RESEARCH ARTICLE

Propagación *in vitro* de materiales seleccionados de *Rubus glaucus* Benth (mora de Castilla) en la provincia de Pamplona, región nororiental de Colombia

Propagation *in vitro* of selected materials of *Rubus glaucus* Benth (mora de Castilla) in the province of Pamplona, northeastern Colombia

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Resumen

En este estudio los materiales fueron seleccionados en 53 fincas pertenecientes a cuatro asociaciones de cultivadores de mora en los municipios de Pamplona y Chitagá (Norte de Santander, Colombia). Se emplearon como explantes segmentos nodales, los cuales permitieron obtener en poco tiempo brotes adventicios adecuados para la multiplicación masiva. Para la etapa de establecimiento se empleó el medio de cultivo Murashige y Skoog, (MS 1962), suplementado con ácido giberélico (GA_3) (0.0-0.1 mg/L) y 6-benzil aminopurina (BAP) (0,0-2,0 mg/L), en la etapa de multiplicación MS ,suplementado con GA_3 (0,0-0,03 mg/L^{-1}) y BAP (0,0-2,5 mg/L) y para la etapa de enraizamiento MS, suplementados con ácido indolbutírico (AIB) (0,0-1,0 mg/L). A los datos generados en las tres etapas, se les aplicó un diseño experimental de bloques completos al azar y se analizaron estadísticamente los promedios de los tratamientos mediante una prueba de Tukey. Los resultados alcanzados indicaron tasas promedio de contaminación (16,5-49,7 %) multiplicación (3,8-4,3 brotes/explante) y enraizamiento (3,3-4,3 raíces/planta) *in vitro* para los diferentes materiales seleccionados y evaluados. Estos resultados, logrados por primera vez en la región Nororiental de Colombia, son importantes por cuanto se contara con materiales seleccionados disponibles para los cultivadores de mora de la región.

Palabras clave: Mora de los Andes, micropropagación, asociaciones de cultivadores, 6-benzil aminopurina.

Abstract

In this study plant materials were selected in 53 farms belonging to four growers associations of blackberry in the municipalities of Pamplona and Chitagá (North of de Santander, Colombia). Nodal segments were used as initial explants of *R. glaucus*. For the establishment stage Murashige and Skoog, 1962 (MS) media was used and, supplemented with of gibberellic acid (GA_3) (0.0 -0.1 mg/L) and 6-aminopurine (BAP) (0.0 -2.0 mg/L); for the multiplication stage MS was supplemented with GA3 (0.0 -0.03 mg/L) and BAP, (0.0 -2.5 mg/L) and for the rooting stage MS was supplemented with acid indolbutirico (0.0 -1.0 mg/L). From the data generated during the three stages, an experimental design of incomplete blocks was randomly applied and the treatments averages were statistically analyzed using the Tukey Test. The results indicated average rates of contamination (16.5-49.7 %), multiplication (3.8-4.3 shoots/explant) and *in vitro* rooting (3.3-4.3 roots/plant) for the different evaluated materials. These results, achieved first in the Northeastern region of Colombia, are important in that they will feature selected materials available for blackberry growers in the region.

Key words: Andean blackberry, micropropagation, growers associations, 6-aminopurine.

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Introduction

The *Rubus* genus is one of the most diverse in morphological and genetic terms. It presents a wide spectrum of wild species and the farmed species are popular for their edible fruits (Jennings, 1988). The fruits are eaten fresh or processed and there is currently great interest in them for their medicinal properties due to their high antioxidant activity (Seeram *et al.* 2006, Cusba Mejia 2011).

The *Rubus* species that is currently farmed on a massive scale in South America is *R. glaucus* Benth. This species is widely distributed around the country from the department of Putumayo to the Magdalena Valley, and it is grown at altitudes between 2,000 and 3,200 m.a.s.l. According to data from the Ministry of Agriculture and Rural Development and the National Administrative Department of Statistics (DANE, for the Spanish original) in November 2013, Colombia had 7,007 planted hectares, out of which, 4,922 were at the age of production, and there was an annual production of 73,856 tons. Additionally, the departments of Cundinamarca, Santander, Norte de Santander and Antioquia have the greatest percentages in area and production (DANE, 2013).

Despite the great potential of blackberry on the international market, it is propagated in Colombia using plant material that is not considered to be elite. Producers and nurseries propagate the regional materials without physiological or sanitary standards, and they do not ensure security of the material's genetic identity (Castro & Díaz, 2001; Castro, Díaz, Montoya & Ríos, 2006).

In this context, the *in vitro* cultivation or micropropagation of plant tissue is a practical for overcoming these limitations. Additionally, it is a quick technique for fruit species and it can produce genetically identical *Rubus* plants free from viruses on a massive scale and in a reduced space without being affected by environmental variations (Zimmerman, 1991; Sobczykiewicz, 1992).

Several *in vitro* culture studies have been published around the world on different species of *Rubus*. The first work of *in vitro* propagation of *Rubus* was carried out by Broome and Zimmerman (1978) in the *thornless* and *hardy* blackberry cultivars. Subsequently, research has focused on the factors that influence *in vitro* response, such as disinfection of the explants and low rates of propagation (McPheeters, Skirvin & Hall 1988; Tian, Duan & Wang, 2005; Wu, Miller, Hall & Mooney, 2009; Clark & Finn, 2011). Additionally, experiments that focus on the micropropagation of new and existing cultivars are of note (Donnelly & Daubeny 1986; Bobrowski, Mello Farias & Peters, 1996; González *et al*. 2000; Zawadzka & Orlikowska, 2006). In Colombia, Marulanda and Márquez (2002) obtained plants originating from the coffee-growing region of Colombia by organogenesis. While Castro and Díaz (2001) have developed methodologies for the micropropagation of blackberry from the selected material. Additionally, Valderrama *et al.* (2009) published the *in vitro* arrangement and validation of blackberry seedlings, as well as *ex vitro* management for delivery to farmers of Silvania, Cundinamarca. The research of Sigarro and García (2011) is also notable, who evaluated micropropagation of thornless *R. glaucus*. However, despite the different research over the years, a large number of cultivars are still resistant to tissue culture. The critical factors that influence *in vitro* response include disinfection of the explant and the low rate of propagation (Daubeny, 1986, McPheeters *et al*. 1988; Donnelly and Daubeny, 1986, and Bobrowski *et al*. 1996; González, López, Valdés and Ordas, 2000; Tian *et al*. 2005; Zawadzka and Orlikowska, 2006; Clark and Finn, 2011).

Therefore, this study aimed to evaluate the micropropagation of selected materials of *R. glaucus* from eight commercial farms in the municipalities of Pamplona and Chitagá, which had been agronomically as well as molecularly characterized (Cancino, Quevedo, Villamizar, Sánchez and Díaz, 2014).

Materials and Methods

Plant Material

Field trips were made between March 2009 and November 2011 for the collection of samples of *R. glaucus* in 53 farms of four blackberry farmers' associations of the municipalities of Pamplona and Chitagá. According to the results of the surveys on the farmers and agronomical characteristics, eight farms were selected in which the best materials of the four associations were found (Figure 1, Table 2). One material was selected per farm for a total of eight materials for the micropropagation evaluation. These materials had previously been agronomically and molecularly characterized (Cancino *et al.* 2011, 2014). The farms are located in 14 rural areas of the municipalities of Pamplona and Chitagá between 7°,7′,60′′ and 7°,28′,15′′ north and 72°,35′,16′′ and 72°,42′,43′′ west (Figure 1). All of the farms own commercial crops of Andean blackberry with thorns (WT) and thornless (TL) *R. glaucus* surrounded by secondary forests and shrubwoods between 2,070 and 2,860 m.a.s.l.

In Vitro Establishment

Field visits were made again to each property for the *in vitro* establishment of explants of the selected material in the different farms. Stem segments were collected from young branches measuring 50 cm in length, they were wrapped in newspaper and taken to the *in vitro* plant culture laboratory of the Universidad de Pamplona. The branches were cut until achieving nodal segments (micro-cuttings), 3-5 cm long, with approximately one axillary bud. The explants were washed in a soapy solution. Then, they were subjected to two

treatments of surface disinfection (Table 1). After the surface disinfection periods, the explants were rinsed five times with sterile distilled water and placed in a solution of 150 mg/l of citric acid to control oxidation while proceeding to planting. The disinfected explants were cut again until a two-centimeter-long explant was obtained (micro-cutting with axillary bud). Each explant was placed in a M&S salts (Murashige and Skoog, 1962) establishment medium supplemented with concentrations of gibberellic acid (GA_3) and 6-benzylaminopurine (BAP). At the end of one week, the meristematic apexes that emerged from each micro-cutting free from any contamination were taken and transferred to the fresh medium in order to ensure quick establishment of the explants, and 30% sucrose. The media with an adjusted pH of 5.8 were sterilized in an autoclave for 15 minutes at 15 pounds of pressure and at 121 °C. Bacteriological agar at 7% was used for solidification of the media. Subsequently, after 20 days of incubation at a temperature of 20 ± 2 °C, a photoperiod of 16 hours of light / 8 hours of darkness, and a light intensity of 2,000 lux, the contaminated explants were discarded and the uncontaminated explants were left for 20 more days. The explants were established under strict conditions of asepsis using a previously conditioned laminar flow cabinet. The explants were established in jelly jars with 20 ml of semi-sold culture medium.

Table 1. Evaluated surface disinfection treatments.

Component	T1	T2
	Minutes (m) Seconds (s)	
Isodine® foam 2%.	10 m	10 m
Mertec fungicide® 0.4%	30 m	60 m
Alcohol 70%	30 s	60 s
Sodium hypochlorite 2% Sodium hypochlorite 3%	10 m	 10 m

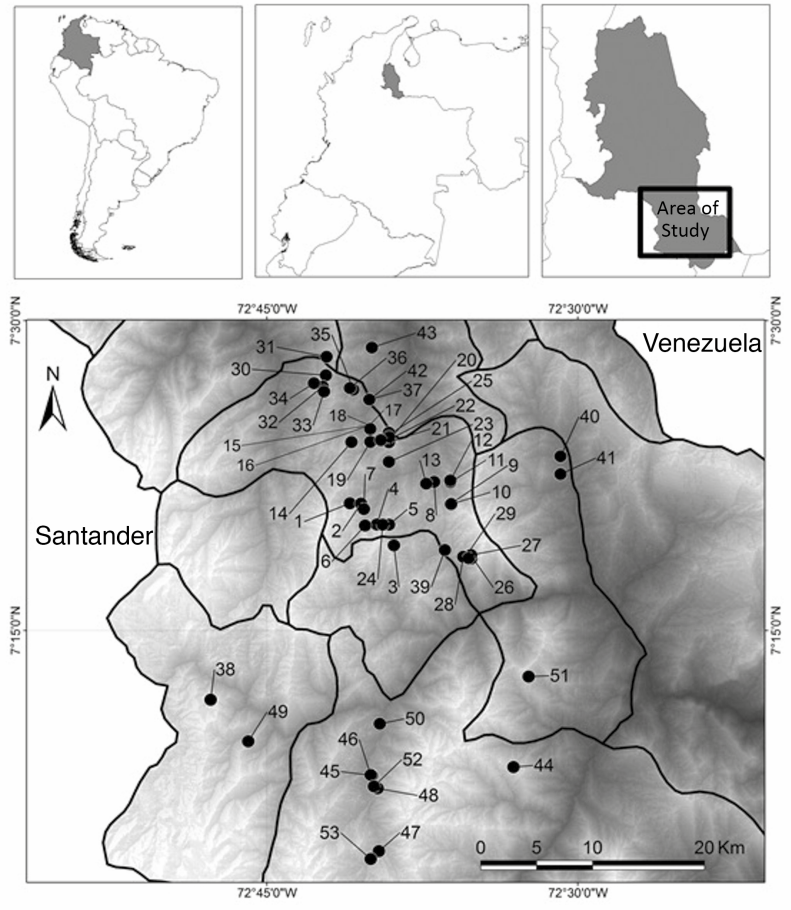

Figure 1. Area of study. Municipalities of Pamplona and Chitagá, Norte de Santander, Colombia. Location of the farms (53) where the samples of *R. glaucus* Benth were collected.

Table 2. Materials selected from the eight farms of this study in the municipality of Pamplona.

Number	Code	Farm	Rural Area	m.a.s.l.[3]
1	PCHA03 SE[1]	Arrayan	Chíchira	2,328
2	PRH305 SE	Higuerón	El Rosal	2,745
3	PCV3016 SE	La Victoria	Cimitarígua	2,585
4	PSS SE	Salado	San Francisco	2,293
5	PSAG002 CE[2]	La Gonzalera	Sabaneta alta	2,585
6	PSAG010CE	La Gonzalera	Sabaneta alta	2,585
7	PSAG011CE	La Gonzalera	Sabaneta alta	2 585
8	PCV102CE	La Victoria	Cimitarígua	2,585

[1] TL. Thornless *R. glaucus* Benth[2]. WT. *R.glaucus Benth with thorns.*[3] *m.a.s.l.: meters* above sea level.

In Vitro Multiplication

The healthy explants free from contaminants were divided and transferred to the M&S multiplication medium, and supplemented with concentrations of GA_3 and BAP, (Table 3, media: M_2, M_3, M_5 and M_6), and 30% sucrose. After four weeks of growth, the new explants were divided and transferred to a fresh multiplication medium until obtaining the number of explants for each evaluated genotype.

In Vitro Pretransplant

The multiplied explants were divided and planted in the M&S pretransplant medium supplemented with Indole-3-butyric acid (Table 3, media: M_8 and M_9) and 30% sucrose. The explants were incubated for four weeks under the same temperature, photoperiod and light intensity conditions established in the establishment and multiplication stages.

Experimental Design and Data Analysis

A design of completely random blocks was carried out with a factorial arrangement of 9 x 3. This arrangement meant there were nine media with three hormone concentrations (in each stage) in M&S salts for each one of the different materials of *R. glaucus* Benth evaluated for a total of 27 treatments. Each treatment had five

Table 3. Effect of BAP, GA_3 and IBA on the *in vitro* propagation stages of selected materials of *R. glaucus* Benth.

Stages	Growth Regulators		
	Benzylaminopurine	Gibberellic Acid	Indole-3-butyricAcid
Establishment	**(BAP mg/l)**	**(GA_3 mg/l)**	**(IBA mg/L)**
M_1	0.0	0.0	0.0
M_2	1.0	0.5	0.0
M_3	2.0	1.0	0.0
Multiplication			
M_4	0.0	0.0	0.0
M_5	2.5	0.03	0.0
M_6	2.5	0.05	0.0
Pretransplant			
M_7	0.0	0.0	0.0
M_8	0.0	0.0	1.0
M_9	0.0	0.0	2.0

repetitions where each replica was represented by a culture flask with an explant. An analysis of variance (ANOVA) was carried out, followed by a Tukey's range test with a level of significance of 5% to determine which of the treatments presented significant statistical differences. The data were collected over eight weeks. The statistical program SPSS Version 19 was used. Statistical analyses were carried out on the data to obtain the average value and dispersion values. Additionally, an analysis of variance was conducted and in the event of statistical differences between locations, Tukey's range test was carried out. The statistical model to carry out the ANOVA was completely at random with an embedded or hierarchical effect (Dytham, 2012).

Results and Discussion

Effect of the Disinfection Treatments on Materials from the Eight Farms Evaluated in the Study

Significant differences occurred at a level of 5% ($p < 0.05$) between the T_1 and T_2 disinfection treatments (Figure 2). Greater contamination was observed in the T_1 in the materials of the eight farms evaluated. This was because the explants were exposed for shorter times to the disinfectant agents than with T_2. However, there was greater oxidation in T_2. Furthermore, it was observed that the genotypes of thornless *R. glaucus* presented lower percentages of contamination than the genotypes with thorns. It is possible that the presence of thorns facilitates that different types of contaminants remain on the explants, increasing surface contamination. The results are different to those observed by Valderrama *et al*. (2009), who recorded percentages between 28 and 57% in thornless *R. glaucus*. In this context, it is important to indicate that the selection and concentration of the disinfectants and the time of disinfection are largely determined by the characteristics of the explant and they are established experimentally by trial and error (Pedraza-Manrique, 2008). The percentage of contamination and therefore, the survival of the explants is strongly influenced by several factors, such as the time of year, the physiological state of the plant, the environmental conditions and the morphology of the explant. In this study, the materials were collected in the dry season (December-March and June-August), which promoted the low rates of contamination. While the collection of explants by Valderrama, Álvarez, Barrero, Robayo and Nuñez (2009) was carried out in different periods of the year, which possibly resulted in greater levels of contamination in these researchers' study materials. It is important to indicate that the use of explants from plants maintained in greenhouse conditions is the most recommendable for *in vitro* establishment of plants, which must be disinfected with fungicides and bactericides. However, for this study, it was not possible to have plants kept in greenhouse conditions. Regarding the phenolization or oxidation of the explants with the two disinfection treatments in the micro-cuttings, there was greater oxidation in all the materials with the T_2 treatment compared to the T_1.treatment. It is important to highlight that the subculture of the micro-cuttings' adventitious buds after one week of cultivation allowed the reduction of endogenous contamination to a minimum level without the need to use strong procedures for the disinfection of plant material.

Effect of the Culture Media: M_1, M_2 and$_3$ on *the Length* and Number of Leaves in the Establishment Stage of Meristematic Buds of R. Glaucus Benth

The effect of the culture media on the development of the explants during the establishment stage in the eight materials is observed in Table 4. Significant differences ($p < 0.05$) were found in the number of leaves between culture media, observing greater growth and development of meristematic apexes in M3 medium,

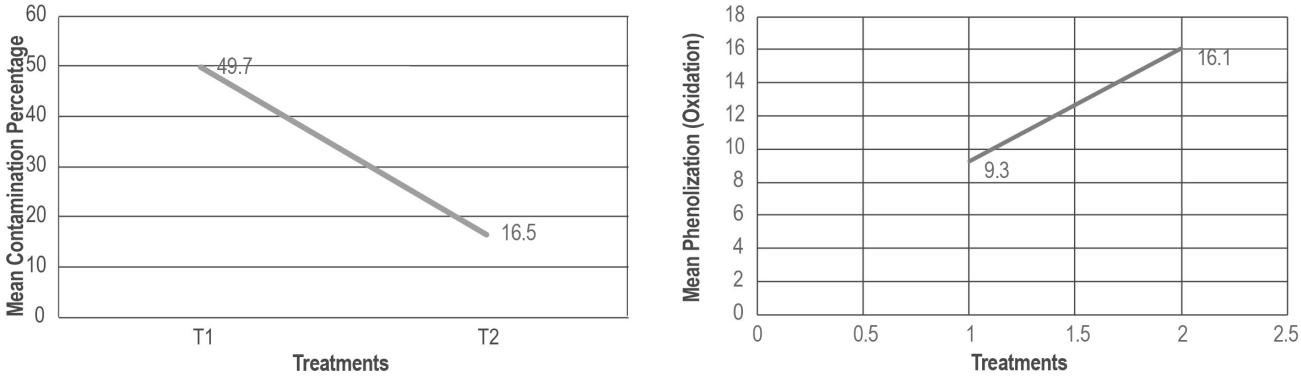

Figure 2. Effect of the disinfection treatments on the *R. glaucus* Benth materials.

which allows greater formation of seedlings prior to the start of the multiplication stage. Several authors (Muñoz and Reyes, 2006, Sigarroa and García, 2011) have observed that the use of GA_3 in the cultivation of *R. glaucus* Benth meristems presents the best development when it is applied in combination with BAP. The number of leaves as well as the growth in length were high compared to the other treatments (17.8 leaves and 1.41 cm, respectively) (Table 4).

Table 4. Tukey's range test on the effect of the culture media on the number of leaves in the establishment stage in meristematic buds of *R. glaucus* Benth of eight evaluated materials.

Treatment	No. of Leaves		Survival Percentage
M_3	14,538	a	82.2
M_2	8,375	b	71.3
M_1	2,975	c	65.3

Values followed by a different letter are significantly different with $p < 0.05$, using Tukey's range test.

Multiplication of Shoots

After four weeks from establishment of the explants in the multiplication media, plants were obtained in the M_5 medium, in which greater lengths and higher numbers of shoots were achieved than in the M_4 and M_6 media ($p < 0.05$) (Table 5). Significant differences were found between the treatments ($p < 0.05$) for the variables of average growth and number of shoots (Table 5). Better results were observed in M_5 due to a lower concentration of GA_3, and an equal concentration of BAP in M_6. It is important to highlight that the number of shoots obtained in all the evaluated materials was less than that found by Sigarroa and García (2011) (7.5), Villa, Gómez, Salles and Pascual (2009) (3.99), and Castro and Díaz 2001 (4.7). In the case of this research, it is evident that the large diversity of TL and WT *R. glaucus* Benth presents a very heterogeneous response in the variable of the number of shoots. The difference in the number of shoots obtained may be the result of the differences in the different evaluated genotypes.

In Vitro Pretransplant

The shoots were divided and planted in the pretransplant medium and remained there for four weeks until the development and formation of roots. The best results were observed in the M_9 medium. The average number of roots in this medium for the different evaluated materials was very homogeneous, observing 4.3 to 3.3 roots per plant by four weeks of cultivation (Table 6). Similarly, the growth in root length was between 4.9 and 2.1 cm. These results are similar to those obtained by Muñoz and Reyes (2006) in which the effect of IBA on root formation was better than the other regulators such as indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid (NAA) for this stage. The results obtained in this study are similar to those described by Castro *et al*. (2006), who conducted pretransplant studies on different species of *Rubus* under the application of indole-3-butyric acid (IBA) in different concentrations, finding that concentrations of IBA between 1 and 3 mg/l induce a rooting percentage of 100%. Similarly, Muñoz and Reyes (2006) indicate a similar response to the previous study, because with 1.0 mg/l of IBA, plants were obtained with a greater number of roots and 100% rooted plants.

Conclusions

The use of micro-cuttings with an axillary bud is an efficient methodology to start a micropropagation process (Figure 3) in different genotypes of *R. glaucus* Benth, facilitating the production of seedlings free from contaminants for the following stages of the micropropagation process. The best results observed for the establishment and multiplication stages of the eight evaluated genotypes of *R. glaucus* Benth were from M_3 (2.0 mg/l 6 benzylaminopurine 6BAP, 1.0 mg/l gibberellic acid GA_3) and M_5 (2.5 mg/l 6BAP, 0.03 mg/l GA_3), respectively, progressively facilitating the length, leaf development and the number of shoots. The application of 2.0 mg/l indole-3-butyric acid (IBA), facilitates root development in the different genotypes of *R. glaucus* Benth evaluated. The eight evaluated genotypes adequately responded to the methodology developed in this study, which makes it promising and appropriate for the mass propagation of these materials in the regions of Pamplona and Chitagá (Norte de Santander, Colombia).

Table 5. Tukey's range test on the effect of the culture media on the average growth and the number of shoots in the multiplication stage in meristematic buds of *R. glaucus* Benth of eight evaluated materials.

Treatment	Average Growth		Culture Medium	Number of Shoots		Survival Percentage
M5	2.156	a	M5	1.800	a	74.3
M4	1.188	b	M6	1.513	b	69.3
M6	1.075	b	M4	0.813	b	63.6

Values followed by a different letter are significantly different with $p < 0.05$, using Tukey's range test.

Table 6. Tukey's range test on the culture media M_7, M_8 and M_9 in the pretransplant stage of nodal segments of *R. glaucus* in materials from eight farms.

Culture Medium	Root Length		Culture Medium	Number of Roots / Plant		Survival Percentage
M_9	3.675	a	M_9	3.713	a	81.3
M_7	2.313	b	M_7	2.263	b	74.4
M_8	1.813	b	M_8	1.800	b	71.5

Values followed by a different letter are significantly different with $p < 0.05$, using Tukey's range test.

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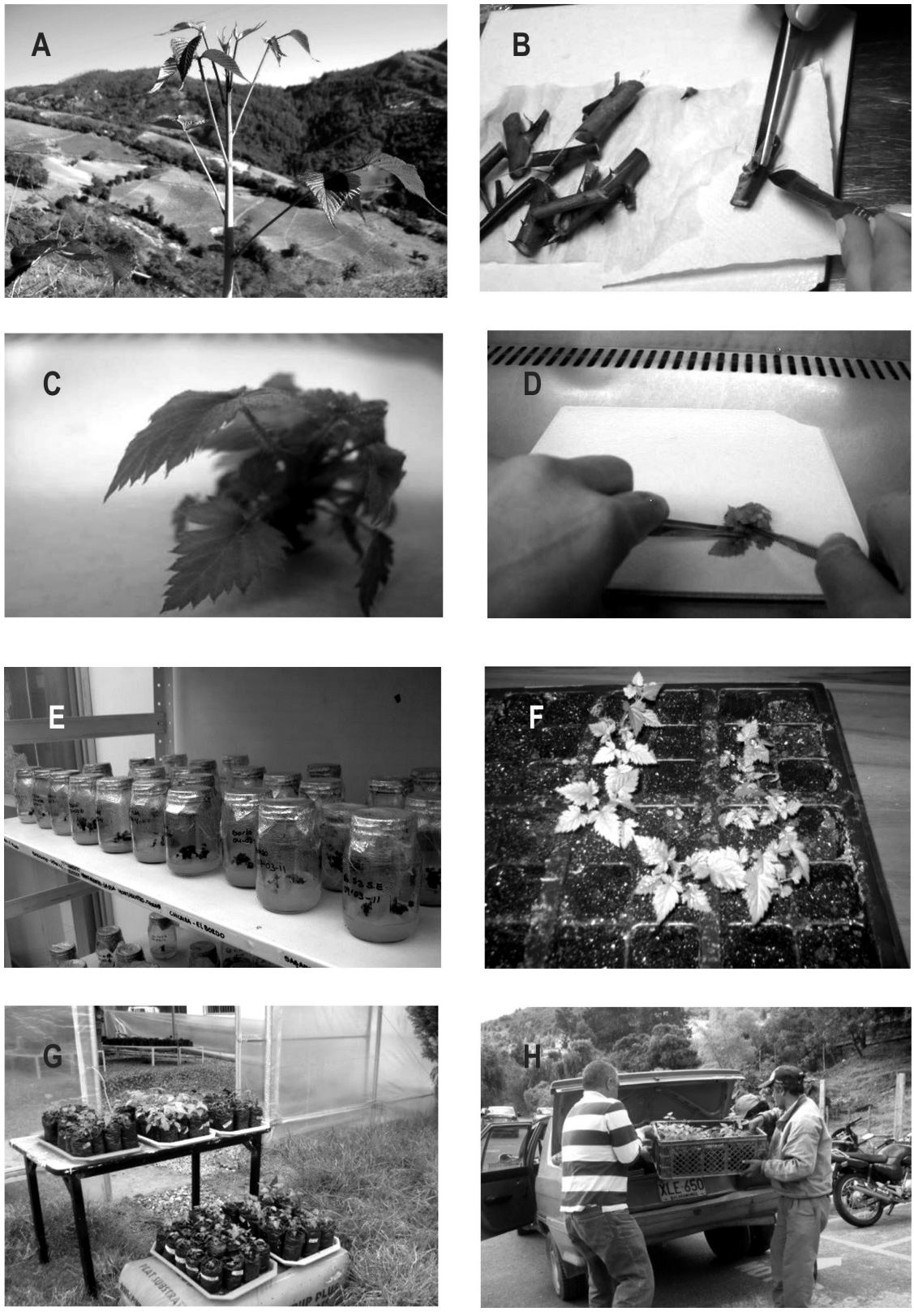

Figure 3. Micropropagation process of selected materials of *Rubus glaucus* Benth. **A.** Stem segments of selected plants. **B.** Cutting of the stem segments to propagate in the laboratory. **C.** Shoots obtained four weeks after planting. **D.** Cutting and selection of shoots. **E.** Development and elongation of *in vitro* shoots. **F.** Establishment in ***ex vitro*** conditions. **G.** Greenhouse conditioned materials. H. Delivery of micropropagated materials to the farmers.

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