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Protocol for the reduction of costs in habanero chili (*Capsicum chinense* Jacq.) micropropagation

Protocolo para la reducción de costos en la micropropagación de chile habanero (*Capsicum chinense* Jacq.)

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Abstract. An alternative method for improving the production of habanero chili is the tissue culture technique; however, the gelling agent, the high salt and sucrose concentrations used in the culture media raise production costs and limit the adaptation of the regenerated plants to greenhouse or field conditions. In this study, the effect of the substrates perlite-coconut fiber, coconut fiber-volcanic rock, vermiculite-perlite, and perlite-volcanic rock in conjunction with various culture media in *in vitro* plant regeneration from embryos was evaluated. The differentiation of adventitious shoots on substrates was scarcely observed or non-existent. Inducing the formation of shoots on agar and their development and rooting on substrates allowed to obtain plants. The greatest number of shoots per explant was observed on media with Murashige and Skoog salts (MS) 100%, 15 or 30 g/L sucrose, 4 mg/L benzylaminopurine, 0.3 mg/L indolacetic acid, and vermiculite-perlite or agar. The highest rooting percentages were obtained for treatments that consisted of MS 50%, MS 100% or Arnon and Hoagland salts (H), 15 g/L sucrose, 1 mg/L indole-3-butyric acid, and perlite-volcanic rock, vermiculite-perlite or agar (88-90%). The highest survival rate, number of leaves, plant length, and stem diameter during acclimation were obtained with media containing MS 50%, MS 100% or H salts, 15 g/L sucrose, 1 mg/L indole-3-butyric acid, and vermiculite-perlite, perlite-volcanic-rock or agar. It is feasible to use culture media less concentrated and substrates for the micropropagation of the habanero chili. The protocol developed significantly reduced production costs.

Keywords: *In vitro* plant regeneration; Habanero chili; Substrates; Culture medium; Arnon and Hoagland Salts; Costs.

Resumen. Un método alternativo para mejorar la producción de chile habanero es la técnica de cultivo de tejidos; sin embargo, el agente gelificante, los niveles altos de sales y concentraciones de sacarosa utilizados en los medios de cultivo elevan los costos de producción y limitan la adaptación de las plantas regeneradas a las condiciones de invernadero o campo. En este estudio, se evaluó el efecto de los sustratos perlita-fibra de coco, fibra de coco-roca volcánica, vermiculita-perlita, y perlita-roca volcánica, en conjunción con diversos medios de cultivo, en la regeneración *in vitro* de plantas a partir de embriones. La diferenciación de brotes adventicios en sustratos fue escasa o nula. Inducir la formación de brotes en agar y su posterior desarrollo y enraizamiento en sustratos permitió obtener plantas. El mayor número de brotes por explante se observó en un medio con sales de Murashige y Skoog (MS) 100%, 15 o 30 g/L de sacarosa, 4 mg/L de bencilaminopurina, 0,3 mg/L de ácido indolacético, y vermiculita-perlita o agar. Los porcentajes de enraizamiento más altos se obtuvieron para los tratamientos que consistían de las sales MS 50%, MS 100% o sales de Arnon y Hoagland (H), 15 g/L de sacarosa, 1 mg/L ácido indol-3-butírico, y perlita-roca volcánica, vermiculita-perlita o agar (88-90%). La tasa de supervivencia más alta, número de hojas, longitud de la planta, y el diámetro del tallo durante la aclimatación se obtuvieron con los medios que contenían sales MS 50%, MS 100% o sales H, 15 g/L de sacarosa, 1 mg/L ácido indol-3-butírico, y vermiculita-perlita, perlita-roca volcánica o agar. Es factible el uso de medios de cultivo menos concentrados y sustratos para la micropropagación del chile habanero. El protocolo desarrollado redujo significativamente los costos de producción.

Palabras clave: Regeneración *in vitro* de plantas; Chile habanero; Medio de cultivo; Sales Arnon y Hoagland; Costos.

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INTRODUCTION

The habanero chili pepper (*Capsicum chinense* Jacq.) is one of the species of the genus *Capsicum* with the highest known degree of pungency. This feature is conferred by the production of compounds known as capsaicinoids, which are used in the food, pharmaceutical, and cosmetic industries (Nuez et al., 1996). In Mexico, the production of this vegetable does not satisfy the internal and external demand, mainly due to the low level of technology used for cultivation, as well as the poor quality of the seeds (Rincones, 2009).

Tissue culture has demonstrated effective results in propagating habanero chili (Santana-Buzzy et al., 2005; López-Puc et al., 2006; Zapata-Castillo et al., 2007; Sanatombi & Sharma, 2007; Valadez-Bustos et al., 2009; Kehie et al., 2012, 2013); however, the application of this technique has some limitations regarding the use of a gelling agent (agar) in the culture medium, which is not only costly but can also reduce the oxygen concentration, makes difficult the assimilation of nutrients by explants, and reduces survival rates in *ex vitro* conditions (Fujiwara et al., 1993; Ichimura & Oda, 1998). An alternative to the use of gelling agents is the use of substrates used in hydroponics, such as vermiculite, perlite, volcanic rock, coconut fiber, sand, peat moss, and sawdust. In this respect, Afreen-Zobayed et al. (2000) used vermiculite and paper pulp to root sweet potato microplants (*Ipomoea batatas* L. (Lam.), cv. Beniazuma) and obtained high percentages of survival during acclimation and high production in terms of dry weight.

On the other hand, it has been observed that some components of the culture medium, like the concentration of basal salts of the MS medium and sucrose concentration commonly used in tissue culture can be higher than that required for some species (Conner & Thomas, 1982; Lapeña et al., 1992; Nhut et al., 2006; González et al., 2012; Raya-Montaño et al., 2011). It has been reported that a high sucrose concentration in the culture medium can negatively affect the metabolism and photosynthesis of plants under *in vitro* conditions (Schaefer et al., 1992; Kozai et al., 1995; Kwa et al., 1995). Habanero chili adventitious shoots cultured on a culture medium with low sucrose and nutrients concentrations was shown to improve the plant's ability to root and survive under greenhouse conditions (Barrales-López et al., 2015). The aim of present study was to determine the effect of culture medium composition as well as hydroponic substrates on the *in vitro* plant regeneration and acclimation of the habanero chili pepper.

MATERIALS AND METHODS

Experiment 1 (E1)

Seed disinfestation. Habanero chili seeds cv. Orange (Green Seeds®, CA, USA) were submerged in a 1% fungicide solution (Daconil®, Química Industrial Agrícola, DF, México) for 5 min; they were later soaked in a sodium hypochlorite solution (v/v) with 1.8% active chlorine (Cloralex®, Alen, NL, Mexico) and 1.0 mL/L Tween 20 for 20 min and rinsed four times with sterilized distilled water.

Seed imbibition. Disinfested seeds were placed in 150 ml jars containing 30 mL of perlite-coconut fiber (PC), perlite-volcanic rock (PR), coconut fiber-volcanic rock (CR) (3:1, particle size 0.5 mm) or agar 7 g/L (Merck, Darmstadt, Germany) moistened with 30 mL of a culture medium with Murashige & Skoog (1962) (MS) 50% salts and 10 g/L sucrose. The pH of the culture medium was adjusted to 5.7 ± 0.1. Medium and substrates were sterilized by autoclaving at 1.05 kg/cm^2 for 20 min.

Adventitious shoot induction. Seeds embedded for five days were cut into two pieces and placed in jars containing 30 mL of substrate (PC, PR, CR or agar) moistened with 30 mL of induction medium, which contained MS basal salts, 30 g/L sucrose, 4 mg/L benzylaminopurine (BAP), 0.3 mg/L indoleacetic acid (IAA) and 2 mg/L $AgNO_3$, according to the protocol established by Valadez-Bustos et al. (2009). After four weeks, the percentage of explants with adventitious shoots and the percentage of surviving explants were evaluated. Plant growth regulators, salts, sucrose and $AgNO_3$ were supplied by Sigma Aldrich (St. Louis, MO, USA.).

Culture conditions. All cultures were incubated in a growth chamber at 25 ± 2 °C with a 16 h photoperiod and light intensity of 25 µmol/m^2/s provided by cool white fluorescent lamps (Philips, Amsterdam, Netherlands).

Experiment 2 (E2)

Adventitious shoot induction. Seeds were disinfested and embedded according to the procedure described in experiment 1. They were cut into two sections and placed in jars containing eight culture media which are shown in Table 1. All media were supplemented with 2 mg/L $AgNO_3$. The pH levels of media were adjusted to 5.7 ± 0.1 and gelled with 7 g/L of agar.

Elongation of adventitious shoots. The explants that formed adventitious shoots were transferred to jars containing 30 mL of the eight media described in Table 1 for shoot induction but with 1 mg/L gibberellic acid (GA_3), 4 mg/L $AgNO_3$ and agar 7 g/L (Valadez-Bustos et al., 2009). After four weeks, the explants were cultured in jars containing substrates PR, VP (vermiculite-perlite) (3:1, particle size 0.5 mm) or agar 7 g/L moistened with the same media. The number of shoots formed by explant and their dry weight were evaluated after 60 days.

Rooting. Shoots (2 cm long, approximately) were placed on the substrates PR, VP or agar moistened with 30 mL of the following media: E1: MS 100%, 15 g/L sucrose, 1mg/L indole-3-butyric acid (IBA); E2: MS 50%, 15 g/L sucrose, 1 mg/L IBA; E3: MS 25%, 15 g/L sucrose, 1 mg/L IBA, and E4: H, 15 g/L sucrose, 1 mg/L IBA, all supplemented with 2

mg/L $AgNO_3$. After one week, the shoots were subcultured to the same media without IBA. The percentage of rooting, shoot height, number of leaves, length and number of roots were evaluated after three weeks.

Acclimation. The roots of regenerated plants were washed with tap water to eliminate residues of the substrates or agar; next, they were submerged in a fungicide solution (Daconil 1 g/L) and transferred to 250 mL polystyrene containers with peat moss-perlite (3:1) previously saturated to field capacity. A polyethylene bag was placed over the containers and removed after three weeks. The plants were fertilized once every two weeks with Miracle-Grow© (Scotts Co., Marysville, OH, USA), fertilizing at 50% of the concentration recommended by the manufacturer. The plant length, number of leaves, stem diameter, and survival were evaluated after seven weeks.

Data analysis. For adventitious shoot induction in E1 a completely random arrangement with four treatments and five repetitions was used; a jar containing five explants was considered as the experimental unit. For elongation of adventitious shoots in E2, a 3 x 8 factorial arrangement (substrates and media) was used with a minimum of three repetitions; a jar with five explants was considered as the experimental unit. For rooting an unbalanced factorial arrangement 3 X 4 was used; a minimum of three repetitions were considered for each treatment, with a jar containing three shoots considered as the experimental unit. For acclimation in E2 a completely randomized unbalanced experimental design with 12 treatments was used. For the variables evaluated during acclimation, one plant was taken as the experimental unit, with a minimum of five repetitions for every treatment. The data obtained from each evaluation were analyzed using the Statistical Analysis System (SAS) and the Tukey test (P<0.05).

Table 1. Culture media for shoot induction from habanero chili explants (Experiment 2).
Tabla 1. Medios de cultivo para la inducción de brotes a partir de explantes de chile habanero (Experimento 2).

Medium	Salts (%)	Sucrose (g/L)	BAP (mg/L)	IAA (mg/L)
1	MS 100	30	4.0	0.3
2	MS 100	15	4.0	0.3
3	MS 50	15	4.0	0.3
4	MS 50	7.5	4.0	0.3
5	MS 25	7.5	4.0	0.3
6	MS 25	3.7	4.0	0.3
7	H 100	30	4.0	0.3
8	H100	15	4.0	0.3

MS: Murashige and Skoog salts; H: Arnon and Hoagland salts (1940); BAP: benzylaminopurine; IAA: indoleacetic acid.

RESULTS

Experiment 1

The substrates had a significant effect on the survival and the formation of adventitious shoots. The highest percentage of survival was observed on perlite-volcanic rock, agar and coconut fiber-volcanic rock (68-80%), and the lowest value was observed in the substrate perlite-coconut fiber (PC) (54%) (Table 2). Likewise, the explants cultured on coconut fiber-volcanic rock (CR) and perlite-coconut fiber (PC) substrates displayed a high extent of oxidation. The formation of shoots was scarce or null for all substrates evaluated (Table 2). Due to the small number of shoots regenerated in the explants cultured on substrates (particularly those containing coconut fiber) and their high extent of oxidation, in experiment 2 (E2) shoot induction and initial shoot development phases were carried out on media gelled with agar. The middle and final phase of shoot development and rooting were carried out on PR and VP substrates.

Table 2. Effect of different substrates on survival and percentage of explants of habanero chili cv. Orange that formed adventitious shoots (Experiment 1).
Tabla 2. Efecto de diferentes sustratos en la supervivencia y porcentaje de explantes de chile habanero cv. Naranja que formaron brotes adventicios (Experimento 1).

Substrate	Survival (%)	Explants with shoots (%)
Agar	80.0 ab	50.0 a
Perlite-volcanic rock	86.0 a	0.0 b
Perlite-coconut fiber	54.0 b	5.0 b
Coconut fiber-volcanic rock	68.0 ab	0.0 b

Values with different letters within column are significantly different (Tukey P<0.05).

Experiment 2

Plant regeneration. Substrates. An analysis of the results showed that the number of shoots formed per explant cultivated on agar (2.7) was not significantly different from the number of shoots formed per explants cultured on substrates; moreover, perlite-volcanic rock (PV) induced a lower response than did vermiculite-perlite (VP) (Table 3). In terms of dry weight, the explants cultured on both PR and VP showed statistically equal values to those that were cultivated on agar (Table 3).

Moreover, no significant differences were observed in the number of roots formed for the shoots grown on substrates and agar, but the roots formed on agar were longer (Table 4). The greatest number of leaves was observed in the shoots cultured on agar (7.2), and the lowest was observed in perlite-volcanic rock (4.0); furthermore, the shoot height was not significantly different between the shoots cultivated on agar and those cultivated on substrates (Table 4).

Table 3. Effect of the substrates on the dry weight of explants and number of adventitious shoots formed per habanero chili explants.
Tabla 3. Efecto de los sustratos sobre el peso seco de los explantes y número de brotes adventicios formados en los explantes de chile habanero.

Substrate	Number of shoots per explant	Dry weight (mg)
Agar	2.70 ab	34.57 a
Perlite-volcanic rock	2.04 b	39.20 a
Vermiculite-perlite	3.16 a	35.23 a

Values with different letters within column are significantly different (Tukey P<0.05).

Table 4. Effect of the substrates on the number of roots, root length, number of leaves and shoots height of habanero chili cv. Orange.
Tabla 4. Efecto de los sustratos sobre el número de raíces, longitud de raíz, número de hojas y altura de los brotes de chile habanero cv. Naranja.

Substrate	Number of roots	Root length (cm)	Number of leaves	Shoot height (cm)
Agar	2.55 a	6.78 a	7.20 a	1.93 a
Perlite-volcanic rock	2.75 a	1.06 c	4.00 c	2.13 a
Vermiculite-perlite	3.40 a	2.88 b	5.30 b	2.03 a

Values with different letters within column are significantly different (Tukey P<0.05).

Culture medium. The number of shoots formed by the explants cultivated on medium 1 used conventionally was statistically higher than that of explants cultivated on media 4, 5, 6, 7 and 8 (Table 5). Regarding the dry weight of the explants, only media 4, 5, 6, 7, and 8 showed values statistically lower those of medium 1 (Table 5).

Likewise, the number of roots was greater in shoots cultured on medium E4 (4.6) (Table 6). The root length of shoots cultivated on E3 and E4 media was significantly different (4.2 and 4.0, respectively) from that of the shoots cultivated on E2 medium (2.6). Moreover, the number of leaves was not significantly affected by the composition of the culture medium, although the shoot height was affected—it was higher for the shoots cultured on E3 medium than for those cultured on E1, E2 and E4 media (Table 6).

Culture medium and substrate interactions. The number of shoots formed by the explants exposed to treatment VP2 (medium 2, vermiculite-perlite) was statistically equal to that of explants exposed to the conventional treatment A1 (medium 1, agar), and statistically higher (6.33) than that of explants submitted to treatments A2, A4, A5, A7, PR2, PR4, PR5, PR6, VP4, VP5, VP6, VP7, VP8 (Table 7). The dry weight of the explants cultured on treatments A2, A3, PR1, PR2, PR3, PR7, VP1, VP2, VP4 and VP7 was statistically similar to that of the explants grown under treatment A1 (Table 7).

The rooting percentage of the shoots cultured under treatments PRE1 (medium E1, perlite-volcanic rock), VPE1 (medium E1, vermiculite-perlite), AE2 (medium E2, agar) and PRE4 (medium E4, perlite-volcanic rock) was statistically higher than that of shoots grown under treatment AE3 (medium E3, agar) (Table 8).

Table 5. Effect of culture media on the number of adventitious shoots per explant and dry weight of habanero chili cv. Orange explants cultured *in vitro*.
Tabla 5. Efecto de los medios de cultivo sobre el número de brotes adventicios por explante y peso seco de los explantes de chile habanero cv. Naranja cultivados *in vitro*.

Medium	Salts (%)	Sucrose (g/L)	BAP (mg/L)	IAA (mg/L)	Number of shoots per explant	Dry weight mg)
1	MS 100	30	4.0	0.3	4.66 a	63.22 a
2	MS 100	15	4.0	0.3	3.22 ab	48.59 ab
3	MS 50	15	4.0	0.3	3.66 ab	49.31 ab
4	MS 50	7.5	4.0	0.3	1.22 c	26.77 dc
5	MS 25	7.5	4.0	0.3	1.44 c	27.75 dc
6	MS 25	3.7	4.0	0.3	2.55 bc	19.97 d
7	H 100	30	4.0	0.3	1.55 c	40.15 bc
8	H100	15	4.0	0.3	2.78 bc	17.93 d

MS: Murashige and Skoog; H: Arnon and Hoagland. Values with different letters within column are significantly different (Tukey P<0.05).

Table 6. Effect of the rooting culture media on the number of roots, root length, number of leaves, and length of habanero chili cv. Orange adventitious shoots rooted *in vitro*.
Tabla 6. Efecto de los medios de cultivo de enraizamiento en el número de raíces, longitud de raíz, número de hojas, y longitud de los brotes adventicios de chile habanero cv. Naranja enraizados *in vitro*.

	Medium			Number of roots	Root lenght (cm)	Number of leaves	Shoot height
	Salts (%)	Sucrose (g/L)	IBA (mg/L)				
E1	MS 100	30	1.0	3.20 b	3.36 ab	6.20 a	2.85 b
E2	MS 50	15	1.0	1.46 c	2.61 b	5.06 a	1.81 bc
E3	MS 25	15	1.0	2.33 bc	4.23 a	5.40 a	4.23 a
E4	H	15	1.0	4.60 a	4.09 a	5.46 a	2.03 b

MS: Murashige and Skoog; H: Arnon and Hoagland; IBA: indole-3-butyric acid. Values with different letters within column are significantly different (Tukey P<0.05).

Table 7. Effect of treatments on the number of adventitious shoots per explant and dry weight of habanero chili explants.
Tabla 7. Efecto de los tratamientos sobre el número de brotes adventicios por explante y peso seco de los explantes de chile habanero.

Treatments	Medium				Substrate	Number of shoots per explant	Dry weight (mg)
	Salts (%)	Sucrose (g/L)	BAP mg/L	IAA mg/L			
A1	MS 100	30.0	4.0	0.3	agar	5.66 ab	65.33 abc
A2	MS 100	15.0	4.0	0.3	agar	2.00 dc	46.26 abcde
A3	MS 50	15.0	4.0	0.3	agar	3.33 abcd	74.13 a
A4	MS 50	7.5	4.0	0.3	agar	1.33 dc	23.63 edf
A5	MS 25	7.5	4.0	0.3	agar	1.00 dc	31.66 bcdef
A6	MS 25	3.7	4.0	0.3	agar	3.33 abcd	4.76 f
A7	H	30.0	4.0	0.3	agar	1.33 dc	21.30 def
A8	H	15.0	4.0	0.3	agar	3.66 abc	9.53 ef
PR1	MS 100	30.0	4.0	0.3	PR	4.00 abc	72.50 ab
PR2	MS 100	15.0	4.0	0.3	PR	1.33 dc	47.10 abcde
PR3	MS 50	15.0	4.0	0.3	PR	4.00 abc	42.56 abcdef
PR4	MS 50	7.5.0	4.0	0.3	PR	0	21.90 def
PR5	MS 25	7.5.0	4.0	0.3	PR	1.00 dc	24.50 cdef
PR6	MS 25	3.7	4.0	0.3	PR	2.00 dc	27.76 cdef
PR7	H	30.0	4.0	0.3	PR	4.33 abc	58.86 abcd
PR8	H	15.0	4.0	0.3	PR	2.66 bcd	18.43 def
VP1	MS 100	30.0	4.0	0.3	VP	4.33 ab	51.83 abcd
VP2	MS 100	15.0	4.0	0.3	VP	6.33 a	52.40 abcd
VP3	MS 50	15.0	4.0	0.3	VP	3.66 abc	31.23 cdef
VP4	MS 50	7.5	4.0	0.3	VP	2.33 bcd	34.80 abcdef
VP5	MS 25	7.5	4.0	0.3	VP	2.33 bcd	27.10 cdef
VP6	MS 25	3.7	4.0	0.3	VP	2.33 bcd	18.40 def
VP7	H	30.0	4.0	0.3	VP	1.33 dc	40.30 abcde
VP8	H	15.0	4.0	0.3	VP	2.00 dc	25.83cdef

MS: Murashige and Skoog; H: Arnon and Hoagland; BAP: benzylaminopurine; IAA: indoleacetic acid; A: agar; PR: perlite-volcanic rock, VP: vermiculite-perlite. Values with different letters within column are significantly different (Tukey P<0.05).

Regarding the number of roots, treatment VPE4 (medium E4, vermiculite-perlite) yielded a significantly greater number of roots than treatments AE1 (medium E1, agar), AE2 (medium E2, agar) and AE3 (medium E3, agar) (Table 8). The root length for the shoots cultured under treatment AE3 (medium 3, agar) was significantly higher than that of shoots cultured under treatments AE1, PRE1, PRE4, VPE1 and VPE4 (Table 8). The shoot height under treatment PRE1 (medium E1, perlite-volcanic rock) was higher than that measured under the rest of the treatments tested, except for VPE1 (medium E1, vermiculite-perlite). Likewise, the number of leaves in shoots exposed to treatment AE4 was statistically higher than that in shoots exposed to the rest of the treatments, except for treatment AE3 (Table 8).

Acclimation. Culture medium and substrates interactions. Treatments AE4 (medium E4, agar), PRE2 (medium E2, perlite-volcanic rock), VPE1 (medium E1, vermiculite-perlite) and VPE2 (medium E2, vermiculite-perlite), displayed the highest values for survival (100%) (Table 9). Moreover, the plant height in treatment VPE2 (medium E2, vermiculite-perlite) and VPE1 (medium E1, vermiculite-perlite) were statistically higher (6.9 and 6.6, respectively) than values in all of the other treatments (Fig 1). With respect to the number of leaves, significant differences were also observed, with treatments VPE2 (medium E2, vermiculite-perlite), PRE3 (medium E3, perlite-volcanic rock), and PRE2 (medium E2, perlite-volcanic rock) showing a greater number of leaves than the other treatments tested (Table 9). Furthermore, the stem diameter of plants exposed to treatment VPE2 (medium E2, vermiculite-perlite) was statistically higher than that of plants exposed to treatments AE3, AE4, PRE1, PRE2, PRE4 and VPE4 (Table 9).

Analysis of production costs. Table 10 shows the cost of a vitroplant of habanero chili regenerated using the conventional method described by Valadez-Bustos et al. (2009), and the protocol proposed in this research using substrates VP, PR and diluted media. Producing a habanero chili plant using the conventional protocol (MS 100%, 30 g/L sucrose and agar) for all stages of micropropagation is 2.2 times more expensive than using the protocol developed in this study.

DISCUSSION

Experiment 1. The low survival of explants cultured on substrates that included coconut fiber (PC, CR) was largely due to the high oxidation rate of explants caused by the decomposition of lignin, cellulose and pectin forming the coconut fiber (Mwaikambo & Ansell, 2002). Similarly, Deb & Pongener (2013) found that germination rate of *Cymbidium iridioides* was lower on coconut fiber compared to medium gelled with agar. Likewise, this substrate did not support regeneration and culture proliferation. Moreover, the scarce or null formation of shoots in the explants cultured on substrates

Table 8. Effect of treatments on the rooting percentage, number of roots, root length, shoot height, and number of leaves of habanero chili adventitious shoots rooted *in vitro*.
Tabla 8. Efecto de tratamientos en el porcentaje de enraizamiento, número de raíces, longitud de raíz, altura del vástago y número de hojas de los brotes adventicios de chile habanero enraizados *in vitro*.

Treatment	Rooting (%)	Number of roots	Root length (cm)	Shoot height (cm)	Number of leaves
AE1	85.72 ab	1.60 de	6.50 bc	2.12 bcd	7.4 b
AE2	90.48 a	2.00 cde	6.90 ab	1.58 cd	6.6 c
AE3	33.33 b	2.20 bcde	7.35 a	1.60 cd	8.0 ab
AE4	66. 67 ab	4.40 abc	6.40 ab	2.42 bc	8.4 a
PRE1	100.0 a	4.00 abcd	2.48 cd	3.63 a	5.6 d
PRE2	0.0	0.0	0.0	0.0	0.0
PRE3	0.0	0.0	0.0	0.0	0.0
PRE4	88.89 a	4.60 ab	1.4 d	2.14 bcd	4.0 e
VPE1	100.0 a	4.00 abcd	1.10 d	2.80 ab	6.4 c
VPE2	80.95 ab	2.40 abcde	0.94 d	2.34 bc	5.2 de
VPE3	83.34 ab	2.40 bcde	5.00 abc	1.48 cd	7.3 b
VPE4	83.34 ab	4.80 a	4.48 bc	1.53 cd	5.8 d

A: agar; PR: perlite-volcanic rock, VP: vermiculite-perlite; E1: MS 100%, 15 g/L sucrose, IBA 1.0 mg/L; E2: MS 50%, 15 g/L sucrose, IBA 1.0 mg/L; E3: MS 25%, 15 g/L sucrose, IBA 1.0 mg/L; E4: H, 15 g/L sucrose, IBA 1.0 mg/L. Values with different letters within column are significantly different (Tukey P<0.05).

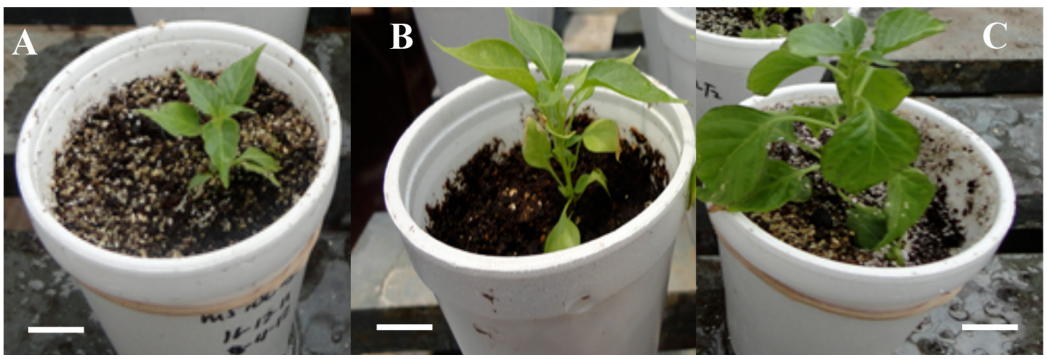

Fig. 1. Habanero chili vitroplants after seven weeks of growing in the greenhouse. A. AE1 (agar, MS 100%, 15 g/L sucrose, 1.0 mg/l IBA); B. PRE2 (perlite-volcanic rock, MS 50%, 15 g/L sucrose, 1.0 mg/L IBA); C. VPE2 (vermiculite-perlite, MS 50%, 15 g/L sucrose, 1.0 mg/L IBA). MS: Murashige & Skoog salts; IBA: indole-3-butyric acid. Scale bar: 1 cm.
Fig. 1. Vitroplantas de chile habanero después de siete semanas de crecimiento en el invernadero. A. AE1 (agar, MS 100%, 15 g/L sacarosa, 1.0 mg/l AIB); B. PRE2 (perlita-roca volcánica, MS 50%, 15 g/L sacarosa, 1.0 mg/L AIB); C. VPE2 (vermiculita-perlita, MS 50%, 15 g/L sacarosa, 1.0 mg/L AIB). MS: Sales Murashige y Skoog; AIB: ácido indol-3-butírico. Barra de escala: 1 cm.

Table 9. Effect of treatments on survival, plant height, number of leaves, and stem diameter of habanero chili plants regenerated *in vitro* after seven weeks of being grown under greenhouse conditions.
Tabla 9. Efecto de tratamientos en la supervivencia, altura de planta, número de hojas, y diámetro de tallo de plantas de chile habanero regeneradas *in vitro*, después de siete semanas de ser cultivadas en el invernadero.

Treatment	Survival	Plant height (cm)	Number of leaves	Stem diameter (mm)
AE1	66.0 abcd	2.4 f	7.8 dc	1.5 abc
AE2	84.0 ab	2.7 e	7.3 dc	1.7 ab
AE3	67.0 abc	1.4 f	7.0 d	0.8 d
AE4	100.0 a	4.6 d	10.5 bc	1.3 bc
PRE1	35.0 bcd	5.7 c	9.4 bc	1.0 c
PRE2	100.0 a	6.2 b	15.0 a	1.3 bc
PRE3	29.0 bcd	5.0 c	16.5 a	1.6 abc
PRE4	13.0 cd	3.0 f	4.0 e	0.9 d
VPE1	100.0 a	6.6 a	13.1 ab	1.7 ab
VPE2	100.0 a	6.9 a	16.1 a	1.9 a
VPE3	0.0	0	0	0
VPE4	75.0 abc	6.1 b	5.1 d	1.0 d

A: agar; PR: perlite-volcanic rock, VP: vermiculite-perlite; E1: MS 100%, 15 g/L sucrose, 1.0 mg/L IBA; E2: MS 50%, 15 g/L sucrose, 1.0 mg/L IBA; E3: MS 25%, 15 g/L sucrose, 1.0 mg/L IBA; E4: H, 15 g/L sucrose, 1.0 mg/L IBA. Values with different letters within column are significantly different (Tukey P<0.05).

Table 10. Approximate cost (US dollars) of habanero chili cv. Orange plants regenerated *in vitro* using different protocols.
Tabla 10. Costo aproximado (dólares americanos) de plantas de chile habanero cv. Naranja regeneradas *in vitro* con diferentes protocolos.

Protocol	Shoots induction medium (30 mL)	Development medium (90 mL)	Rooting medium (60 mL)	Total	Number of shoots per explant	Cost per plant
Conventional with agar	0.19	0.57	0.37	1.13	6.0	0.18
Proposed with vermiculite-perlite or perlite-volcanic rock mixtures	0.19	0.28	0.04	0.51	6.0	0.08

could be due to the heterogeneous surface of the substrates because of the shape and size of their particles, which reduced the contact between the explant and culture medium contained within their pores.

Experiment 2. The fact that the number of shoots formed on the explants cultured on vermiculite-perlite was not significantly different from that of the explants cultured on agar indicates that once the adventitious shoots are differentiated and begin their development on a medium with agar, their later growth in the presence of substrates is possible, particularly in vermiculite-perlite. Likewise, the substrates did not limit the biomass accumulation of the explants because the explants cultured on agar or on substrates showed similar values for this variable (dry weight).

Furthermore, substrates (vermiculite-perlite and perlite-volcanic rock) were as efficient as the agar to promote rooting of the shoots. These results coincide with those reported by Jay-Allemand et al. (1992), who observed that the shoots of four walnut tree (*Juglans regia*) hybrids (MR9, MR8, HA2-13, and HA 3-1) had the highest rooting percentages (91-100%) and root length in treatments conformed by vermiculite and semi-solid medium (250:200) than the shoots cultured on agar. Further, Druart (1997) reported 90-100% *in vitro* rooting of *Malus domestica* shoots on vermiculite.

Moreover, the number of roots formed in shoots cultured on the mixtures of vermiculite-perlite and perlite-volcanic rock were significantly higher from those of the shoots cultured on conventional treatment. The higher number of root per shoot could allow easier absorption of nutrients from the culture medium and better growth (Mohan et al., 2004).

The advantage of using substrates over agar for root formation may be due to the physical characteristics of substrates, such as porosity (Pai), which helps maintain a better water and oxygen balance in substrates than agar, enhancing the diffusion of nutrients for the shoot and therefore the formation and development of the radical system (Hillel, 1982; Ansorena, 1994; Miller & Donahue, 1995). George (1993) stated that aeration of the tissues and roots cultured on porous substrates is better than that on agar. In this regard, Newell et al. (2003) tested supports with different percentages of porosity, porous agar (10%), white sand-agar (15%) and a mixture of sphagnum, river sand and perlite in a ratio of 0.5:2:2 (25%), with respect to the plant development and rooting of *Grevillea thelemanniana* and *Verticordia plumosa* x *Chamelaucium uncinatum*. The authors found that the mixture with the highest porosity helped form more vigorous roots in less time. Likewise, Labrousse et al. (2012) observed better cell organization of the root cap, longer radical hairs, and greater dry weight accumulation in roots formed in *Nemesia* sp. shoots cultured on paper pulp than in shoots cultured on agar.

The fact that the number of shoots formed in the explants cultured on the conventional medium 1 (MS 100%, 30 g/L sucrose, 4 mg/L BAP, 0.3 mg/L IAA) was higher than that of explants cultured on media which contained the same concentration of BAP and IAA but lower salts and sucrose concentrations (media 4, 5, 6 7, 8) indicates that the differentiation of shoots is greatly affected by salts and sucrose concentrations. In *Digitalis obscura*, Lapeña et al. (1992) observed that low concentration of MS salts (25 and 50%) did not affect the formation of shoots but did affect their final size.

Likewise, the H salts did not meet the nutritional requirements for the development of adventitious shoots when it was combined with levels of sucrose lower than 30 g/L. This response could be partially due to the concentrations of N and K being 48.2% and 49.7% lower, respectively, than that of MS.

Moreover, the highest percentages of hyperhydrated explants were found in the media with the lowest concentrations of MS salts and less than 15 g/L sucrose; this feature had a negative effect on the growth and development of adventitious shoots. In contrast, no hyperhydration was observed in shoots cultured on media containing H salts, but they showed chlorosis, a symptom of nutritional deficiency.

As in the case of the number of shoots, the accumulation of dry weight in the explants was strongly related to the levels of salts and sucrose in the medium and was lower in the explants cultured on media with the lowest concentrations of salts and sucrose. Having observed no significant differences in the dry weight of the explants cultured on medium 3 and that of the explants cultured on conventional medium (medium 1), it is possible to infer that the explants had a similar photosynthetic rate under both culture conditions, given that the dry weight is broadly related to the use of incident radiation and the efficiency to transform it (Jarma et al. 2010).

High rooting percentage and number of roots were observed for shoots cultured on medium E4 (H, 15 g/L sucrose, 1 mg/L IBA). A similar effect of the combination of H salts with 1.5 % sucrose on the number of roots formed in habanero chili adventitious shoots was reported by Barrales-López et al. (2015). This response may be due to both the P content and the form of nitrogen that makes up the salts H, which allows a balance between anions and cations; this balance, in turn, increases the pH of the radical surface and the absorption of P required in diverse functions of the plant (Abel et al., 2002; Vance et al., 2003; Fernández, 2007).

Furthermore, the number of leaves was not significantly affected by the composition of the culture medium, although the shoot height was affected. The fact that the height of shoots cultured on medium E3 (MS 25%, 15 g/L sucrose, 1 mg/L IBA) was higher than media E1, E2 and E4 could suggest that growth once roots are formed, could be sustained with lower concentrations of N and K than those of MS 100%, MS 50% or H salts.

Several of the treatments involved media 2, 3, 7 and 8 (PR2, PR3, PR7, VP2, VP3) and substrates showed a response similar to that observed for the conventional treatment (medium

1, agar) in terms of the number of shoots, although treatment VP2 (medium 2, vermiculite-perlite) surpassed this response. This finding suggests that treatment VP2 could replace the conventional treatment in the middle and late stage of shoot development with a high probability of success. The effect of the treatments on the dry weight of the explants was similar to that of the culture media; that is, the treatments that combined media 2 and 3, as well as 4 and 7, with vermiculite-perlite or perlite-volcanic rock (PR3, PR7, VP2, VP4, VP7) were similar to the conventional treatment.

The high rooting percentages observed for the treatments involving media E2 and E4 and agar or perlite-volcanic rock (AE2, PRE4) suggest that for the formation of roots, the use of concentrated nutrient solutions and high sucrose levels is not essential. It is worth noting that treatment VPE4 (medium 4, vermiculite-perlite) not only promoted a high percentage of rooting but also induced the formation of a greater number of roots than did AE1 treatment (medium 1, agar). This result indicates that, although the differentiation of the adventitious shoots did not occur optimally in the presence of substrates, the developmental morphology stage before rooting and the rooting itself were favored by the substrates and less concentrated media than the conventional one.

The high survival values obtained with treatments AE4 (medium E4, agar), PRE2 (medium E2, perlite-volcanic rock), VPE1 (medium E1, vermiculite-perlite) and VPE2 (medium 2, vermiculite-perlite) indicate that substituting agar for substrates and using culture media with lower concentrations of salts and sucrose than those used in the conventional medium 1 improves the adaptation of regenerated plants to greenhouse conditions. Similar survival values were observed for vitroplants of *Ipomoea batatas* L. (Lam.) cv. *Beniazuma Grevillea thelemanniana* and *Verticordia plumosa x Chamelaucium uncinatum*, and *Nemesia* when they were cultured on vermiculite and sugarcane bagasse during acclimation (Afreen-Zobayed et al., 2000; Labrousse et al., 2012). Further, Fujiwara and Kozai (1995) and Jeong et al. (1995) found that high concentrations of sucrose and mineral salts in the culture medium and low concentrations of CO_2 in the container can limit the photosynthesis of regenerated plants.

Moreover, treatment VPE2 not only allowed for high survival percentages of the plants under greenhouse conditions but also high values for shoot height, number of leaves, and stem diameter. It is worth mentioning that the number of leaves lost during acclimation by the plants that were rooted on substrates was lower than in plants that were constantly kept in agar.

The use of substrates as agar substitutes has been studied with respect to the *in vitro* rooting of different species but not that of habanero chili (Jay-Allemand et al., 1992; Dethier 1993; Kirdmanee et al., 1995; Afreen-Zobayed et al., 2000). Likewise, the effect of media less concentrated on the response *in vitro* has been reported for *Solanum tuberosum* and *Digitalis obscura* (Lapeña et al., 1992; Nhut et al., 2006). Although the effect of culture medium composition was studied for habanero chili by Barrales-López et al. (2015), it was only tested on rooting. Therefore, to the best of our knowledge, this is the first investigation on habanero chili to study the effect of the composition of the culture medium in combination with substrates on adventitious shoots induction, rooting and acclimation.

The results of this investigation showed that inducing the formation of habanero chili cv. Orange shoots was more efficient when medium 1 (MS 100%, 30 g/L sucrose, 4 mg/L BAP, 0.3 mg/L IAA) and agar were used; however, for many of the variables evaluated both *in vitro* and *ex vitro* (number of shoots per explant, plant height, number of leaves, rooting, stem diameters and survival), the combination of media containing MS 50% or 100%, 15 g/L sucrose, and vermiculite-perlite or perlite-volcanic rock promoted similar or higher values than those obtained with the conventional propagation method proposed by Valadez-Bustos et al. (2009).

One of the drawbacks of micropropagation is the cost of vitroplants relative to that of plants obtained by seeds or by the method conventionally used to propagate species. The agar used as a support for the culture media is the component that contributes most to the cost, constituting until 70% of the total cost. Likewise, the concentrations of the salts (MS) and sucrose used conventionally not only contribute (to a lesser degree) to the cost of vitroplants but can surpass the requirements of tissues cultivated *in vitro*; therefore, it is feasible to reduce this cost without significantly affecting the *in vitro* response (Lapeña et al., 1992; Raya-Montaño et al., 2011; González et al., 2012).

Regenerating habanero chili cv. Orange plants is possible with a protocol in which adventitious shoots induction and the beginning of the shoots development occur on medium containing MS 100%, 30 g/L sucrose, 4 mg/L BAP, 0.3 mg/L IAA or 1 mg/L GA and agar, in which the middle and final stages of development of shoots occurs on a medium containing MS 100% with 15 g/L sucrose, 1 mg/L GA and vermiculite-perlite or perlite-volcanic rock, and in which rooting occurs on media containing MS 50% or H salts, 15 g/L sucrose, 1 mg/L IBA with vermiculite-perlite or perlite-volcanic rock.

Producing a habanero chili plant using the protocol described in this paper is less expensive than using the conventional protocol in which MS 100% salts, 30 g/L sucrose and agar are used for all stages of micropropagation, which is mainly due to the substrates accounting only 13 to 17% of the total cost of production. Likewise, substrates could be recycled while agar could not (Deb & Pongener, 2013). Moreover, using culture media less concentrated also contributes to reducing costs.

CONCLUSIONS

Using culture media less concentrated and substrates during the development of adventitious buds (middle and late phases) and rooting allowed for the regeneration of plants with the same efficiency observed for the conventional method for *in vitro* propagation. The plant regeneration protocol developed in this study not only allowed for an adequate response of the explants *in vitro* and improved the behavior of regenerated plants under greenhouse conditions but significantly reduced production costs.

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