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RESEARCH

In vitro regeneration of Mortiño plants (*Vaccinium floribundum* Kunth) by induced callogenesis

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ABSTRACT

Mortiño (*Vaccinium floribundum* Kunth) is an Andean fruit tree, increasingly popular due to its flavor, nutritional value, and it is regarded as a functional food. However, there are no effective methodologies for its propagation, and tissue culture technologies for its availability need to be developed despite prior studies on the micropropagation of Mortiño seedlings. Therefore, in this work, callogenesis was evaluated as an alternative for *in vitro* regeneration of Mortiño plants. Callus formation was induced starting from foliar tissue of Mortiño vitroplants. Explants were grown in Murashige & Skoog-MS ½ and Woody Plant Medium (WPM) culture media, supplemented with cytokinins Thidiazuron (TDZ, 1.5 and 3.0 mg/L), Zeatin (ZEA) and Trans-zeatin (TZR), both at 2.0 and 4.0 mg/L. Plant material was incubated at either 16 h light/8 h dark or full darkness. A favorable response was observed in WPM supplemented with 1.5 mg/L TDZ, with 65.0 and 81.7 % of adventitious shoots developed at 16h light/8 h dark and full darkness, respectively. These results show promising for the regeneration of Mortiño plants by indirect organogenesis.

Keywords: Callogenesis, adventitious shoots, growth induction, Mortiño plants

RESUMEN

Regeneración *in vitro* del Mortiño (*Vaccinium floribundum* Kunth) mediante callogénesis inducida. El Mortiño (*Vaccinium floribundum* Kunth) es un frutal andino de creciente demanda por su agradable sabor, sus propiedades antioxidantes y su valor nutritivo como alimento funcional. Sin embargo, es necesario desarrollar metodologías efectivas para la propagación eficiente del Mortiño, a pesar de estudios anteriores mediante la aplicación de tecnologías de cultivo de tejidos. En este trabajo se evaluó la callogénesis como una alternativa de regeneración *in vitro* del Mortiño. La formación de los callos se indujo a partir del tejido foliar de vitroplantas. Los explantes crecieron en los medios de cultivo Murashige & Skoog½ y Medio para Plantas Leñosas (WPM), suplementados con las citoquininas Thidiazuron (TDZ, 1.5 y 3.0 mg/L), Zeatina (ZEA) y Trans-zeatin (TZR), ambas a 2.0 y 4.0 mg/L. El material vegetal se incubó en fotoperiodos de 16 h de luz/8h de oscuridad, y en total oscuridad. Se obtuvo una respuesta favorable de regeneración de callos con el medio WPM suplementado con TDZ a 1.5 mg/L, con 65.0 y 81.7 % de brotes adventicios para los fotoperiodos de 16 h de luz/8h de oscuridad y de total oscuridad, respectivamente. Tales resultados son promisorios para la regeneración de plantas de Mortiño mediante la organogénesis indirecta.

Palabras clave: Callogénesis, brotes adventicios, inducción, Mortiño

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Introduction

Mortiño (*Vaccinium floribundum* Kunth) is an Andean fruit tree belonging to the Ericaceae family found in the Ecuadorian wastelands, mainly at 1800-3900 m above sea level [1]. It grows in template and cold climates at 8-16 °C [2, 3], as a small bush (3.5-m high) which produces a spherical, dark blue berry of 10-mm diameter and sweet-and-sour taste [1, 4]. Traditionally, this fruit has been highly demanded in national and foreign markets, since it is regarded as a functional food due to its high content of vitamins, anthocyanins, antioxidants and minerals [5]. Functional foods are those being beneficial for human health or decreasing the risks of suffering a disease, because of their active biological components and basic nutrients [5-7]. Mortiño fruits kept under refrigeration maintain its physical and chemical properties without varying the berry weight and volume, while preserving its organoleptic and nutritional properties. That is why Mortiño has a great potential in the food, nutraceutical and pharmaceutical industries.

Currently, there are no commercial Mortiño plantations and the populations are exploited in the wild [1-3, 8]. This is conditioned by the limited knowledge on this plant ecology, phenology and reproduction, with ongoing efforts to establish methodologies for its multiplication [9, 10]. However, conventional propagation methods have been insufficient to establish production parcels for this plant, due to difficulties in seed germination and the unknown plant physiology [11].

In order to solve this problem, the organogenic plant regeneration techniques have been considered. One of them, denominated direct organogenesis, implies the generation of shoots and/or roots from in vitro cultivated explants. The other, known as indirect organogenesis, regenerate plants from callus formation [12]. In fact, the latest results on *V. floribundum* regeneration have been obtained by direct organogenesis from vegetative buds and/or sexual seeds [9, 10]. In those studies, a method was reported for *in vitro*

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plant regeneration using axillary shoots, leading to optimized processes to propagate seedlings. Therefore, this work was aimed at evaluating the morphogenic regeneration capacity of *V. floribundum* from the *in vitro* induction of foliar tissues.

Materials and methods

Facilities and experimental procedures

The study was conducted at the Tissue Culture Laboratory of the Biotechnology Department, in the INIAP's Santa Catalina Experimental Station, in Quito, Ecuador.

The starting vegetal material comprised leaves of *in vitro* cultured plants, which were obtained from germinated seedlings. Explants were transferred into callus-induction media, by placing four leaves on 5-cm in diameter Petri dishes containing 10 mL of medium. Two basal media were tested: 50 % Murashige-Skoog medium [13] and Woody Plant medium (WPM) [14]. Three cytokinins were evaluated: Thidiazuron (TDZ; 1.5 and 3 mg/L), Zeatin (ZEA; 2 and 4 mg/L) and Trans-zeatin (TZR; 2 and 4 mg/L). Basal medium supplemented at 1.5 mg/L TDZ was used as control condition. Also, the effect of 16 light/8 h dark and full darkness photoperiods was analyzed. The explants were kept at 18 ± 2 °C and 40 % relative humidity. Explants' oxidation and death were determined when their color changed from brown to dark, and tissue necrosis developed. Callus formation was recorded when the vegetal tissue displayed green or reddish irregular cell growth (Figure 1). All the tested variables were assessed every 10 days for 60 days after planting (dap).

Experimental design and statistical analysis

Factors were arrayed with their levels, following a Complete Random Design, with a 2 × 3 × 2 factorial array and 5 repeats. The factors followed the order: comprised culture medium (M), growth regulators (R), and their dosages (D). The statistical significance level was set at α (0.05) LSD. Statistical analyses were done using the INFOSTAT package [15].

Results

Calluses were successfully obtained from foliar tissues (Table 1). There were no statistical differences in foliar tissue oxidation among culture media, growth regulators and dosages. The M × R × D interaction provoked the explants' oxidation in respect with leaf number, when subjected to the experimental 16 h light/8 h dark and 24 h darkness photoperiods. The highest oxidation averages were obtained with the MS ½ medium, in both photoperiods (1.61 and 1.82 oxidized leaves, respectively), as shown in Figure 2.

The lowest oxidized leaves' average (1.46) was detected at the 16 h light photoperiod with TDZ. Leaves tissues showed the lowest oxidation (1.51) at low concentrations of growth regulators. A positive interaction between WPM and TDZ was observed at 1.5 mg/L, which led to a lower amount of oxidized foliar tissue (1.16), as compared to the higher TZR dosage (4 mg/L). Likewise, necrotic explants progressively increased over time, the highest area of necrotic tissue achieved after 10 days and stabilizing after 50 days.

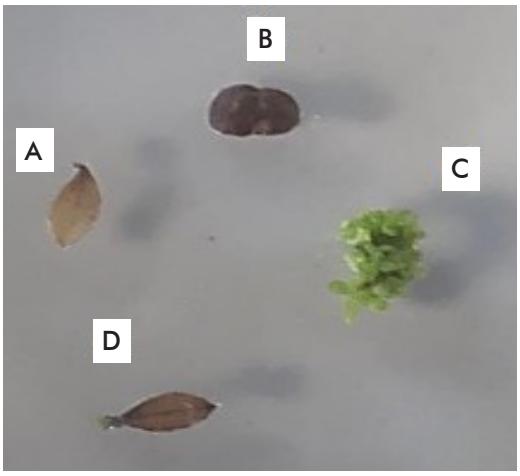

Figure 1. Sample foliar tissues of *V. floribundum*. A) Oxidized leaves. B) Oxidized callus. C) Callus with adventitious shoots. D) Oxidized leaves with shoots.

Table 1. Effect of the photoperiod, culture medium, growth regulators and cytokinin dosages on the callogenesis of the *V. floribundum* induced foliar tissue*

Factors	Photoperiods			
	16 h light/8 h dark		Total darkness (24 h)	
	Folliar tissue oxidation (No. of leaves)	Callus formation (%)	Folliar tissue oxidation (No. of leaves)	Callus formation (%)
Culture media (M)				
MS ½	1.61 a	27.50 b	1.86 a	28.33 b
WPM	1.45 a	45.00 a	1.77 a	40.83 a
Growth regulators (R)				
TDZ	1.46 a	58.75 a	1.74 a	50.00 a
ZEA	1.51 a	30.00 b	1.85 a	27.50 b
TZR	1.62 a	20.00 b	1.87 a	26.25 b
Dosage (D)				
High	1.55 a	32.50 a	1.86 a	29.17 b
Low	1.51 a	40.00 a	1.78 a	40.00 a
M × R × D				
MS ½ + TDZ + 3.0 mg/L	1.72 b	40.00 bc	1.72 abc	30.00 cd
MS ½ + TDZ + 1.5 mg/L	1.72 b	45.00 bc	2.14 d	25.00 cd
MS ½ + ZEA + 4.0 mg/L	1.54 ab	10.00 c	2.14 d	30.00 cd
MS ½ + ZEA + 2.0 mg/L	1.51 ab	35.00 bc	1.37 a	40.00 bc
MS ½ + TZR + 4.0 mg/L	1.45 ab	25.00 bc	1.97 cd	10.00 d
MS ½ + TZR + 2.0 mg/L	1.69 b	10.00 c	1.84 bcd	35.00 cd
WPM + TDZ + 3.0 mg/L	1.23 a	60.00 ab	1.49 ab	65.00 ab
WPM + TDZ + 1.5 mg/L	1.16 a	90.00 a	1.62 abc	80.00 a
WPM + ZEA + 4.0 mg/L	1.60 ab	50.00 b	1.89 cd	20.00 cd
WPM + ZEA + 2.0 mg/L	1.37 ab	25.00 bc	1.99 cd	20.00 cd
WPM + TZR + 4.0 mg/L	1.78 b	10.00 c	1.93 cd	20.00 cd
WPM + TZR + 2.0 mg/L	1.58 ab	35.00 bc	1.72 abc	40.00 bc

Average values with similar letters indicate statistically similar values according to the LDS test (0.05)

These results indicated that strategies can be implemented at these periods of time in order to reduce the phytotoxic effects of tissue phenolization. Besides, treatments applied at full darkness promoted a higher oxidation (1.82) in comparison with those obtained in the 16 h light/8h dark photoperiod (Figure 3).

Conversely, callus formation showed statistically significant differences among culture media, growth regulators, dosages and their interaction, both in light and dark periods. Best results were found at 16 h of light with the WPM medium, for 45 % of explants. TDZ (1.5 mg/L) was the cytokinin more efficiently inducing callogenesis (40 %). Similarly, a positive effect

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was found by combining WPM and TDZ (1.5 mg/L both), with up to 90 % calluses and indirect organogenesis for the production of adventitious shoots (Table 1; Figures 4 and 5).

The *V. floribundum* calluses obtained showed continuous growth and development during the whole study. Otherwise, tissue necrosis and cell death was observed from day 30 for calluses growing in full dark, and at day 40 for those growing at 16 h light. Similarly, adventitious shoots successfully developed after 10 days, which significantly increased until day 30. This is a key information for efficiently transplant calluses into a new culture medium to preserve them. The highest callus formation average (36.25 %) was achieved in explants subjected to 16 h light (Figure 6), with the top adventitious shoots (81.6 %) found in explants developed at full darkness.

Discussion

The common problems found in *in vitro* culture explants concerning oxidation and darkening is a recurrent phenomenon during the culture of different woody species, with significant practical implications. They are triggered by several factors, including light intensity, the cuts of explants, the composition and concentration of culture media and growth regulators. In this sense, MS medium has been previously identified as inducing partial or total necrosis of stems and leaves in *V. corybosum* [16]. In fact, the report emphasized that the most severe symptoms with the medium containing growth regulators [16]. MS is regarded as a salt-enriched medium, due to its higher content of ions (20.6 mM NH_4^+, 39.4 mM NO_3^-, 6.0 mM Cl^-) [15]. On the contrary, the lowest salt concentration of the WPM medium (5 mM NH_4^+, 9.7 mM NO_3^-, 1.3 mM Cl^-) determines its recommendation for *in vitro* culture of woody plants, due to salt toxicity developing at higher salt concentrations [17]. This determines the profound osmotic stress than can be provoked in plant metabolism with a salt-enriched medium, causing the release of molecules easy to get oxidized and, therefore, turning into phytotoxic compounds [18].

There has been also mentioned that the production of polyphenols is influenced by growth regulators, and that a hormonal imbalance can lead to oxidative stress. Consequently, the reactive oxygen species produced could alter the different metabolic pathways and physiological responses in the explants, even killing the plant [19].

Oxidation is the main cause of explant deterioration in *Vaccinium* and other woody plants during its initial growth *in vitro* [20]. Our results evidence that the phenolic oxidation of the *V. floribundum* foliar tissue is highly influenced by salt composition and concentration in the culture medium, and by the dosage of cytokinins added, in agreement with a previous study [21].

A plausible strategy to lower the oxidation process in *in vitro* cultured explants consists on keeping them under low light intensity or full darkness. Despite, the conditions not always eradicates oxidation [17]. That is the case for some forest species and bushes such as *Eucalyptus tereticornis* [22], *Hamamelis* sp. and *Garrya elliptica* [23], in which oxidation did not decrease in spite of plants were grown *in vitro* under full darkness since start the culture. On the contrary, oxidation was completely neutralized in an herbaceous species as strawberry in the light, and all the explants survived [24].

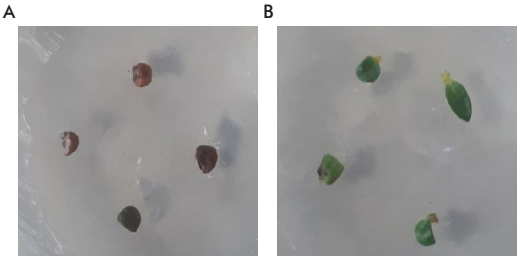

Figure 2. Foliar tissues of *V. floribundum*. A) Leaves incubated in Murakige & Skoog medium showing severe symptoms of oxidation. B) Leaves incubated in Woody Plant medium showing healthy growth.

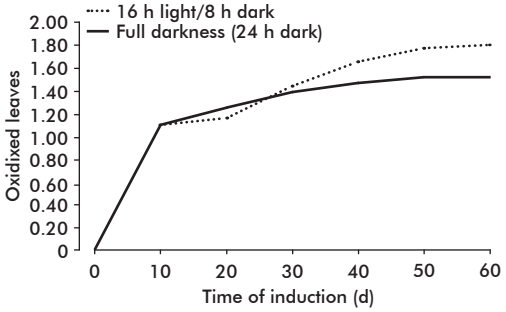

Figure 3. Oxidation of foliar tissues of *V. floribundum* after 60 days of induction at two different photoperiods. Data values comprise the means of all the treatments.

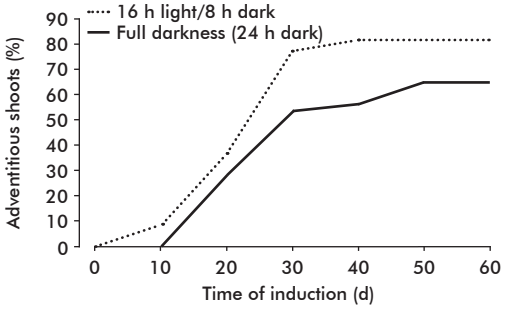

Figure 4. Adventitious shoots (%) of *V. floribundum* after 60 days of induction at two different photoperiods. Data values comprise the means of all the treatments.

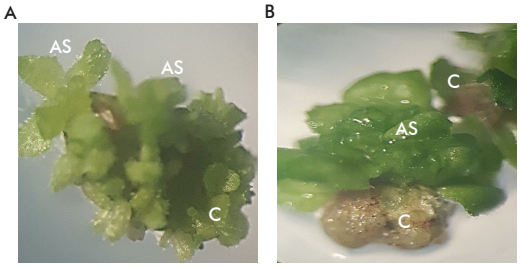

Figure 5. Morphogenesis of foliar tissues of *V. floribundum*. A and B) Two samples of treated plant material showing foliar tissues obtained in Woody Plant medium (WPM) supplemented with Thidiazuron (TDZ). AS: Adventitious shoots. C: Callus.

Regarding callus formation, a positive trend was found, with WPM and the TDZ cytokinin. The WPM is recommended for different *Vaccinium* species in several studies. It favors the adequate development and growth of tissues and organs from an explant, due to its low salt content [20, 25]. Likewise, the effect of TDZ in the absorption and assimilation of nutrients by the explants influences on a number of physiological and biochemical aspects, including those related to the morphogenic response, such as callus formation and shoot regeneration [26, 27]. It was shown that, in a study of culturing *V. angustifolium* at 2.3 µM TDZ, this cytokinin significantly influenced callus formation and shoot regeneration [28].

Our results are in agreement with those by Brenes *et al*. [18], who microcalluses with shoots larger than 2 mm, from foliar segments for four cultivars of *V. ashei* and *V. corymbosum*, using WPM supplemented with 1.5 mg/L TDZ [20].

Furthermore, it was shown that adventitious shoots grew when calluses developed under light and in the dark, with differences in the respective shoots. In this regard, Scalzo *et al*. [29] reported callus formation from explants exposed to 35 µE/m^2·s for 16 h. They concluded that light exposure derived in an increased vegetal biomass with thick shoots of green leaves and higher grade architecture, in contrast to explants developed in the dark, of thinner shoots with pale leaves and aqueous appearance [29]. Coincidently, the properties of calluses obtained in our study coincide resemble those of that report (Figures 4 and 7; summarized in Table 2).

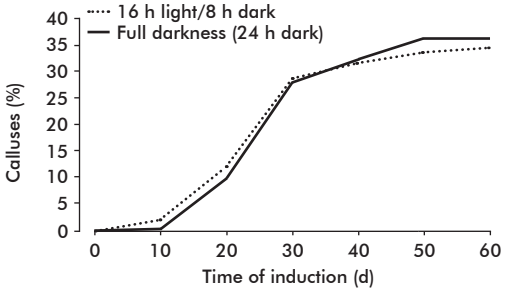

Figure 6. Formation of *V. floribundum* calluses after 60 days of induction at two different photoperiods. Data values comprise the means of all the treatments.

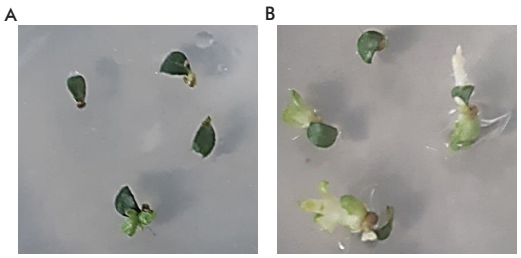

Figure 7. Development of adventitious shoots of *V. floribundum*. A) 16 h light/8 h dark photoperiod. B) 24 h dark (full darkness) photoperiod.

Table 2. Summary of best results on callus induction from folliar tissues of of *V. floribundum*

Component	Best variant	Results
Culture medium (M)	WPM	45.00 % calluses
Growth regulator (R)	TDZ	58.75 % calluses
Dosage (D)	1.5 mg/L	40.00 % calluses
Photoperiod	16 h light/ 8 h dark	36.25 % calluses
M × R × D + Photoperiod		1.53 % oxidation 90.00 % calluses
Photoperiod	24 h dark (full darkness)	81.60 % adventitious shoots

Conclusions

The regeneration of *V. floribundum* via indirect organogenesis is feasible by callus induction and followed by generating adventitious shoots from foliar tissues. The WPM medium supplemented with 1.5 mg/L TDZ cytokinin under 16 h light showed the most efficient to induce callus formation. This knowledge is fundamental to implement attainable procedures for the efficient propagation and cultivation of *V. floribundum* by morphogenetic regeneration techniques. This could impact in a more feasible strategy for the efficient cultivation of Mortiño species, providing a reliable source of economically relevance crops.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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