the culture medium reduced auxin/cytokinin ratio stimulated by boric acid, thereby inducing non-statistically significant differences for number or microtubers.

Furthermore, this fact can be explained through the work of Tanaka and Fujiwara (2008). This author argues that the ascending transport of boron solutes through the xylem depends on the transpiration, and that boron taken by roots accumulates at sites of high breathability as in the margins of mature leaves. However, plants from *in vitro* culture lack functional root and stomata systems (Cañal *et al.*, 2001).

Under these conditions, we suggest that the plants did not need this micronutrient, and therefore no boron solutes flowed to the aerial parts of the plants, finding no statistically significant differences in both conditions for the number of microtubers. On the contrary, boric acid enhanced the biomass of the microtubers. This could be due to the transport of sugars through the membranes and the synthesis of starch (Gutiérrez-Soto & Torres-Acuña, 2013). Therefore, much research still needs to be done with regard to the use of boric acid in protocols for micropropagation.

According to a significant level of 5%, the interaction between factors for the biomass of microtubers was significant (p=0.0065). This indicates that the effect of the condition depends on the level of H_3BO_3 concentration and vice versa. Therefore, the test for the individual effects are valid, showing a significant condition effect (F=6.01, p=0.0168) and a significant effect for concentration (F=3.59, p=0.0179; Figure 3).

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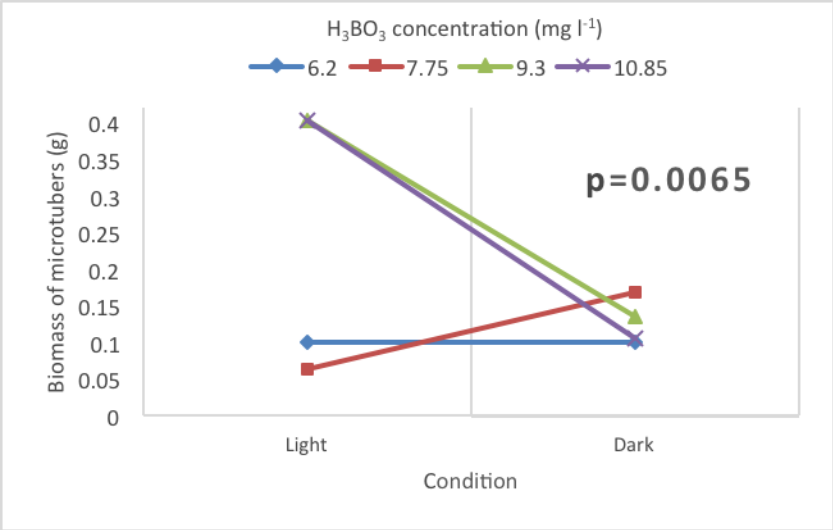

Figure 3. The effect of the condition and the concentration of boric acid on biomass of microtubers *of Solanum tuberosum* L. "Floresta" under *in vitro* culture conditions. Source: own research

Other factors can influence the tuber growth, but they were not evaluated in this work. Among these are: genotype, explant type, temperature, saccharose concentration, radiation (Li *et al.*, 2005), ABA concentration (Gopal, Chamail, & Sarkar, 2004), the concentration of gibberellic acid (Xu, van Lammeren, Vermeer, & Vreugdenhil, 1998), tolerance to salt (Zhang *et al.*, 2005), the concentration of carboxylic acids (Sharma, Chanemougasoundharam, Sarkar, & Pandey, 2004), the control exercised by genes on hormones through polygenic mapping (Ewing, Simko, Omer, & Davies, 2004), the cytokinin concentration, and the use of growth retardants such as chloride of (2-chloroethyl) trimethylammonium chloride (CCC) or Paclobutrazol (Donnelly, Coleman, & Coleman, 2003).

Conclusions

The results showed a greater number of microtubers under the dark condition and a higher biomass under light ones, regardless of the concentration of boric acid, sucrose, and BAP. However, for this experiment, the treatment three has the potential to increase the number and biomass of the microtubers in the "Floresta" cultivar showing the higher values for both variables.

The potato microtuberization is a multifactorial development process which requires the optimization of genotype-specific protocols, which are needed to improve high genetic and phytosanitary quality seed production techniques.

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