Research article

Establishment and in vitro multiplication of thornless blackberry (*Rubus glaucus* Benth.) by shoot apical meristems

Establecimiento y multiplicación in vitro de mora de castilla (*Rubus glaucus* Benth.) variedad sin espinas, mediante ápices meristemáticos

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Abstract

We evaluated a technique of micropropagation in a thornless variety of blackberry (*Rubus glaucus*) from shoot apical meristems. In the establishment phase, we evaluated a disinfection protocol: soapy solution (commercial detergent and water) for 5 minutes + 70% alcohol for 2 minutes + 3% hypochlorite with two different exposure times: T_1 for 5 minutes and T_2 for 10 minutes. While the microcuttings were disinfected, the meristematic shoots were removed and established in vitro in a completely random design to evaluate two cultivation mediums: M1 and M2. From the developed seedlings, the multiplication was performed in three cultivation mediums: M1, M3 and M4. The two disinfection treatments were 100% effective on each of the explants. The planted apex meristem allowed the establishment of aseptic cultivation and adequate development of the explants after six weeks of cultivation with rates of germination of 83.4% for M1 and 66.6% for M2. The analysis of variance (ANOVA) and multiple range test with Fisher's method (LSD), showed that M1 medium favored multiplication with a better growth and development of the explant with a multiplication coefficient of 7.5 shoots per seedling, and an average height of 1.95 cm.

Key words: In vitro cultivation, meristems, micropropagation, *Rubus glaucus*, thornless blackberry.

Resumen

Se evaluó una técnica de micropropagación de plantas de mora (*Rubus glaucus*) de la variedad sin espinas, a partir de ápices meristemáticos. En la fase de establecimiento se evaluó un protocolo de desinfección utilizando por 5 min solución de jabón detergente comercial y agua + alcohol 70% por 2 min + hipoclorito 3% con dos tiempos de exposición diferentes: T_1 por 5 min y T_2 por 10 min. Después de desinfectar las microestacas se extrajeron los ápices meristemáticos y se establecieron in vitro bajo un diseño completamente al azar para evaluar dos medios de cultivo: M1 y M2. A partir de las plántulas desarrolladas se efectuó la multiplicación en los medios de cultivo M1, M3 y M4. Ambos tratamientos de desinfección resultaron efectivos alcanzando 100% de desinfección de los explantes con cada uno de ellos. La siembra de ápices meristemáticos permitió el establecimiento de cultivos asépticos y un adecuado desarrollo de los explantes después de seis semanas de cultivo, con prendimiento de 83.4% para M1 y 66.6% para M2. El análisis de varianza (Anova) y la prueba de rangos múltiples mostraron que la multiplicación fue mejor en el medio M1 con una mayor tasa de crecimiento y desarrollo del explante, al obtener coeficientes de multiplicación de 7.5 brotes/plántula y una altura promedio de 1.95 cm.

Palabras clave: Cultivo de meristemas, cultivo in vitro, micropropagación, mora sin espinas, *Rubus glaucus*.

Introduction

The blackberry (*Rubus glaucus* Benth.) is one of the most popular fruits grown in Colombia, cultivated by small and medium farmers, and it is one of the main sources of income, rural employment generation, food supply and input for agroindustry (Barrero, 2009). Due to its importance, it is expected to increase the cultivated area with 10,000 new hectares with a production of 104.265 tons of fruit harvested in 2020 (Tafur et al., 2006). Despite the wealth and potential of the blackberry, cultivation has major limitations as lack of certified seeds, low adoption of new varieties, and phytosanitary problems related to asexual propagation by cuttings and graftings, which favors pest and disease transmission (Avilan et al., 1989; Angle, 2003, cited by Barrero, 2009). The multiplication of thornless blackberry is increasing due to its high production capacity of fruits, associated with a higher number of branches and tillering, producing between 15 and 20% more than traditional blackberry thorns (Bernal and Diaz, 2006).

Colombia has increased the use of fruit meristem micropropagation by tissue culture technique. This system, besides allowing a mass propagation of specific clones, free of pathogens, ensures high quality, more uniform and clean seeds (Marulanda et al., 2000). Many studies in vitro micropropagated plants are capable of producing fruits cleaner, with a greater size, weight and the homogeneity (Barrero, 2009).

The aim of this study was to evaluate a technique for micropropagation of blackberry (*Rubus glaucus*) thornless variety, by culturing meristematic apices.

Materials and Methods

Collection of plant material

The collection of plant material, with the collaboration of the Municipal Unit of Agricultural Technical Assistance (UMATA), was carried out initially for a survey and analysis of the production areas of Labateca, Norte de Santander. As a source of mother plants, the farm Miralindo was selected. This farm is located in the village of San Bernardo Balsa at 1850 MASL. The plant material was collected in the early morning (06:00 am) from actively growing plants, vigorous development and without visible signs of illness. Once collected, the material was wrapped in newspaper before placing it in a sealed container to be moved to the Plant Biotechnology Laboratory of the University Francisco de Paula Santander, Los Patios, Norte de Santander.

Establishment phase

Micro-cuttings of 1 cm length with an axillaries bud were extracted from field selected material. Those were introduced into a solution of 150 mg/l of ascorbic acid (antioxidant). In sterile area, a disinfection protocol was evaluated with two tissue exposure times (Table 1). Once concluded the exposure times for each disinfectant, three rinses with sterile distilled water were performed before placing the micro-cuttings in a solution of 150 mg/l ascorbic acid for preservation, while proceeding to the extraction and planting the meristem apices. The extraction of these apexes was performed with the aid of a stereoscope, and incubation was performed at 20°C ± 2°C and a photoperiod of 16 h light /8 dark.

Table 1. Evaluated disinfection treatments.

Component	T_1 (min)	T_2 (min)
Soap solution (commercial detergent + water)	5	5
Alcohol 70 %	2	2
Sodium hypochlorite 3%	5	10

The selection of the assessed culture media (Table 2) was made in accordance with previous results obtained by Crossbow (2008). This experiment used 30 meristematic apices (experimental unit) per treatment, with three replicates, each in a randomized block design. At this stage of six weeks, were measured length (cm), number of sheets and percent of disinfection, taking into account the phenolization and the survival rate.

Multiplication phase

In this phase, seedlings developed in the culture medium M1 at the establishment phase were used because of their adequate overall conformation, with an average height of 1.2 cm, and abundant foliage development of

Table 2. Evaluated culture media in establishment phase of thornless blackberry.

Component (mg/lt)	**Media M1**	**Media M2**
Salts MS	100%	100%
Myo-inositol	100	100
Thiamine	1	1
L-cysteine	50	50
Saccharose	30000	30000
Agar	6000	6000
6 BAP	**1**	**1**
GA3	**0.5**	–
IAA	–	**0.5**
pH	6	6

internode elongation. On the other hand, the seedlings in the medium M2 performed a lower height <0.6cm and little leaf development. The multiplication process was an elimination of leaves and the basal necrotic area, and finally the selection of microcuttings with an axillary bud and a length between 0.3 and 0.5 cm.

In this phase, the length (cm) and the multiplication coefficient (number of survived seedlings /number of explants established) were evaluated in three culture media adapted from Najaf-Abadi and Hamidoghli (2009); Marulanda et al. (2000), and Erig et al. (2002) (Table 3).

For four weeks it was evaluated the callus formation from explants in each of the culture media, and the results were analyzed according to the inferential, completely randomized model, using the Statgraphics Centurion XVI (2009) and analysis of variance for comparison of means using the F test (P <0.05).

Results and discussion

Establishment phase

The use of apex meristems as primary explants allowed the establishment of fully aseptic cultures; both treatments achieved 100% disinfection because no endogenous contamination was evident in any of the explants. Under the same conditions of this laboratory and, using the same protocol for disinfection but taking microcuttings of 7 mm as explants, Ballesta (2008) observed up to

Table 3. Evaluated culture media in multiplication phase of microcuttings of thornless blackberry.

Component (mg/lt)	**Media M1**	**Media M3**	**Media M4**
Salts MS	100%	100%	100%
Myo-inositol	100	100	100
Thiamine	1	1	1
L-cysteine	50	50	50
Saccharose	30000	30000	30000
Phytagel	3000	3000	3000
6 BAP	**1**	**1.5**	**1.5**
GA3	**0.5**	–	–
IAA	–	**0.75**	–
IBA	–	–	**0.75**
pH	6	6	6

Sources: Adapted from Najaf-Abadi and Hamidoghli (2009); Marulanda *et al.* (2000), and Erig *et al.* (2002).

70% of endogenous contamination. This suggests that the use of apex meristems as explants, reduces endogenous contamination to a minimum level without using aggressive protocols for disinfection of plant material.

The effect of culture media in the development explants during the establishment phase is observed in Table 4. Significant differences (P <0.05) in length and number of leaves were present across culture media, showing a higher growth and development of apex meristem in medium M1, which allows seedlings development before the start of the multiplication phase (Figure 1). Munoz and Reyes (2006) found that GA3 applied in combination with BAP shows the best development results in meristem culture of Castilla blackberry (*R. glaucus*), resulting in increased production of leaves and propagule for mass multiplication. Marulanda et al. (2000) used a medium with BAP (1 mg/l) and AG3 (1mg/l) for in vitro *R. glaucus* plants. They obtained explants with good development and an average of three buds on each, which were successfully transferred to multiplication medium. The above statements confirm one more time that the types of plant growth regulators, as well as their combination in a culture medium, are necessary for obtaining viable seedlings in the initiation process of micropropagation.

Table 4. Effect of culture media on length and number of leaves in the establishment phase of apex meristems of thornless blackberry, after 6 weeks.

Culture media	Average growth		Phenolization		Surviving
	Lenght (cm)	Leaves (no.)	(+)	(-)	percentage
M1 (6BAP 1 mg/lt; GA3 0.5 mg/lt)	1.21 a*	5.58 a	16.6%	83.4%	83.4
M2 (6BAP 1 mg/lt; IAA 0.5 mg/lt)	0.57 b	2.45 b	6.6%	93.4%	66.6

* Mean with different letters indicates significant differences according to Fisher test (P < 0.05).

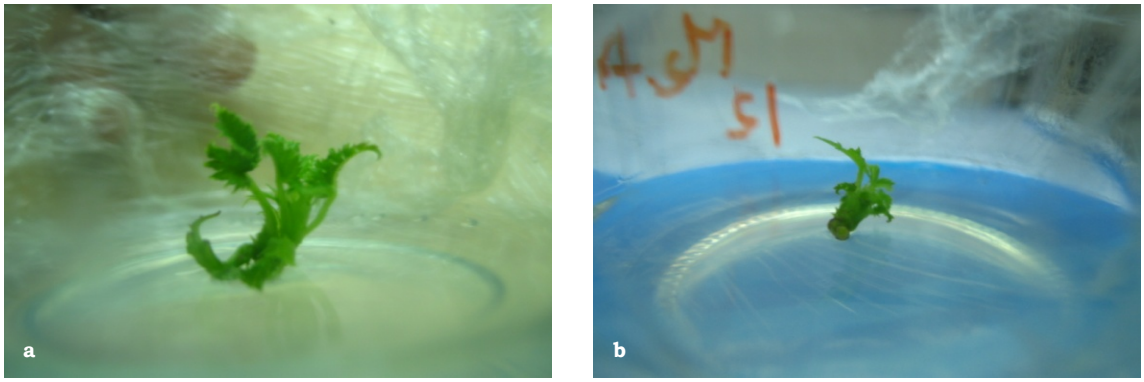

Figure 1. Blackberry thornless seedlings of 6 weeks growth. (a) Seedling developed in M1. (b) Seedling developed in M2.

In both studied culture media, the apex meristems were developed by the presence of cytokinin 6BAP, an hormone that induces cell division. However, the greatest elongation in M1 medium was due to the presence of AG3. Physiologically, gibberellins affect cell elongation, mainly in internodes and increase the production of endogenous auxin. According to Torres *et al.* (1996), the optimal concentration range is 0.1-1 ppm. Since the growth of *R. glaucus* is mainly produced in the internodes, GA3 was the most suitable for growing seedlings with indoleacetic acid (IAA). The combination of 6BAP (1 mg/l) with a non-toxic concentration of GA3 (0.5 mg/l) produced seedlings with higher and better leaf development (Figure 2).

Phenolization phenomenon occurred in a very low percentage for both culture media (Table 4). This is explained by the use of antioxidants (ascorbic acid and L-cysteine) in the rinsing and culture medium, and by the location of the explant. Since the apical meristem is protected by the foliar primordia and it is not directly exposed to a disinfectant, the extraction procedure does not involve many cuts that may facilitate the release of phenolic compounds. Azofeifa (2009) pointed out that oxidation processes are mainly caused by the abrasive effect of the disinfectant applied during aseptic procedure of the explant, cuts in the explant, and composition of the culture medium, highlighting as the major antioxidants ascorbic acid and L-cysteine.

Multiplication phase

After 4 weeks of growth, the seedlings generated from M1 medium reached higher lengths and more number of outbreaks than the M3 and M4 mediums (P <0.01) (Table 5). The best results in M1 medium were due to the hormonal combination of 6BAP + AG3, in contrast to the mediums M3 and M4. The M3 and M4 medium contain 6BAP, besides the IAA hormone from the auxin group for M3

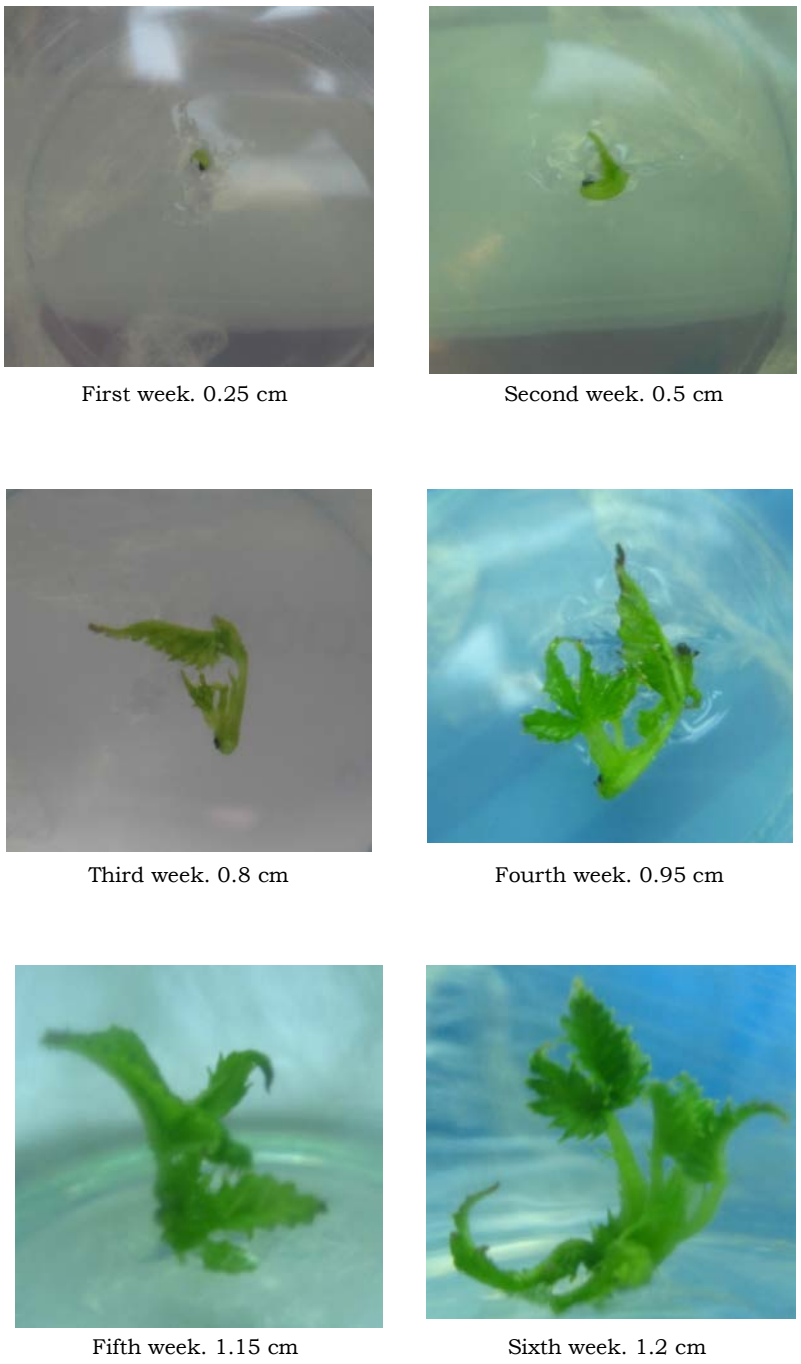

Figure 2. Apical meristem growth evolution in culture medium M1. The most appropriate for seedling establishment phase of thornless blackberry.

medium, and indolebutyric acid (IBA) for M4 medium. When Villa et al. (2005) evaluated the effect of different concentrations of 6BAP on the in vitro multiplication of blackberry (Rubus sp.), they found the highest number of shoots (3.99) in the culture medium with 1 mg/l of BAP. They concluded that this growth regulator is not responsible for stem elongation. On the other hand Najaf-Abadi and Hamidoghli (2009), in studies of micropropagation of thornless blackberry, found a maximum height of 5.87 cm in a cul-

Table 5. Effect of culture media on the multiplication phase of microcuttings of thornless blackberry after 4 weeks.

Culture media	Average growth lenght (cm)	Multiplication coefficient (no. of buds)
M1 (6BAP 1 mg/lt; GA3 0.5 mg/lt)	1.95 a*	7.5
M3 (6BAP 1.5 mg/lt; IAA 0.75 mg/lt)	0.78 b	1.1
M4 (6BAP 1.5 mg/lt; IBA 0.75 mg/lt)	0.83 b	1.1

* Mean with different letters indicate significant differences according to Fisher test (($P < 0.05$).

ture medium with 2 mg/l of BA and 0.5 mg/l of GA3, but with a fewer sprouts.

Culture medium M3 and M4 showed no satisfactory results, as indicated by the low coefficients of multiplication and seedling length, basically by hormone combinations of cytokinin + auxin. Erig et al. (2002), when assessed 6BAP and AIB from in vitro multiplication of raspberry (*R. idaeus*), observed a reduction of stem length in culture media with increasing levels of BAP and varying concentrations of AIB. The use of AIB is toxic at high levels of cytokinin, which is manifested by the lack of elongation and rosette-shaped growth of the seedlings. These characteristics were evident in the M3 and M4 media culture of the present study (Figure 3).

During multiplication phase, all culture media formed callus, but with different intensities. Most seedlings from the media M3 and M4 developed calluses (81% and 84%, respectively). However, the callus population was very low in the media M1 (10%), proving once again that the hormonal combination in M1 is correct. Marulanda et al. (2000) worked with two culture media compounds: 1.5 mg/l BA + 1.5 mg/l GA3, and 2 mg/l BA + 2 mg/l GA3. They found that increasing of BA cytokinin dose significantly increases callus production. This phenomenon is not desirable in a mass micropropagation process because it increases the risk of somaclonal variants (Perez et al., 1998). The obtained results show that it is possible to decrease the concentration of BA until 1 mg/l to reduce callus formation, maintaining good multiplication coefficients, and acceptable seedling length.

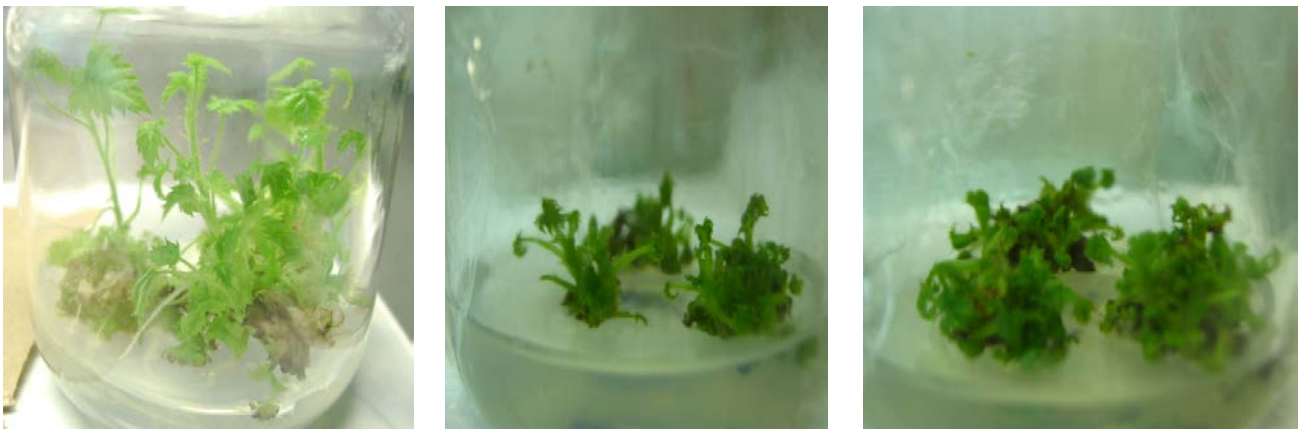

Figure 3. Growth of thornless *R. glaucus* on the evaluated media. From left to right: M1, M3, M4 growth respectively.

Conclusions

- The apical meristem is effective as a primary explant to initiate a process of micropropagation of thornless blackberry. Its cell division allows a rapid growth, only if the explant is in the appropriate culture medium. It also facilitates obtaining plantlets that are free of endogenous contaminants for multiplication phase.
- Disinfection with soap solution (water + commercial detergent), five minutes; 70% ethanol, two minutes; and 3% sodium hypochlorite (5 or 10 minutes), allows 100% disinfection of apex meristems.
- For establishment and multiplication phases, the best culture medium was M1 (6 benzylaminopurine 6BAP 1 mg/l and gibberellic acid GA3 0.5 mg/l). This allows a progressive increase of length, leaf development and number of buds.
- For the micropropagation process of *R. glaucus* thornless, the hormone combination cytokinin/gibberellin performs significantly better than the combination cytokinin/auxin.
- The studied methodology is appropriate and promising for obtaining thornless blackberry seedlings by micropropagation.

References

Azofeifa, Á. 2009. Problemas de oxidación y oscurecimiento de explantes cultivados In vitro. Agron. Mesoam. 20(1):153 - 175.

Barrero, L. 2009. Caracterización, evaluación y producción de material limpio de mora con alto valor agregado. Corporación Colombiana de Investigación Agropecuaria (Corpoica). Regional 1. Cundinamarca. p. 5.

Ballesta, *M. 2008*. Establecimiento y multiplicación in vitro de mora de castilla (*Rubus glaucus*) en el Laboratorio de Biotecnología Vegetal de la Universidad Francisco de Paula Santander. Tesis de grado. Universidad Francisco de Paula Santander. Cúcuta, Norte de Santander. 75 p.

Bernal, J. and Díaz, C. 2006. Materiales locales y mejorados de tomate árbol, mora y lulo sembrados por los agricultores y cultivares, disponibles para su evaluación en Colombia. Corporación Colombiana de Investigación Agropecuaria (Corpoica)-La Selva. Bol. Div. no. 7. p. 10 - 13.

Erig, A; Rossi, A.; and Luces, G. 2002. 6-benzilaminopurina e ácido indolbutírico na multiplicação in vitro da amoreira preta (*Rubus idaeus* L.) cv. Tupy. Ciência Rural 32(5):765 - 770.

Marulanda, M.; Carvajalino, M.; and Vento H. 2000. Establecimiento y multiplicación in vitro de plantas seleccionadas de *Rubus glaucus* Benth. para el departamento de Risaralda (Colombia). Actual. Biol. 22(73):121 - 129.

Muñoz, I. and Reyes, H. 2006. Efecto de reguladores de crecimiento, L-cisteína y ácido ascórbico en el cultivo in vitro de mora de Castilla (*Rubus glaucus* Benth.). Tesis de grado. Universidad Nacional Agraria, Managua, Nicaragua. 27 p.

Najaf-Abadi, J. and Hamidoghli, Y. 2009. Micropropagation of thornless trailing blackberry (*Rubus* sp.) by axillary bud explants. Aust. J. Crop Sci. 3(4):191 - 194.

Pérez, J.; Alvarado, Y.; Gómez, R.; Jiménez, E.; y Orellana., P. 1998. Propagación y mejora genética de plantas por biotecnología. Santa Clara, Cuba. Instituto de biotecnología de las plantas. p. 105 - 116.

Statgraphics Centurion. 2009. Versión XVI. StatPoint Technologies, Inc. Warrenton, U.S.A.

Tafur, R.; Toro, J.; Perfetti, J.; Ruiz, D.; and Morales, J. 2006. Plan Frutícola Nacional (PFN). Ministerio de Agricultura y Desarrollo Rural, Fondo Nacional de Fomento Hortofrutícola, Asohofrucol, SAG. 43 p.

Torres, A. C.; Teixeira, A.; and Campos, M. 1996. Medio de cultivo. Centro Brasileiro Argentino de Biotecnología. Brasilia. 21 p.

Villa, F.; Gomes, A.; Salles, L.; and Pasqual, M. 2005. Multiplicação In vitro da amoreira-preta 'Ebano' em diferentes concentrações de meio MS e BAP. Ciência e Agrotecnologia 29(3):582 - 589.