Development of a germination protocol for blueberry seeds (*Vaccinium meridionale* Swartz)

Establecimiento de un protocolo para germinación de semillas de agraz (*Vaccinium meridionale* Swartz)

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ABSTRACT

The blueberry is a plant with potential for national and international markets because its fruits have food and medicinal uses and it is an important source of sugars, antioxidants, vitamins and minerals. We developed a germination protocol of blueberry seeds, morphologically characterized the seeds, and assessed germination *in vitro* in four culture media (MS 1/3, 1/8 y 1/16 and distilled water) and ground mulch for *ex vitro* germination, taking into account: light conditions, temperature, seed storage periods and fruit ripening stage. We also assessed the hardening of the seedlings germinated *in vitro* in substrates with and without ground mulch. Our results showed fruits with little seeds with 1.27±0.11 mm for length and 0.784±0.08 mm for width; and big seeds with 1.61±0.14 mm for length and 1.03±0.10 mm for width. The optimal conditions for seed germination were: *in vitro* culture using distilled water, continuous light and a temperature of 18±2°C, using stored seeds of a period less than one week from ripe fruits. The hardening and development of the seedlings was better using ground mulch.

Source photographic Figs. 1-3: Castro, C., Y. Olarte, L. Rache, and J. Pacheco.

Key words: *in vitro*, *ex vitro*, hardening, promising fruit.

RESUMEN

El agraz es una planta con potencial en los mercados nacionales e internacionales debido a que sus frutos tienen usos alimenticios, medicinales y son una fuente importante de azúcares, antioxidantes, vitaminas y minerales. Se estableció un protocolo de germinación de semillas de agraz, caracterizando morfológicamente las semillas y evaluando su germinación *in vitro* en cuatro medios de cultivo (MS 1/3, 1/8 y 1/16 y agua destilada) y en tierra de capote para su germinación *ex vitro*, teniendo en cuenta: condiciones de iluminación, temperatura, periodos de almacenamiento de semillas y estado de madurez del fruto. Además, se evaluó el endurecimiento de las plántulas procedentes de semillas germinadas *in vitro* en sustratos con y sin tierra de capote. Los datos obtenidos indicaron que los frutos contienen semillas pequeñas de 1,27±0,11 mm de largo por 0,784±0,08 de ancho y grandes de 1,61±0,14 mm de largo por 1,03±0,10 mm de ancho. Las condiciones óptimas para germinación de semillas fueron: cultivo *in vitro* en agua destilada, con iluminación continua y temperatura de 18±2°C, utilizando semillas almacenadas durante un periodo menor a una semana y provenientes de frutos muy maduros. El sustrato tierra de capote fue más adecuado para el endurecimiento y desarrollo de plántulas.

Palabras clave: *in vitro*, *ex vitro*, endurecimiento, frutales promisorios.

Introduction

Vaccinium meridionale, known in Colombia as blueberry, belongs to the Ericacea family. It occurs in Jamaica, Venezuela, Colombia and Peru; growing at 1,800 (Toro *et al.*, 2002) to 3,500 m above sea level (m a.s.l.) (Avila *et al.*, 2007). *V. meridionale* is abundant in forests of pine and cypress, presenting an excellent development in acid soils with mycorrhizal associations (Arjona, 2001).

This species has a huge potential for the market because its fruits are an important source of sugar, antioxidants, vitamins B and C and minerals such as potassium, calcium and phosphorus. This fruits are used commercially (*i.e.* juices, jams, among others), medicinally (control of diabetes and digestive problems) in general by people and is a keystone species in restoration processes and soil remediation (Vallejo, 2000; Magnitskiy and Ligarreto, 2007; Avila *et al.*, 2007; Rache and Pacheco, 2010).

Under natural conditions, the processes of seed germination and seedling development of the genus *Vaccinium* are hampered by several factors such as fluctuating humidity, temperature and light; generating long germination processes and low numbers of seedlings (Baskin *et al.*, 2000). For satisfying the demand for seedling production

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of *Vaccinium*, due to the fact that the organoleptic and nutritional properties of the fruit have attracted great interest in the establishment of commercial crops, it is necessary to develop new procedures for production of vegetative materials of this species.

Few studies have been reported on propagation from sexual seeds of this genus. Studies on *V. myrtillus, V. vitis-idaea* (Baskin *et al*., 2000), and *V. reticulatum* (Yoshinaga, 1998) are known. On the other hand, for *V. meridionale,* there are no known *in vitro* studies on seed germination. Currently, only some aspects have been studied about the morphology, anatomy and germination of the seeds (Valencia and Ramírez, 1993) and the effect of nitrate potassium, gibberellic acid and indoleacetic acid on the germination of Colombian blueberry seeds under controlled conditions (Magnitskiy and Ligarreto, 2007).

In this study, a protocol for the germination conditions *in vitro* and *ex vitro* of Colombian blueberry seeds is reported for the first time, and appropriate conditions for the hardening of seedlings in a greenhouse were identified.

Materials and methods

Seeds. Used in the different tests were obtained from blueberry fruits collected from plants in the municipality of Ráquira (Boyacá Department). All samples were carried to the plants tissue culture laboratory (Bioplasma) at Universidad Pedagógica y Tecnológica de Colombia (UPTC) in Tunja c ity. Fruit pulps were fermented for 48 h to extract the seeds. To prevent contamination with fungi, all seed sets were cleaned for 10 min with Carboxamide + Dithiocarbamate (Vitavax®) solution at 1 g L^{-1} with constant shaking. After that, we assessed the seed condition using a stereoscope, discarding the empty and smaller seeds. Finally, the selected seeds were collected in glass containers and kept at ambient temperature (15-18°C daytime and 9-12°C nighttime).

Based on a set of 200 fresh extracted seeds, an amorphological analysis was carried out using an optic microscope (objective of 3.2, ocular x10 and micrometer). We assessed features such as: length, width, color, shape, and seed shell texture. Furthermore, we took 100 fruits at random in order to determine the mean diameter of the fruit and mean amount of seeds per fruit. Subsequently, the germination of the blueberry seeds was tested both *in vitro* and *ex vitro* as well as seedling hardening.

In vitro germination. Seed surface disinfection was carried out in a laminar flow chamber as follows: rinsed with Tween® 20 solution (0.1%) for one min with constant shaking, rinsed with ethanol 70% (v/v) for one min, dipped in a mixed solution of sodium hypochlorite (0.525% w/v) and Tween® 20 for 15 min, and finally two consecutive rinses with sterile distilled water.

We carried out six tests as shown (Tab. 1). The best processing for each test, that is the one that showed the highest percentage of germination and the least time of germination on the basis of the assessed variables (culture media, light conditions, seed storage, temperatures and state of fruit maturity), was used in the next test and so on, in order to determine the five most suitable conditions for seed germination.

For each *in vitro* germination test, 600 seeds were randomly taken (big between 1.61 mm in length and 1.03 mm in width and small between 1.27 mm in length and 0.784 mm in width) and seeded in 12 glass bottles of 100 mL, with 50 seeds each. Each test was repeated two times. The culture medium used was MS (Murashige and Skoog, 1962) with macroelements at 1/3 (Rache and Pacheco, 2010), 1/8 and 1/16 of its original concentration (NH_4NO_3: 1650 mg L^{-1}, KNO_3: 1900 mg L^{-1}, KH_2PO_4: 170 mg L^{-1}, $CaCl_2H_2O$: 440 mg L^{-1} and $MgSO_4.7H_2O$: 370 mg L^{-1}). The medium was adjusted to pH 5.8 and autoclaved 15 nain at 15 psi (121°C).

In the first test, the germination in 4 different culture media was assessed: sterile distilled water, MS 1/16, MS 1/8, MS 1/3 and ground-mulch (forest humus, Tab. 1). In each culture medium, the seeds taken from very ripe fruit (referred fruit color) with one week of stored were seeded. These were kept with continuous light at 18±2°C. After five weeks, for each container with seeds seeded on MS, 2 mL of sterile distilled water was added.

In the second test, the germination was assessed under 3 light conditions: continuous light, 16 h photoperiod (70-80 µmol m^2 s^{-1}; both provided by 75 W Silvana lamps) and darkness. Based on the results of the first test, the seeds were seeded in sterile distilled water; the other variables (temperature, seed storage and mature fruits) were the same as the first test (Tab. 1).

In the third test, germination was evaluated taking into account three periods of seed storage: one, four and 16 weeks under environmental laboratory conditions. The seeds were seeded in sterile, distilled water, were kept in continuous light with a temperature of 18±2°C; and seeds extracted from fruits very ripe were used (Tab. 1).

In the fourth test, we assessed the effects of four temperatures: 6, 18, 22 and 29±2°C, the seeds were seeded in sterile, distilled water and with continuous light. We used seeds stored for 1 week and extracted from very ripe fruits (Tab. 1).

In the fifth test, seeds from fruits in three maturity stages were used: immature (stage 1), mature (stage 2) and very ripe (state 3; Fig. 1, Tab. 1). The seeds were seeded in sterile, distilled water, with continuous light, a temperature of 18±2°C and stored for 1 week (Tab. 1).

In the sixth test, the best assessed conditions were determined for the five above tests, taking into account the highest germination percentage and the least time of germination; namely: sterile, distilled water, seeds extracted from very mature fruit, stored for 1 week, with continuous light and a temperature of 18±2°C (Tab. 1).

Ex vitro germination. 600 seeds were planted in six polystyrene trays (17 x 17 x 4 cm), with ground-mulch and kept under greenhouse conditions (75-85% RH, day and nighttime mean temperatures of 18 and 12°C, respectively).

In both *in vitro* and *ex vitro* germination tests, the seeds were considered germinated when the radicle length reached 2 mm. We assessed the beginning of the germination (day when the first seed germinates) and its end (last day on which germinated seeds were recorded) and we calculated the total percentage of germination (PTG). The mean germination time (TMG) was calculated according to the equation 1 proposed by Thompson (1970):

$$TMG = \sum niti / \sum ni \quad (1)$$

where, ti is the time elapsed in the culture and ni the number of germinated seeds in the time interval ti - ti-1.

We also recorded the period from seeding to the fall of the seed shell and cotyledon release. At the end of this period, the length of the germinated seedlings, the presence of root hairs, and the color of the hypocotyl and cotyledon were recorded.

Seedling hardening. We assessed the effect of three substrates carrying out replicates of each one:

- Only soil with abundant organic matter
- Land and soil with abundant organic matter, 3:1
- Land, sand, and rice hulls, 2:1:1

From 200 vigorous seedlings of 1.0 to 1.5 cm of height which showed features such as fibrous roots, with a length of about 0.7 cm, colorless, undeveloped and without root hairs were replanted in each substrate in polystyrene trays (17 x 17 x 4 cm). The trays with seedling were covered with extensible, perforated paper and incubated for 12 weeks (in an incubation room) at a temperature of 22±2°C, with continuous illumination and irrigated every 48 h. In this study, we quantified features such as: percentage of viable seedlings, number of leaves, number of internodes, and seedling length. After 12 weeks, the seedlings that reached a length between 3 and 4 cm, with 8 to 10 short internodes and 10 to 13 leaves were transferred to a greenhouse.

TABLE 1. Tests for germination of Colombian blueberry seeds.

Treatments tested	Test						Suitable conditions
	1	2	3	4	5	6	
Culture medium							
Sterile distilled water	X	X	X	X	X	X	X
MS/16	X						
MS/8	X						
MS/3	X						
Ground-mulch	X						
Light conditions							
Continuous light	X	X	X	X	X	X	X
16 h photoperiod		X					
Darkness		X					
Seed storage (weeks)							
One	X	X	X	X	X	X	X
Four			X				
16			X				
Temperature (°C)							
6 ± 2				X			
18 ± 2	X	X	X	X	X	X	X
22 ± 2				X			
29 ± 2				X			
State of maturity of fruit							
Immature					X		
Mature					X		
Very mature	X	X	X	X	X	X	X

Once transferred to a greenhouse, after one week, the extensible paper was gradually retired from the trays. Subsequently, the seedlings were replanted in 8 x 15 cm plastic bags with substrate 3 (land: sand: rice hulls, 2:1:1), and irrigated weekly. After 12 weeks, we quantified the percentage of viable seedlings and the number and length increase of axillary shoots.

Statistical analysis. The set of experiments was conducted using a completely randomized design (DCA). The obtained data were analyzed by ANOVA and the F-test using the statistical package Statgraphics® Plus Professional version 2.0 for Windows. When the ANOVA showed differences, a DHS Tukey test (Honestly Significant Difference) was applied with a confidence level of 95%.

Results

Morphological analysis showed four Colombian blueberry seed shapes: oblong or ovoid, elliptical, orbed, and apiculate, with predominance from the oblong-elliptical shape (60%). Given its size, a seed can be classified into two distinct groups: small with a mean dimension of 1.27 by 0.78 mm and big of 1.61 by 1.03 mm. In total, each fruit had 28 seeds (13.69 small ones and 14.59 big ones) on average (Tab. 2); the fruits had a diameter from 7 to 11 mm with 28 seeds on average (maximum 64 and minimum 7).

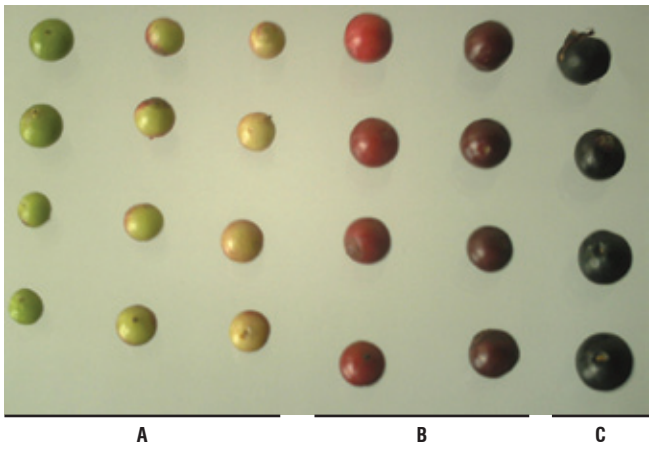

FIGURE 1. Colombian blueberry fruits at different stages of maturity. A, immature (state 1, green fruits); B, mature (stage 2, purple fruit); C, very mature (state 3, dark purple or black fruit).

In vitro germination. The results obtained in the germination test are shown in Tab. 3.

In the first test, the total percentage of germination (TPG) and mean germination time (MGT) quantified in seeds cultured in different media and in a substrate with abundant organic matter showed differences that were statistically significant ($P \leq 0{,}05$). The highest TPG was obtained in seeds cultured in sterile, distilled water followed by MS/16

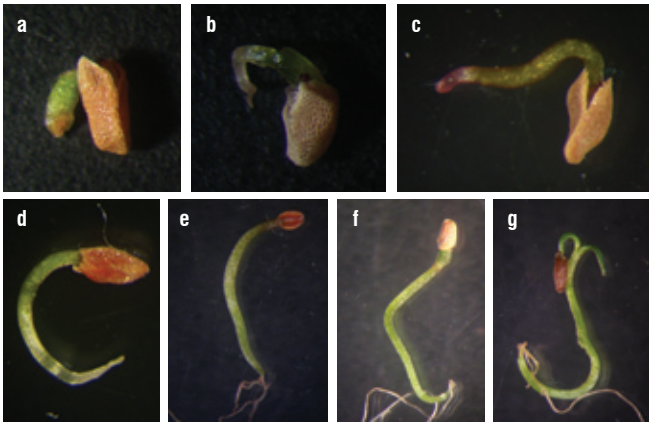

FIGURE 2. Chronological development of the germination process of Colombian blueberry seeds. a and b: after day 14 of the culture, emergence of the radicle and hypocotyl elongation. c and d: after day 21, hypocotyl arcing formation and hypocotyl elongation. e and f: after day 28, hypocotyl arch straightens and cotyledons expand and begin rooting and g: after day 35 of the culture, seed coat falls, cotyledon leaves unfold and become green.

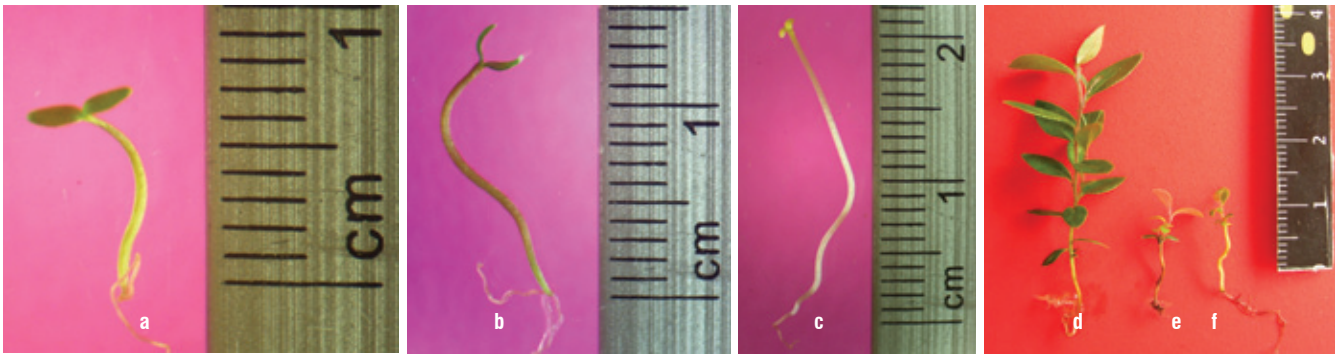

FIGURE 3. a, b and c. Seedlings from newly germinated seeds. a. Seedlings from small seeds (size one: 0.5 to 1.0 cm), b. Seedlings from big seeds (size two: 1.0 to 1.5 cm), c. Etiolated seedlings, these were gterminated from seeds in continuous darkness (size three: 1.5 to 2.0 cm). d, e and f. Seedlings germinated in different substrates: d. Substrate with abundant organic matter (1), e. Land: Substrate with abundant organic matter, 3: 1 (2) and f. Land: Sand: Rice hulls, 2:1:1 (3). Notice the differences in length and leaf development of seedlings in a substrate with abundant organic matter.

TABLE 2. Size classes and number of seeds per Colombian blueberry fruit.

Size classes	Length (mm)			Width (mm)			Seeds per fruit		
	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max
Small	1.0	1.27±0.11	1.44	0.44	0.784±0.08	0.9	0	13.69	46
Big	1.44	1.61±0.14	2.21	0.9	1.03±0.10	1.35	0	14.59	39

TABLE 3. *In vitro* germination of Colombian blueberry seeds with different physical and chemical conditions.

Treatments tested	Onset of germination time (d)	End of germination time (d)	TPG (%)	MGT (d)
Culture medium				
Sterile distilled water	14	56	640.00 c	35.35 a
MS/16	42	105	63.50 c	61.01 c
MS/8	42	105	63.00 c	64.28 d
MS/3	42	77	48.00 b	58.48 b
Substrate with abundant organic matter	35	126	20.60 a	67.03 e
Light conditions				
Continuous light	14	84	62.6 NS	34.38 a
16 h photoperiod	14	105	67.16 NS	36.03 a
Darkness	21	105	50.75 NS	45.51 b
Seed storage (weeks)				
One	14	98	72.66 c	36.94 NS
Four	14	91	37.50 b	38.24 NS
16	21	98	22.00 a	39.86 NS
Temperature (°C)				
6 ± 2	28	112	54.66 b	65.52 c
18 ± 2	14	91	81.00 c	33.50 a
22 ± 2	14	91	60.50 b	35.96 a
29 ± 2	14	119	10.83 a	58.62 b
State of maturity of fruit				
Immature	21	84	66.66 a	43.86 b
Mature	14	105	80.16 b	42.97 b
Very mature	14	84	83.50 b	33.26 a

and MS/8, with a germinative expression embryo capacity inversely proportional to the concentration of salts in the medium. Furthermore, the MGT in sterile, distilled water was the shortest (35.35 d), germination began at 14 d and ended at day 56, whereas in the *ex vitro* treatment with a substrate with abundant organic matter the MGT was 67.03 d, and germination began at day 35 and finished at day 126.

In the second test, the light conditions positively affected the TPG ($P \leq 0.05$) but not the MGT, with continuous light giving a MGT shorter (34.38 d) than in darkness (45.51 d). Germination began in continuous light and the photoperiod of 16 h at day 14 and in darkness at day 21 since these was cultured, whereas germination finished after day 84 in continuous light and at day 105 in the other photoperiod and darkness.

In the third test, the different periods of seed storage significantly influenced the TPG ($P \leq 0.05$), which decreased drastically as the storage period increased. On the other hand, the MGT values were not affected by storage periods. The germination in seeds stored for 1 and 4 weeks began at d 14 of the culture and for 16 weeks, it began at day 21. In seeds stored for 1, 4 and 16 weeks, germination finished between days 91 and 98.

In the fourth test, the data showed an significant effect of the temperature on the TPG and MGT ($P \leq 0.05$); 18°C showed the least performance of TPG and MGT than the others values assessed, in which TPG decreased and MGT was longer. The germination began at day 14 in treatments of 18, 22, and 29±2°C, and at day 28 for the treatment of 6±2°C. In seeds cultured at 18, 22 and 29±2°C, the germination finished between days 91 and 119 and for 6±2°C, it finished at day 112.

In the fifth test, the seeds obtained from fruits in any of the three stages of maturity reached TPG up to 65%. Significant differences were obtained between TPG and MGT ($P \leq 0.05$) (Tab. 3), showing a positive correlation with the TPG and a negative relationship with the MGT. The germination began at day 14 in seeds of fruits in the 2nd and 3rd maturity states and day 21 in the 1st maturity state. In seeds of fruits of the first and third states, the germination finished at day 84and with the second maturity stage, at day 105; we observed that the seeds of fruits in the third maturity stage began and finished germination faster than the other stages.

TPG, total percentage of germination; MGT, mean germination time. Different letters within a column indicate significant difference ($P \leq 0.05$). NS, Not significant.

TABLE 4. Effects of substrate type on seedling viability, length, production of leaves and internodes.

Substrate type	Viability (%)	Leaves per seedling	Internodes per seedling	Length (cm) per seedling
1	94 c	10.77±4.29 c	8.68±2.93 c	2.58±1.13 b
2	36 a	6.15±1.92 b	3.77±1.90 b	0.68±0.32 a
3	44 b	4.47±1.59 a	2.27±1.61 a	0.46±0.36 a

Different letters within a column indicate significant difference ($P \leq 0.05$).

According to Valencia and Ramírez (1993), blueberry seeds show epigeal germination. In appropriate physicochemical conditions, the germination process begins with the emergence of the radicle between 14 and 21 d of culture and the cotyledon leaves are observed after 35 d (Fig. 2). When the seed coat falls, the hypocotyl and cotyledon leaves exhibit a green color that changes to red. The roots were colorless, tiny, undeveloped and without root hairs.

Newly germinated seedlings reached a length between 0.5 and 2.0 cm and can be grouped as follows: from 0.5 to 1.0 cm (seedlings from small seeds, size 1, Fig. 3a), 1.0 to 1.5 cm (seedlings from big seeds, size 2, and Fig. 3b) and etiolated seedlings of 1.5 to 2.0 cm (size 3, Fig. 3c). Seedlings of the third size were obtained in treatments for germination in continuous darkness, showing elongated hypocotyl, a cotyledon leaf with limited development and almost complete absence of chlorophyll (yellowish seedlings). These abnormal characteristics disappeared when etiolated seedlings were exposed to light conditions, but some did not recover the color and died.

Seedling Hardening. After 12 weeks in the incubation room, the viability percentage was higher in seedlings transplanted to substrate one (94%) than substrates two (36%) and three (44%). We found significant differences among the three assessed treatments, the seedlings transplanted to substrate 1 had the highest means in all the quantified variables (Tab. 4).

After 12 weeks in the greenhouse, seedlings showed vigorous growth with 99.5% of viability with a mean production of 1.12±1.39 of shoots and an increase in mean length of 4.31±2 cm. Among the substrates used for hardening, the substrate with abundant organic matter showed a strong superiority in feasibility and development of seedlings than the other tested substrates (Fig. 3d, e and f).

Discussion

The size and shape variation observed in the Colombian blueberry seeds agrees with that reported by Valencia and Ramírez (1993) for this species. According to Dalling (2002), the variety of seeds of a species is the result of a series of adjustments to achieve a successful dispersion as well as of the seed space limitations for its development inside the fruit. Therefore, the form of the blueberry seeds is related to space limitations within the fruit, with it being common to find a lot of seeds ($\bar{X}$= 27 per fruit) in relatively small fruits (7 to 11 mm in diameter). Besides, according to Dalling (2002), fruits with several seeds have a higher likelihood of having at least one viable mature seed.

McGinley (1993) indicates that environmental conditions can affect the number of seeds per plant and but not the size. On the other hand, Dalling (2002) states that intraspecific variation of the seed size may be due to the number of seeds produced per fruit and particular environmental conditions.

According to Correa (2002), seeds of this species could be classified as not affected by light because they germinate in light and dark conditions. However, the differences between TPG and MGT quantified in continuous light, photoperiod and continuous darkness, indicate that the light factor affects the germination process. In turn, Finch and Leubner (2006) show that in most seeds, the germination is faster in conditions of light due to the presence of phytochrome in the seed embryonary axis. This pigment is presented two ways in the seed, Pr (P660) is inactive and Prf (P730) which induces germination.

The TPG for the Colombian blueberry was similar to that reported in previous studies in other species of the same genus such as *V. myrtillus* and *V. vitis-idaea* (TPG of 62 to 100%) in the presence of light and a temperature of 10 to 20°C (Baskin *et al*., 2000).

Vadillo *et al*. (2004) highlighted the temperature and moisture of stored seeds as relevant variables to maintain seed viability. Clearly, laboratory storage conditions are not suitable for seed maintenance for over a week, because when the storage period is prolonged, viability decreases. This decrease can be related, according to Correa (2002), to the seminal deterioration during the storage, which produces strong changes in enzymatic amount and activity, degrading the nutritional reserves; according to Pérez and Martínez (1994), because of alterations in the substances responsible of metabolism. These effects may be related

to the constant metabolic activity inside the seeds in the conditions of the study, which somehow, negatively influenced the process of seed germination of blueberries stored longer than one week.

In previous studies carried out by Medina and Lobo (2006), the Colombian blueberry was quantified in greenhouse conditions with 70% germination in seeds stored for four months in a cold room at 0°C and humidity of 5%, after 60 d of culture; Magnitskiy and Ligarreto (2007) in the same species, quantified 29 and 38% germination in seeds without pretreatment (seeds without imbibition in solutions with 200-500 mg L^{-1} of potassium nitrate or gibberellic acid) and stored during 30 d at 5°C, within the fruit or not, after 35 d of culture. Yoshinaga (1998) recorded seeds of *V. reticulatum* with a good germination percentage after a storage period of 6 to 12 months to 1.1°C.

Obviously, temperature proved to be the most influential physical factor on blueberry seed germination. Its effect is due to the ability to influence the activity of enzymes that regulate the rate of biochemical reactions that occur inside the seed after its rehydration. In blueberry seeds, 18°C was the most suitable temperature at which the enzyme activity was carried out efficiently, stimulating the highest percentage of germination. We found that seeds exposed to 29±2°C showed a relative dormancy and that a transfer to a temperature of 22±2°C, rebooted their germination.

The physiological maturation of seeds is related to the PTG (Correa, 2002). With the results obtained in this study, we evidenced a clear relationship between fruit maturity and seed physiological maturity. This result agrees with Otegui *et al*. (2007), due to the fact that the germination rate increases with fruit maturity. Bridgen (2001) highlights the importance of finding the optimum state for seed collection, because an immature embryo does not have an accurate development. However, some seeds are able to germinate long before reaching full maturity (Correa, 2002). The PTG obtained in seeds from green blueberry fruit showed an early ripening even with an immature appearance.

During seedling hardening, it was observed that the composition of the substrate is an important factor for the development of the cauline and radical. Clearly, the substrate with abundant organic matter was the most suitable for hardening of Colombian blueberry seedlings. These results can be associated with the features found in this kind of substrate such as high porosity, high water retention, good aeration, low pH and fine texture along with mycorrhizae, largely responsible for seedling vigor (Valencia and Ramírez, 1993). Furthermore, these characteristics are part of the natural ground where Colombian blueberry grows (subparamo and paramo) (Rangel, 2000). Moreover, the feasibility of Colombian blueberry seedlings in the substrate with abundant organic matter during hardening is probably due to the facility to develop a fibrous, thin, shallow root system that is characterized by a shortage of root hairs (Malik and Cawthon, 1998). This helps to anchor and expand the roots among the fine particles.

The protocol effectiveness of our work for hardening of Colombian blueberry seedlings was evidenced by the high proportion (99.5%) of viable seedlings with vigorous development characterized by an adequate number of axillary shoots with sufficient elongation.

Conclusions

The optimum conditions for *in vitro* germination of Colombian blueberry seeds were: use of sterile, distilled water, continuous illumination and a temperature of 18±2°C, seeds stored for 1 to 7 d and from very mature fruit.

The *ex vitro* establishment and development of Colombian blueberry seedlings was successful when a substrate with abundant organic matter was used.

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