In vitro propagation of Vaccinium floribundum Kunth from seeds: promissory technology for mortiño accelerated production

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ARTICLE

In vitro propagation of *Vaccinium floribundum* Kunth from seeds: promissory technology for mortiño accelerated production

L.S. Meneses, L.E. Morillo, and W. Vásquez-Castillo

Abstract: The Andean mortiño (*Vaccinium floribundum* Kunth) grows wild in the northern paramos of South América. The berries present potential opportunities for agribusiness, but an efficient technology for the production of plants and berries is required. On the other hand, the development of plant production methods from mortiño seeds will allow the plants' accessibility and its potential use in breeding programs. The objective of this study was to develop an efficient in vitro protocol for accelerated production using seeds. We carried out the research in four phases: seed germination, plant multiplication, rooting, and acclimatization in the greenhouse. For in vitro seed germination, we studied the effect of two culture media [woody plant medium (WPM) and Murashige and Skoog medium (MS], two photoperiods (16 and 24 hours of light), and two temperatures (18 and 28 °C). The best treatment was found to be WPM + 24 h light + 18 °C. In the micropropagation of seedlings, the effect of two concentrations of three cytokinines [(trans-zeatin riboside (TZR), zeatin (ZEA), and 2-isopentenyl adenine (2iP)], and two photoperiods (16 and 0 h light) was evaluated, whereby the concentration of 0.5 mg L^{-1} of TZR was the best treatment. For rooting, two doses of three auxins [indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA), and indole-3-acetic acid (IAA)] were evaluated, resulting in the 2 mg·L^{-1} concentration of IBA giving the best root induction. Finally, the in vitro rooted plants were acclimatized in a greenhouse. We found that peat was the best substrate. These results show that the technology developed here is useful for in vitro production of *V. floribundum* using seeds.

Key words: mortiño, plant production, germination, seeds, Andean paramos.

Résumé : Le bleuet des Andes ou *mortiño* (*Vaccinium floribundum* Kunth) pousse à l'état sauvage dans le *paramos* boréal de l'Amérique du Sud. Ses baies pourraient être exploitées par les agriculteurs, mais on a besoin d'une technologie efficace pour cultiver la plante. Parallèlement, l'élaboration de techniques de croissance à partir des graines facilitera l'hybridation et l'usage éventuel de la plante dans les programmes d'amélioration génétique. Les auteurs souhaitaient développer un protocole in vitro efficace pour accélérer la production au moyen des semences. Pour cela, ils ont axé leurs recherches sur quatre aspects : la germination, la multiplication, l'enracinement et l'acclimatation en serre. Pour faire germer les semences in vitro, ils ont examiné les effets de deux milieux de culture (substrat de plantes ligneuses ou milieu de Murashige et Skoog), de deux photopériodes (16 et 24 heures d'éclairage) et de deux températures (18 et 28 °C). Ils ont obtenu les meilleurs résultats avec le substrat de plantes ligneuses, un éclairage de 24 h et une température de 18 °C. Pour la micromultiplication des plantules, les auteurs ont étudié les effets de trois cytokines (TZR, ZEA et 2iP) à deux concentrations et de deux photopériodes (16 et 0 h d'éclairage). Une concentration de 0,5 mg de TZR par litre est le meilleur traitement. Pour l'enracinement, ils ont évalué trois auxines (IBA, NAA et IAA) et obtenu la meilleure induction des racines avec 2 mg d'IBA par litre. Enfin, les plantes enracinées in vitro ont été acclimatées en serre. Le meilleur substrat pour cela était la mousse de sphaigne. D'après les résultats obtenus, la technologie mise au point par les auteurs faciliterait la production de *V. floribundum* in vitro à partir de graines. [Traduit par la Rédaction]

Mots-clés : mortiño, production végétale, germination, semences, paramos Andin.

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Introduction

The genus *Vaccinium* belongs to the Ericaceae family and worldwide it comprises approximately 4500 species (Luby et al. 1991). In Ecuador, the most important species of the genus is *V. floribundum* Kunth, known as 'mortiño', mountain grape, or in English Andean blueberry (National Research Council-NRC. 1989); Ballington et al. 1993). This fruit tree is found in the Andes between 1400 and 4350 m above sea level (asl) (NRC 1989; Sanjinés et al. 2006; Coba et al. 2012). In Ecuador, it is found only between 2800 and 4000 m above mean sea level (msl) (Racines-Oliva et al. 2016). While in Colombia, the species *V. meridionale* is also known as mortiño and *V. floribundum* is not used for human consumption (Coba et al. 2012), in Ecuador, *V. floribundum* berries are harvested twice a year manually for human consumption (NRC 1989; Vasco et al. 2009). Its chemical composition and phenolic compound profile show a high antioxidant content. The presence of anthocyanins, such as cyanidin and delphinidin derivatives, suggest its high potential for the agro-industry due to their potential health benefits (Vasco et al. 2009; Schreckinger et al. 2010; Coba et al. 2012).

V. floribundum plant production currently presents various obstacles, such as low seed viability. Previous studies have pointed out the difficulty of propagating *V. floribundum* using sexual seeds. Gutiérrez and Camacho (2011) considered that production from seeds is problematic due to small seed sizes, and the dependence on strict light and temperature conditions for seed germination; they suggested dormancy as an additional factor. Muñoz (2004) stated that, in his experiments, neither microshoot propagation using a sterilized substrate nor germination from botanical seeds were efficient processes. More recently, Cobo et al. (2018) reported a useful protocol for the propagation and multiplication of *V. floribundum* seedlings from axillary buds. As Gutiérrez and Camacho (2011) concluded, the use of seeds through tissue culture techniques must be considered an alternative propagation system in *V. floribundum*. While seed tests with tetrazolium reported 95% viability, germination was almost null after 30 d of evaluation. Based on these results, these authors suggested that the seeds of *V. floribundum* have physical or physiological dormancy. The development of plant production methods for mortiño from seeds will improve the plants' accessibility, increasing the genetic variability for its potential use in breeding programs. Using seeds from different *V. floribundum* populations will assist in establishing experimental field trials for agronomic practices and production for this species. In addition, it will constitute the basis of the re-establishment of the plants in disturbed páramos (Gutiérrez and Camacho 2011). Páramos are highmountain neotropical ecosystems with humid grasslands and patches of low forests (Chuncho and Chuncho 2019). In this regard, the objective of this study was to develop an efficient in vitro protocol for *V. floribundum* multiplication from seeds.

Materials and Methods

The study was conducted at the INIAP-EESC biotechnology laboratories, located in Quito, Ecuador, at 3100 m asl (0° 22′8″S and 78° 33′24″W). The research was carried out in four experimental phases: (*i*) seed germination, (*ii*) micropropagation of seedlings, (*iii*) induction for rooting, and (*iv*) acclimatization of the in vitro plants.

In vitro seed germination

V. floribundum seeds were obtained from ripe fruits harvested in the Andean páramos. The seeds were washed and sieved to discard foreign material. Subsequently, the seeds were left to dry on paper towels at room temperature for a period of five days. Seeds in good condition were selected by stereomicroscope. Cracked, hollow and damaged seeds were discarded. The disinfection of selected seeds was accomplished following the protocol by Torres et al. (2010). For each treatment, 20 seeds were placed in a Petri dish that contained 20 ml of culture media (woody plant medium (WPM) or Murashige and Skoog medium (MS), 2% sucrose, 0.58% agar, pH 5.2].

The variables studied were (1) germination (%), determined by the relationship between the number of germinated seeds and the total number of seeds; and (2) germination time (d), counted from the day the radicle appeared and the hypocotyl of the first seed was elongated until the day this process ended. The evaluation was carried out over 80 days in a plant growth chamber (Thermo Sientific model 844).

In vitro propagation

Shoots grown from seeds were transferred into another culture medium with cytokinins to promote plant growth. Ten-millimeter shoots were placed in 15 × 1 cm test tubes containing 5 ml of culture medium (WPM, 3% sucrose, 0.6% agar, pH 5). The effect of cytokinins on seedling growth was evaluated using two concentrations of each plant growth regulator: trans-zeatine riboside (TZR) (1 mg·L^{-1} and 0.5 mg·L^{-1}); Zeatine (ZEA) (1 mg·L^{-1} and 0.5 mg·L^{-1}), and 2-isopentenil adenine (2iP) (10 mg·L^{-1} and 5 mg·L^{-1}). The explants were placed in growth rooms [18 ± 2 °C, 40% relative humidity (RH)] with a photoperiod of 16 h of light (1600 lux) or in total darkness. The variables evaluated were (1) shoot length (mm), which was determined by the difference between the initial and final shoot length at 60 d using a graduated Vernier; and (2) multiplication index (%), determined based on the relationship between the final and initial number of shoots. The evaluation was carried out at the end of each subculture, which had an interval of 60 d.

Treatments were arranged in a completely random design (CRD) with three growth regulators, two doses and under two photperiods. Treatments were replicated 10 times.

In vitro root development

To induce the formation of roots, the effect of auxins was evaluated. Plantlets obtained at the in vitro propagation stage were transferred into tubes containing 5 ml of WPM culture medium (3% sucrose, 0.6% agar, pH 5). The effect of auxin on rooting was evaluated using two concentrations: indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) 4 $mg \cdot L^{-1}$, 2 $mg \cdot L^{-1}$). The plants were kept in a growth room at 18 ± 2 °C, 40% RH and 16 h light (1600 lux). The variables studied were (1) rooting (%), through the relationship between the number of explants with roots and the total number of planted explants; and (2) root length (mm), determined by measuring from the base of the in vitro plant to the apex of each root, using a graduated Vernier. The evaluation of the variables was carried out at 60 d.

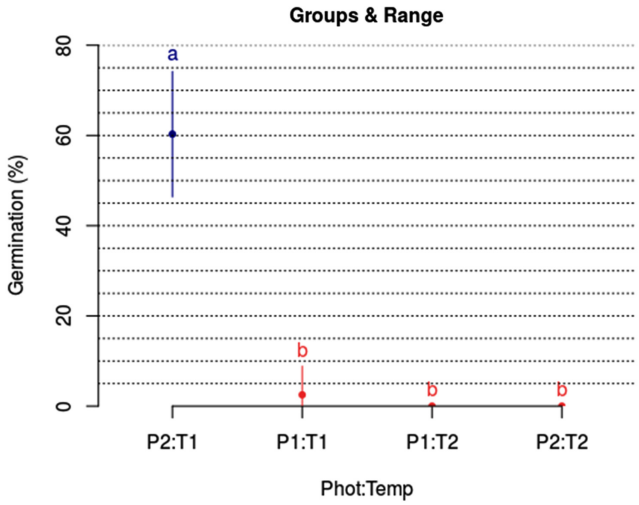
Acclimatization of in vitro plants

For acclimatization tests, in vitro plants with roots were placed into a greenhouse and covered with a 35% shade polyethylene black mesh (Zaram-EMPAQUIM CIA.LTDA). The temperature was 28 ± 2 °C during the day and 10 ± 2 °C at night; RH was 60 % and 12 h of natural daylight. The light intensity fluctuated between 20 000 and 10 000 lux in the morning and afternoon respectively. For transplantation, the roots of the in vitro plants were washed to remove the culture medium. The in vitro seedlings were then placed into 295 cc plastic cups containing not sterilized black paramo soil where *V. floribundum* usually grows [5.9 pH; 6.7% organic matter (OM), 0.28% Nitrogen (N), 7 ppm phosphorus (P) and 0.23 cmol/kg potassium (K); Racines-Oliva et al. 2016] or Promix peat (Canadian *Sphagnum* peat moss 75%–85%, vermiculite, dolomitic and calcitic limestone, wetting agent). The container was then covered with a 265 cc plastic white cup to create a high relative humidity environment to prevent tissue dehydration. Irrigation of the seedlings was with or without fertilizer, which contained foliar dissolution of 5 ml L^{-1} of 10% N – 4% P_2O_5 – 7% K_2O (Nitrophoska®). The variables evaluated at 30 d were as follows: (1) increase in plant length (mm) was determined by measuring from the base to the apex. Data of this variable were transformed using the square root to comply with the Gaussian assumptions of normality and variance homogeneity; (2) seedling survival (%) was evaluated 15 and 30 d after the in vitro plants were transplanted.

Statistical analysis

Statistical analysis of the data was carried out using the "R" program. The experiments were analized employing a CRD in factorial arrangement of treatments.

Fig. 1. Output from ANOVA analysis in R showing the effects of photoperiod (P1 = 16 h light; P2 = 24 h light) and temperature (T1 = 18 °C; T2 = 28 °C) on *V. floribundum* seed germination. Treatments followed by the same letter are statistically equal. The bar segment in each treatment represents the standard deviation. [Colour online.]



Five replicates and three factors were used for in vitro seed germination experiment: (1) two culture mediums (WPM and MS); (2) two photoperiods (16 and 24 h light); and (3) two temperatures (18 and 28 °C). Treatments for in vitro propagation were arranged with three growth regulators, two doses under two photperiods with 10 replicates. For the in vitro root development experiment we employed three growth regulators, two doses, and 10 repetitions per treatment. Finally for adaptation plants experiment the treatments were two substrates and two fertilization methods with 10 repetitions. Mean separation as required used the least significant difference (LSD) at the 5% level.

Results

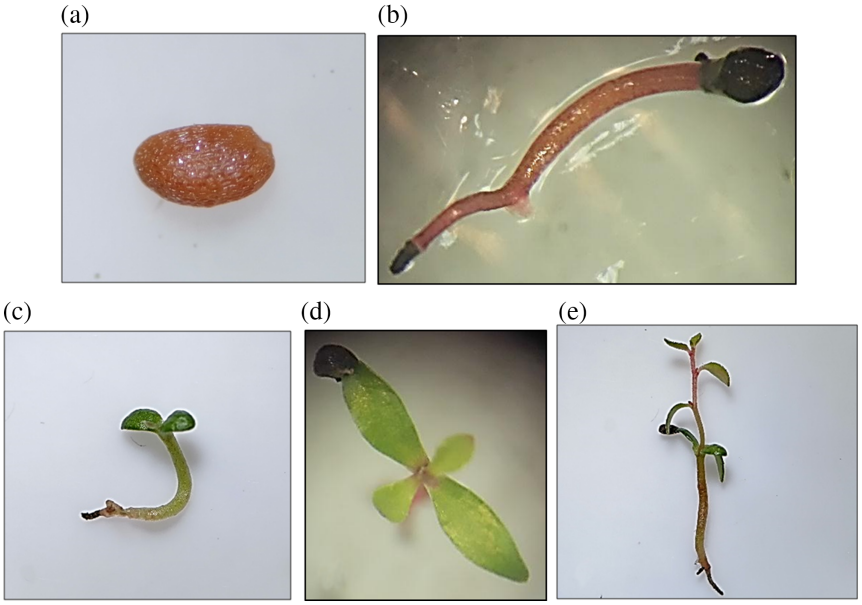
In vitro seed germination

There were differences in vitro seed germination between photoperiod and temperature, yet not for culture media. Differential responses between photoperiod (P) × temperature (T), were evident in seed germination (Fig. 1). The highest germination of mortiño seeds occurred with the 24 h light + 18 °C treatment, with 60.3% germinated; different phases of the germination process of the mortiño seeds are shown in Fig. 2.

In vitro propagation

TZR induced the most developed shoots in the first and second subcultures (4.20 mm and 8.49 mm, respectively) (Figs. 3 and 4). Regarding the TZR doses, differences were obtained in the second subculture, where the high concentrations favored the growth of shoots (6.13 mm). When evaluating the photoperiod, differences were detected in the second subculture,

Fig. 2. In vitro seed germination process of *V. floribundum*. (*a*) Selected seeds; (*b*) germination after 10 d, showing the radicle; (*c*) plumule appearance after 30 d; (*d*) after 40–45 d true leaves appear; (*e*) plantlet ready for in vitro shoot propagation. [Colour online.]



whose photoperiod was 16 h light, and resulted in longer shoots (5.80 mm); a differential result between the studied factors was also detected. The treatment ZEA + 0.5 mg·L^{-1} + 16 h light produced longer shoots in the first subculture (5.80 mm), while in the second subculture the longest shoots (10.10 mm) resulted from a treatment of TZR + 1 mg·L^{-1} + 16 h light (Fig. 3*a*). The cytokinin TZR produced a higher number of shoots per explant (5.3 and 5.88 in the first and second subculture, respectively) than the ZEA and 2iP. Likewise, it was observed that the number of shoots per explant increased with low concentrations of cytokinin, (4.10 and 4.93 in the first and second subculture, respectively). The 16 h light photoperiod favored the number of explants in the second subculture (4.87 shoots/explant). Furthermore, the interaction of TZR in a low dose (0.5 mg·L^{-1}) with 16 h light induced the highest number of shoots per explant (6.50) as shown in Fig. 3*b* and Fig. 4.

In vitro rooting

The in vitro rooting results are shown in Table 1 and Fig. 5. The highest in vitro rooting (100%), number of roots (4.05), and root length (9.15 mm) were induced with the IBA auxin, followed by NAA and IAA. A low dose (2 mg·L^{-1}) of the phytohormones favored rooting (73.3%), number of roots (3.37), and length of roots (5.67 mm) compared with the high dose (4 mg·L^{-1}). The phytohormone x dose interaction presented the best results with a low IBA dose (2 mg·L^{-1}), producing the highest number of roots per plant (4.5 roots) with an average of 10.2 mm in length.

Acclimatization of in vitro plants

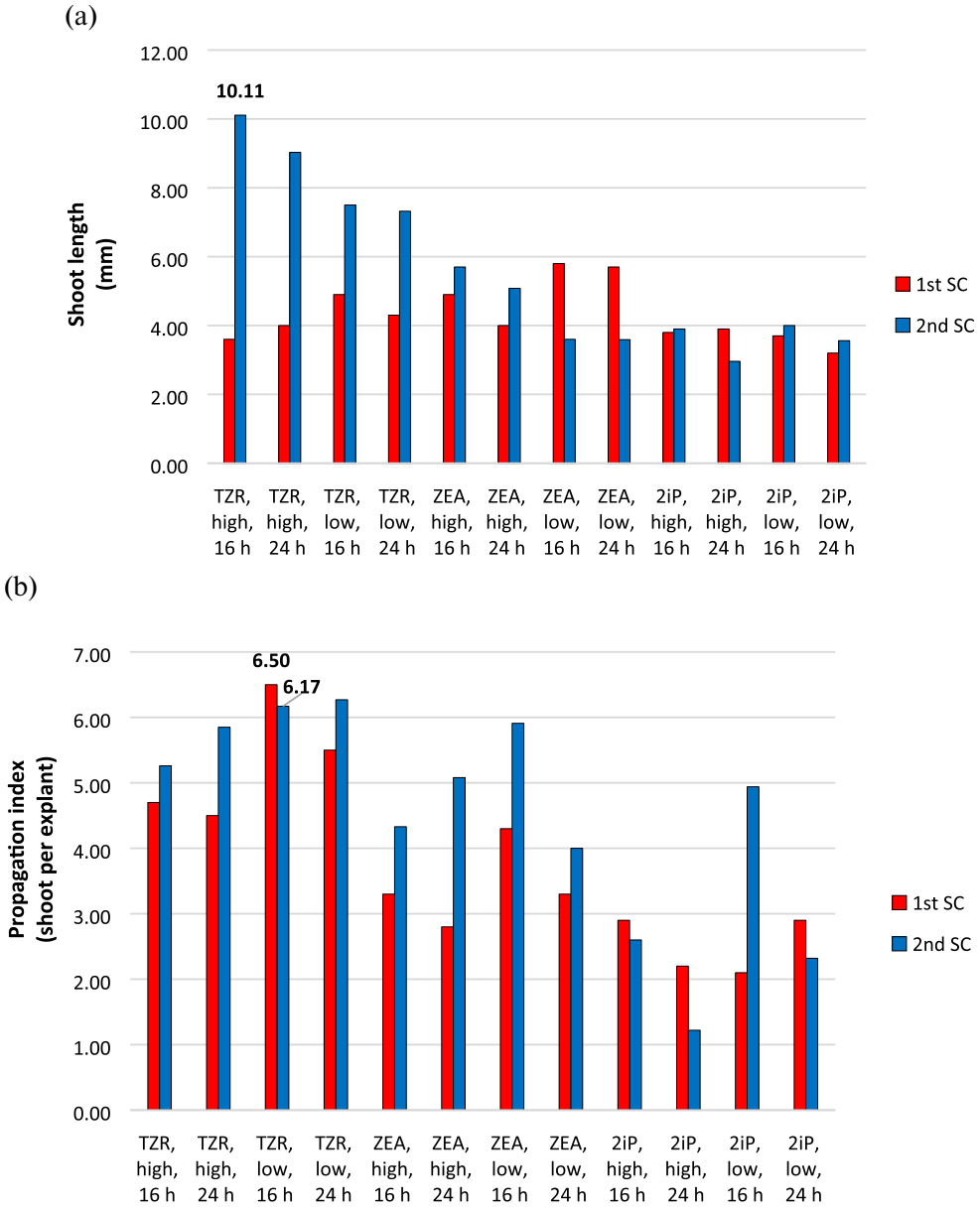
Acclimatization of *V. floribundum* plantlets in the greenhouse are shown in Table 2 and Fig. 6. The highest percentage of seedling survival was obtained in the substrate composed of peat (100%), followed by black páramo soil (70%). The same effect was seen for the length of the seedlings, as the peat substrate plantlets were more than double that of the paramo soil: 3.22 and 1.30 mm, respectively. There was a differential response of survival and length of seedlings with the highest survival and length of the seedlings obtained in the substrate composed of peat with and without fertilizer.

Discussion

In vitro seed germination

Seed germination is regulated by its viability, while dormancy, a characteristic that controls seed germination, is controlled by physical, chemical and environmental barriers (Gutiérrez and Camacho 2011). The nutrients of the culture media used (MS and WPM) did not have a clear effect on the germination of *V. floribundum* seeds. This may be due to the fact that in the early stages of germination, the growth and development of the embryo uses the seed reserves (energy and chemical compounds) (Caroca et al. 2016). Temperature is another factor that directly influences seed germination (Kim et al. 2004; Sorgato et al. 2015). The effect of temperature on germination is related to the enzymatic activity in the biochemical reactions of the seed after its hydration (Finch and Leubner 2006).

Fig. 3. Cytokinin doses and photoperiod effects upon *in vitro V. floribundum* plantlets. (*a*) Shoot length and (*b*) propagation index. [Colour online.]

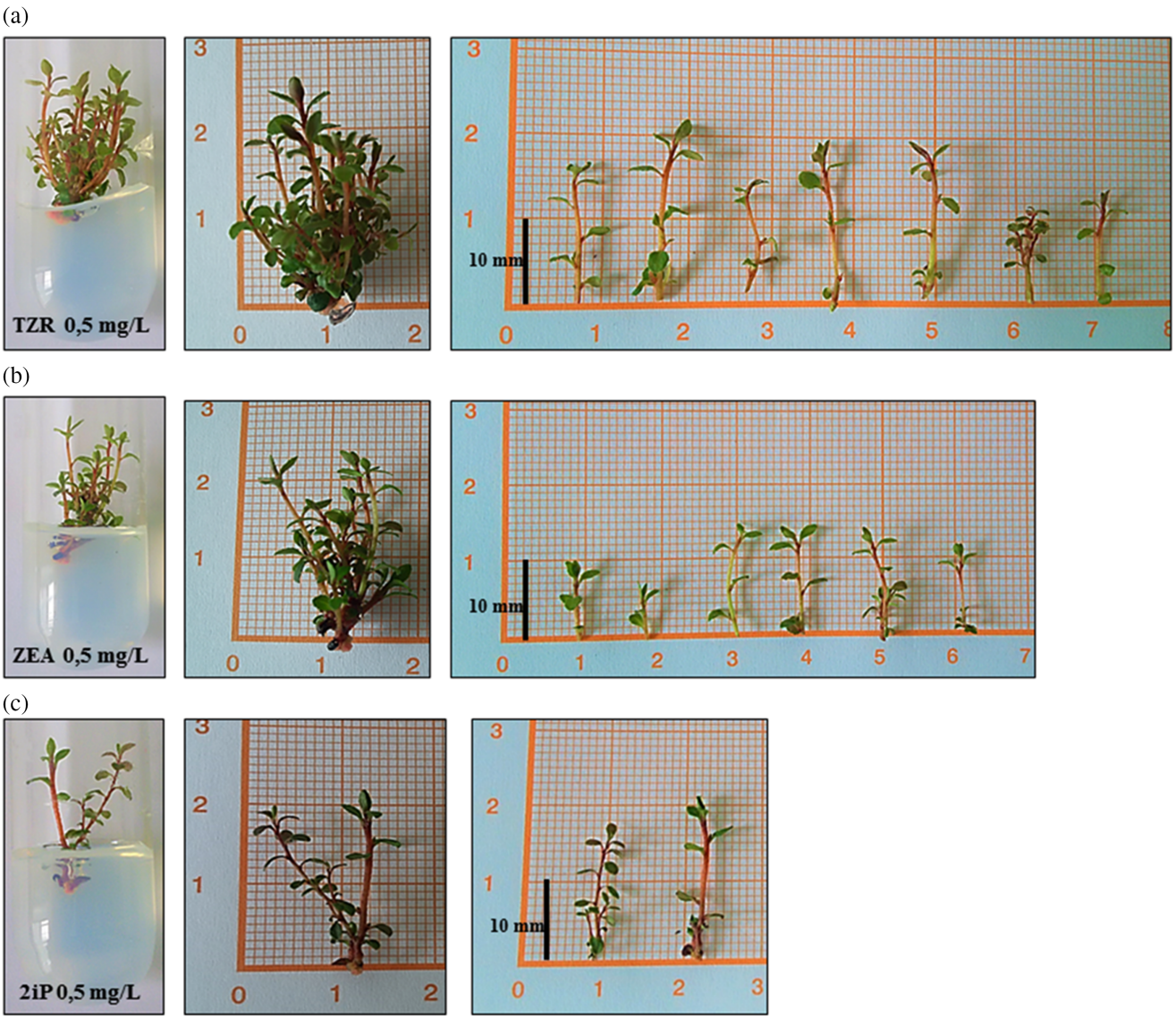


Furthermore, the seeds germinate within a temperature range that is specific to each species (Baskin et al. 2000). In this study, the highest germination of *V. floribundum* seeds occurred at 18 °C, while there was no germination at 28 °C. These results are consistent with previous studies that recommend an optimum temperature for *V. meridionale* seed germination of 18 °C, while at 29 °C the seed showed relative dormancy. However, at 22 °C, the seeds began to germinate (Castro and Álvarez 2013).

In vitro propagation

Clearly, it was observed that the cytokinins TZR and ZEA were the most efficient in the multiplication and growth of mortiño shoots. This agrees with previous studies carried out in blueberries which indicate that these regulators favor the growth of explants (Ruzic et al. 2012). Furthermore, Rowland and Ogden (1992) discovered that the use of TZR was more efficient in the proliferation of shoots in *V. corymbosum* than ZEA and 2iP. The concentration of growth regulators is important in inducing shoot formation and morphogenesis. Gajdošová et al. (2006) indicated that low concentrations of ZEA favor the formation of shoots in meristem culture of *Vaccinium* spp. Likewise, Debnath and McRae (2002) reported that in blueberry sprouts, high concentrations of cytokinins favor hydration and limit growth.

Fig. 4. Influence of cytokinins on the induction of *V. floribundum* shoots after 8 wk with different hormones. (*a*) Trans-zeatin; (*b*) zeatin; (*c*) 2iP. [Colour online.]



The propagation index in mortiño seedlings using TZR 0.5 mg·L^{-1} obtained in our study was 6.5 shoots per explant in 6 wk. Although our index was lower than that of Cobo et al. (2018), who reported 9 shoots per explant in 16 wk in seedlings from axillary buds using 2iP in combination with NAA, the time required for multiplication was reduced by more than half. This allowed the number of subcultures of *V. floribundum* to double in the same period of time.

In vitro rooting

Magnitskiy et al. (2011) described how IBA directly and indirectly influences morphogenesis in microcutting rooting. Castro and Álvarez (2013) in *V. meridionale* obtained between 66% and 80% rooting depending on the genotype. Similarly, Rache and Pacheco (2010) reported almost 90% of *V. meridionale* microstems with the use of IBA. In other species, such as *V. corymbosum* and *V. vitis-idaea*, in vitro rooting with the use of IBA was at 80% (Baskin et al. 2000; Debnath 2003; Debnath 2005; Ostrolucká et al. 2007; Ruzic et al. 2012). Torres et al. (2010) reported up to 60% rooting on in vitro *V. floribundum* seedlings using IBA. For their part, Cobo et al. (2018) studied the ex vitro rooting of *V. floribundum* seedlings using IBA. In our study, the use of IBA (2 mg·L^{-1}) promoted 100% rooting in *V. floribundum* plantlets. Ruzic et al. (2012) stated that low concentrations of IBA ($\leq$ 1 mg·L^{-1}) in in vitro *V. corymbosum* seedlings are suitable for the production of clonal plants. This suggests that exogenous auxin concentrations favor root induction.

Table 1. Auxins and dosage effect on three characteristics on in vitro *V. floribundum* explants.

Treatment	Rooting (%)	Root No.	Root length (mm)
Plant growth regulator (R)			
IBA	100.00a	4.05a	9.15a
NAA	70.00b	0.95b	1.45b
IAA	30.00c	0.80b	2.60b
Dose (D)			
High (4 mg·L^{-1})	60.00a	1.50b	3.13b
Low (2 mg·L^{-1})	73.33a	3.37a	5.67a
R × D			
IBA (4 mg·L^{-1})	100.00a	3.60a	8.10a
IBA (2 mg·L^{-1})	100.00a	4.50a	10.20a
NAA (4 mg·L^{-1})	80.00ab	0.90bc	1.30c
NAA (2 mg·L^{-1})	60.00b	1.00bc	1.60c
IAA (4 mg·L^{-1})	0.00c	0.00c	0.00c
IAA (2 mg·L^{-1})	60.00b	1.60b	5.20b

Note: Means are based on 10 replications. Means followed by the same letter in each column are statistically equal (LSD 5%). Original data were transformed using square root prior to analysis. Abbreviations: IBA, indole-3-butyric acid; NAA, 1-naphthaleneacetic acid; IAA, indole-3-acetic acid.

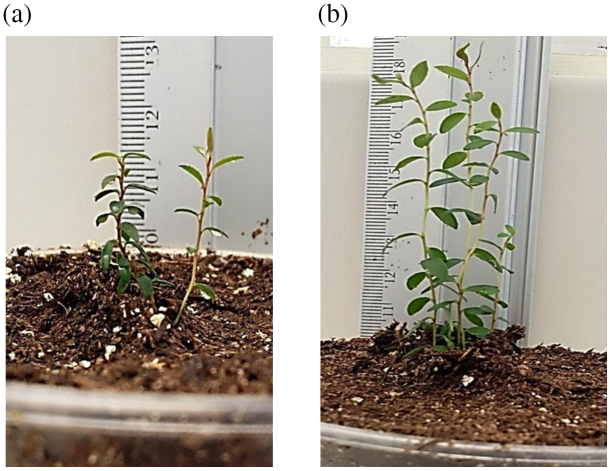
Fig. 5. Influence of auxins (IBA) on roots induction after eight weeks of culture in *V. floribundum*. [Colour online.]

Table 2. Substrate (S) and fertilization (F) effects upon *V. floribundum* plantlets in terms of establishment and growth in greenhouse conditions.

Treatment	Survival (%)	Plantlet length (mm)
Substrate (S)		
Black paramo soil	70.00b	1.30b
Peat	100.00a	3.22a
S × F		
Black paramo soil (with fertilizer)	70.00b	1.60b
Black paramo soil (without fertilizer)	70.00b	1.00b
Peat (with fertilizer)	100.00a	3.16a
Peat (without fertilizer)	100.00a	3.28a

Note: Means are based on 10 replications. Means followed by the same letter in each column are statistically equal (LSD 5%). Original data were transformed using square root prior to analysis.

Fig. 6. *V. floribundum* under ex vitro conditions. (*a*) Plantlet establishment (1 d); (*b*) plants after 6 mo. [Colour online.]



Acclimatization of *V. floribundum* in vitro plants

Environmental conditions (temperature, relative humidity, and light brightness) and substrate (Gil et al. 2017) are important factors for the establishment of plantlets in ex vitro conditions (greenhouses). In previous studies with *V. floribundum* (Noboa 2019), it has been stated that the best cutting establishment (86.6%), shoot number per cutting (2.67) and shoot length (1.2 cm) were obtained on black paramo soil. In our study, the best establishment (100% plantlet survival with a shoot length increase of 3.2 mm) was obtained with a peat substrate, as previously reported by Cobo et al. (2018). This might be explained by the physical and chemical characteristics of peat (its fine structure, lightness, porosity, pH (5.2–5.8) and inert nature free of toxic elements and contaminants) allowing for good root

Table 3. Summarized results for in vitro seed *V. floribundum* plant production.

Step	Results
Seed germination WPM (24 h + 18 °C)	59.60% germination
In vitro propagation TZR (0.5 mg·L^{-1} + 16 h light)	6.50 shoots/explant
In vitro rooting IBA (2 mg·L^{-1})	100% rooting 4.50 roots 10.20 mm root length
Acclimatization Peat (without fertilizer)	100% survival 3.28 mm plantlet length

Note: WPM, woody plant medium; TZR, trans-zeatin riboside; IBA, indole-3-butyric acid.

system growth and development. Meanwhile, substrates enriched with organic matter might promote good results (Hidalgo et al. 2009).

The results from the fertilization experiments suggest that there is no need for fertilization during the first stages of mortiño growth. This is probably explained by the fact that in vitro plants have limited functionality in an autotrophic root system (Kumar and Rao 2012), which limits the plantlet's ability to absorb exogenous nutrients. It is likely that fertilization will become a key factor in later *V. floribundum* growth stages once the plant has developed a functional root system.

Conclusion

In vitro germination of *V. floribundum* seeds must be carried out in controlled conditions (24 h light and 24 °C). TZR and ZEA were more efficient than 2iP for the multiplication of *V. floribundum* seedlings by organogenesis. Rhizogenesis was achieved using a low dose of IBA (2 mg·L^{-1}). To harden the in vitro plants, they must be transplanted into peat as the soil substrate as opposed to local soil. Results presented in Table 3 show that the technology developed for the production of *V. floribundum* plants from seeds is efficient. Our method can also be used in a breeding program, since seed plant utilization increases the genetic variability of the species. Furthermore, our system can be used for the restoration of *V. floribundum* populations in disturbed Andean paramos in Ecuador.

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