

Effect of culture medium consistence and silver nitrate on micropropagation of two potato (*Solanum tuberosum*) cultivars

Efecto de la consistencia del medio de cultivo y del nitrato de plata en la micropropagación de dos cultivares de papa (*Solanum tuberosum*)

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Abstract

Potato (*Solanum tuberosum* L.) is one of the main crops of the Andes region in Venezuela where some *S. tuberosum* ssp. *andigena* cultivars are used, including Arbolona negra. Since 1946, the Andean cultivars have been slowly substituted with foreign potato seeds, for example cv. Granola from Germany. Potato micropropagation is an excellent alternative for the conservation of native cultivars, for the massive production of potato seeds and for the production of plantlets with adequate growth parameters that allow the study of potato-pathogen interactions. However, potato's vitroplants frequently show symptoms caused by ethylene accumulation in the culture flasks. In this work, we compare the *in vitro* response of Granola and Arbolona negra cultivars using MS semi-solid or liquid medium supplemented or not with AgNO₃. These potato cultivars did not show epinasty or hyperhidricity symptoms caused by ethylene when were cultivated on MS (1962) semi-solid medium supplemented with AgNO₃ 2 mg l⁻¹. Under these conditions, leaf area shows the highest values for both cultivars, but there were no differences in others growth parameters such as stem length or leaf number in comparison with plantlets cultivated on medium without AgNO₃. These results allowed us to recommend the use of semi-solid medium supplemented with AgNO₃ for the micropropagation of these two cultivars.

Key words: native potato, ethylene, vitroplantlets.

Resumen

La papa (*Solanum tuberosum* L.) es uno de los principales cultivos de la región de los Andes de Venezuela, donde algunos cultivares de *S. tuberosum* ssp. *andigena* se utilizan, entre ellos, Arbolona negra. Desde 1946, los cultivares andinos han sido poco a poco sustituidos por cultivares comerciales, por ejemplo Granola de Alemania. La micropropagación es una excelente alternativa para la conservación de cultivares nativos de papa, para la producción masiva de semillas y para la producción de vitroplantas con parámetros de crecimiento adecuados que permitan el estudio de la interacción papa-patógeno. Sin embargo, las vitroplantas de papa frecuentemente muestran síntomas causados por la acumulación de etileno en los envases de cultivo. En este trabajo, se compara la respuesta *in vitro* de los cultivares Granola y Arbolona negra en medio MS semisólido o líquido suplementado o no con AgNO₃. Estos cultivares no presentaron síntomas de hiperhidricidad o epinastia causados por el etileno cuando se cultivaron en medio MS (1962) semisólido suplementado con AgNO₃ 2 mg·l⁻¹. Bajo estas condiciones el área foliar mostró los valores más altos para ambos cultivares, pero no hubo diferencias en los otros parámetros de crecimiento medidos tales como la longitud del tallo o el número de hojas en comparación con vitroplántulas cultivadas en medio sin AgNO₃. Estos resultados

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nos permiten recomendar la utilización del medio semisólido suplementado con AgNO_3 para la micropropagación de estos dos cultivares.

Palabras clave: papa nativa, etileno, vitroplántulas.

Recibido: noviembre 30 de 2012

Aprobado: noviembre 5 de 2013

Introduction

In 2010, the national potato production reached 187435 Hg/Ha in a surface of 23000 Ha in Venezuela (FAOSTAT, 2011). The altitude of the areas where the potato crop is grown, is between 400 and 3,000 meters above sea level, where average temperatures range from 10 to 20°C and annual rainfalls from 1,000 to 1,800 mm. However, these climatic conditions are also very favorable for potato late blight (causal agent *Phytophthora infestans* (Mont) de Bary), which is the main limitation for local potato production, causing losses of up to 100% in some years (Lozada-García *et al.*, 2008).

In the Andes region some *S. tuberosum* ssp. *andigena* cultivars are used, which at higher altitudes (above 2000 meters above sea level) are generally more productive than ssp. *tuberosum* cultivars. Important cultivars of this group include Guantiva, Pastusa, Purace (of Colombian origin) and Arbolona and Merideña (of Venezuelan origin) (FONAIAP, 1983). Since 1946 the Andean cultivars have been slowly substituted with foreign potato seeds, known as white potatoes, for example cv. Granola from Germany. This cultivar produces tubers after 90 days but is susceptible to many potato diseases whereas Andean cultivars, which take 6 to 9 months to produce tubers, are resistant to these diseases (Moreno, 1968). Specifically, Arbolona negra is resistant to most of the potato diseases but produces tubers after 9 months. However, these tubers can be stored for more than one year and can be used as qualified seed after this period.

The success of white potato cultivars was responsible for the diminution and almost elimination of black potatoes in Andean regions of Venezuela (Romero and Monasterio, 2005). Data taken from 4 communities show that modernization has introduced these new white cultivars carrying diseases which attack the native cultivar. Foreign cultivars have replaced the native's ones which are no longer in demand nor cultivated. Arguments in favor of re-establishing the black potato as a commercial venture and as a home grown product are set forth (Romero and Monasterio, 2005b).

Using *in vitro* culture techniques, it would be possible to rescue the potato Andean germplasm. Also, *in vitro* cultured plantlets are the best source of healthy leaves to be used in experiments designed for the studies of plant-pathogen interactions. These experiments have been performed in the field, to study the response of potato's resistant and susceptible cultivars to the infection with *P. infestans*; however, in many cases, the results were inconclusive because the environmental influence (Singh and Birhman, 1994). Many authors have reported different protocols for *in vitro* culture of potato using different explants and different cultivars (Roest and Bokelman, 1976; Martel and de García, 1992; de García and Martínez, 1995; Seabrook and Douglass, 2001; Vargas *et al.*, 2005); however, ethylene produced by tissue, callus and plantlets in closed vessels may lead to abnormal plantlet growth, hyperhydricity, abnormal branching *in vitro*, epinasty, leaf and flower bud abscission, diminution of foliar area (Turhan, 2004; Mullins *et al.*, 2006; Zobayed *et al.*, 2001; Zobayed, 2005; Hazarika, 2006; Steinitz *et al.*, 2010; Giridhar, 2004; Dang and Wei, 2009; Jackson *et al.*, 1991).

Since 1976 it had been known that Ag^+ applied foliarly as AgNO_3 , effectively blocked the ability of exogenously applied ethylene to elicit the classical "triple" response in intact etiolated peas; stimulate leaf, flower, and fruit abscission in cotton; and induce senescence of orchids. This property of Ag^+ surpasses that of the well known ethylene antagonist, CO_2 , and its persistence, specificity, and lack of phytotoxicity at effective concentrations should prove useful in defining further the role of ethylene in plant growth (Beyer, 1976).

In this sense, plant regeneration *in vitro* is often improved by adding silver ions to the culture media as AgNO_3 or silver thiosulfate (Steinitz *et al.*, 2010). As Ag^+ ions can prevent a wide cultivar of ethylene-induced plant responses, including growth inhibition and senescence, the effect is assumed to be mediated via the inhibition of the physiological action of ethylene (Zhang *et al.*, 2001). The effect of AgNO_3 added to the *in vitro* culture medium has been studied in many plant species; Fuentes *et al.* (2000), studied the respon-

se of five *Coffea canephora* Pierre genotypes with regard to somatic embryogenesis on media containing silver nitrate and different carbohydrates (sucrose, fructose, maltose and glucose). They concluded that this compound acts as a direct inhibitor of the ethylene action, which in turn regulates the availability of ethylene in the culture vessel during specific stages of coffee embryogenesis. To improve the regeneration efficiency of cassava (*Manihot esculenta* Crantz) *in vitro*, the effect of silver nitrate (AgNO_3) on shoot organogenesis from cotyledons was assessed. Adding AgNO_3 to the regeneration medium improved the regeneration frequency and reduced callus formation in all tested cultivars. Both the extent of the response to and the optimum concentration of AgNO_3 were cultivar dependent (Zhang *et al.*, 2001). Giridhar *et al.* (2004) established direct somatic embryogenesis from hypocotyl explants of *in vitro* regenerated plantlets of *C. arabica* and *C. canephora* on modified MS medium containing 1.7 - 11.9 $\text{mg}\cdot\text{l}^{-1}$ silver nitrate supplemented with 0.2 $\text{mg}\cdot\text{l}^{-1}$ N^6 benzyl adenine and 0.5 $\text{mg}\cdot\text{l}^{-1}$ indole-3-acetic acid. A maximum of 144.1 ± 7.3 and 68.7 ± 3.3 embryos per explants were produced at 6.8 $\text{mg}\cdot\text{l}^{-1}$ silver nitrate in *C. canephora* and *C. arabica* respectively. Dang and Wei (2009), reported an efficient regeneration system for *Phaseolus vulgaris*. The addition of AgNO_3 enhanced the frequency of the shoot formation from 61.3 to 87.6%. Gutiérrez-Miceli *et al.* (2010), reported the optimum concentrations of naphthalene acetic acid (NAA) and 6-benzyladenine (BA) to stimulate callus growth and the optimum concentrations of NAA; kinetin and AgNO_3 for callus redifferentiation in *Dianthus caryophyllus* L. Results showed that high NAA and AgNO_3 concentrations increased shoot and root induction.

The objective of this work was to improve the micropropagation of potato cultivars Granola and Arbolona negra in order to obtain *vitroplantlets* that allow the establishment of an adequate system for the study of potato-pathogen interaction.

Materials and methods

Plant Material

Native Arbolona negra (*Solanum tuberosum* ssp. *andigenum*) and Granola (*Solanum tuberosum* ssp. *tuberosum*) cultivars obtained from Universidad de los Andes and INIA-Mérida (Instituto Nacional de Investigaciones Agrícolas), respectively, were used in this study. These cultivars were maintained *in vitro* through microshoots culture.

Maintenance of *in vitro* plant material

The plant material was maintained as axillary shoots culture on MS sales (Murashige and Skoog, 1962) supplemented with 100 $\text{mg}\cdot\text{l}^{-1}$ myo-inositol, Mörel vitamins, sacarose 25 $\text{g}\cdot\text{l}^{-1}$ solidified with 6 $\text{g}\cdot\text{l}^{-1}$ agar (MS1 semi-solid medium), pH 5.6, at 18 ± 1 °C under 16 h photoperiod and subcultured at 8-week intervals. This culture medium was also used for the studies of the effect of the medium consistence and the silver nitrate test.

Effect of the medium consistence on micropropagation

To evaluate the influence of the medium consistence on micropropagation, 30 stem sections with 1 axillary bud, obtained from each cultivar were cultured on the same MS1 semi-solid medium, 1 stem section per tube. In the case of liquid media, 30 explants of each cultivar were cultured on 15 ml of MS medium (Murashige and Skoog, 1962) in 250 ml erlenmeyer flasks, 5 explants per erlenmeyer. Cultures were incubated at 125 rpm on a shaker New Brunswick Scientific®, at 18 ± 1 °C under 16 h photoperiod.

Silver nitrate test

MS1 semi-solid medium supplemented with AgNO_3 was used for assessing the effect of silver nitrate. A stock solution of AgNO_3 was filter sterilized, stored at 4 °C and added into the autoclaved medium. The effect of various silver nitrate concentrations (0, 1, 2 and 5 $\text{mg}\cdot\text{l}^{-1}$) on shoot regeneration and plantlet development was tested. Thirty explants per cultivar were cultured per treatment. Five long stem section of 1 cm with one bud were cultured per flask. All flasks were incubated under diffuse ventilation (Zhang *et al.*, 2006): Flask caps were perforated with four holes of 1 cm diameter each; between the cap and the flask, 2 whatman filter papers and a polypropylene mesh were placed. Cultures were incubated at 18 ± 1 °C under 16 h photoperiod.

After six weeks shoot length (mm), leaves number, fresh weight (g), dry weight (g), branch number and foliar area (cm^2) were recorded.

Statistical analysis

Significance of treatment effects was determined by analysis of variance using SPSS 12th version Statistical Packet. Significant differences between the means were assessed by Duncan's multiple range test at $P = 0.05$ (Duncan, 1955).

Results

Effect of the medium consistence on micropropagation

Table 1 shows the results of growth parameters for Granola and Arbolona negra cultured on semi-solid or liquid MS (1962) medium. Plantlets growing on semi-solid medium for eight weeks were taller than plantlets obtained on liquid medium, for both cultivars, but they look fragile and with a foliar area of 12.50 mm² (figure 1a, figure 2a). Plantlets growing on liquid medium had less leaves number than plants growing on solid medium, for both cultivars; however, leaf area of plantlets developed on liquid medium was 6 to 8 times higher than leaf area of plantlets cultured on semi-solid media. Arbolona negra plantlets showed larger stems than Granola plantlets when cultured on liquid media

(figure 1b, figure 2b). However, after 8 weeks of culture, necrosity, leaf epinasty, leaf abscission and hiperhidricity were observed in both, Granola and Arbolona negra cultures both in semi-solid and liquid culture.

Silver nitrate test

We tested different concentrations of silver nitrate for both cultivars. Table 2 shows the results for the growth parameters estimated. As the AgNO₃ concentration increased, leaf number diminished, stem length diminished and leaf area increased for both cultivars. Plantlets growing on MS medium supplemented with 2 mg·l⁻¹ AgNO₃ show an adequate stem length and a high leaf area. After eight weeks of culture, these plants did not show symptoms of ethylene growth inhibition: epinasty or hiperhidricity (figures 3 and 4).



Figure 1. *S. tuberosum* cv. Granola plantlets after 8 weeks of culture. a) Solid medium. b) Liquid medium.

Table 1. Effect of the medium consistence on growth parameters determined for Granola and Arbolona Negra after 8 weeks of culture

Cultivar	Treatment	Leaves number	Shoot length (cm)	Stem fresh weight (g)	Root length (cm)	Root fresh weight (g)	Leaf area (mm ²)
Granola	Solid	14.47±0.48 ^a	12.17±0.58 ^a	0.06±0.00 ^a	3.85±0.19 ^a	0.01±0.00 ^a	12.50±0.67 ^a
	Liquid	8.33±0.39 ^b	10.65±0.65 ^a	0.32±0.03 ^b	2.23±0.19 ^b	0.04±0.00 ^b	75.00±6.61 ^b
Arbolona negra	Solid	15.70±0.84 ^a	15,13±0.83 ^a	0.12±0.00 ^a	6.30±0.39 ^a	0.05±0.00 ^a	12.50±0.96 ^a
	Liquid	7.50±0.47 ^b	12.00±0.76 ^b	0.32±0.05 ^b	2.85±0.24 ^b	0.03±0.00 ^a	100.00±5.35 ^b

Values are means ±SE of 30 cultured explants. For each column and cultivar, means followed by the same letter are not significantly different according Duncan's multiple comparison test ($p < 0,05$)

Table 2. Effect of silver nitrate on growth parameters for Granola and Arbolona Negra after 8 weeks of culture.

Cultivar	Treatment	Leaf number	Shoot length (cm)	Stem fresh weight (g)	Root length (cm)	Root fresh weight (g)	Leaf area (mm ²)
Granola	0	8.60±0.44 ^a	12.23±0.74 ^a	0.17±0.02 ^a	4.19±0.21 ^b	0.03±0.00 ^a	24.75±2.22 ^b
	1	8.07±0.22 ^{a,b}	6.07±0.41 ^c	0.12±0.01 ^b	7.03±0.42 ^a	0.02±0.00 ^b	74.31±4.59 ^c
	2	8.37±0.29 ^a	5.12±0.39 ^c	0.17±0.01 ^a	6.47±0.42 ^a	0.01±0.00 ^b	122.85±7.19 ^a
	5	7.23±0.29 ^b	3.36±0.22 ^b	0.16±0.01 ^{a,b}	5.99±0.49 ^a	0.01±0.00 ^b	103.19±6.78 ^d
Arbolona negra	0	8.97±0.37 ^c	14.31±0.65 ^c	0.34±0.04 ^b	8.97±0.35 ^{a,b}	0.07±0.01 ^b	32.02±2.53 ^a
	1	8.83±0.18 ^b	9.29±0.42 ^b	0.16±0.01 ^a	8.04±0.31 ^a	0.01±0.00 ^a	92.78±7.24 ^b
	2	7.31±0.28 ^b	7.40±0.51 ^b	0.17±0.02 ^b	9.41±0.75 ^b	0.02±0.00 ^a	123.174±8.39 ^c
	5	6.28±0.24 ^a	4.53±0.36 ^a	0.21±0.02 ^b	9.04±1.03 ^{a,b}	0.03±0.01 ^a	167.74±11.95 ^d

Values are means ±SE of 30 cultured explants. For each column and cultivar, means followed by the same letter are not significantly different according Duncan's multiple comparison test (p<0,05)

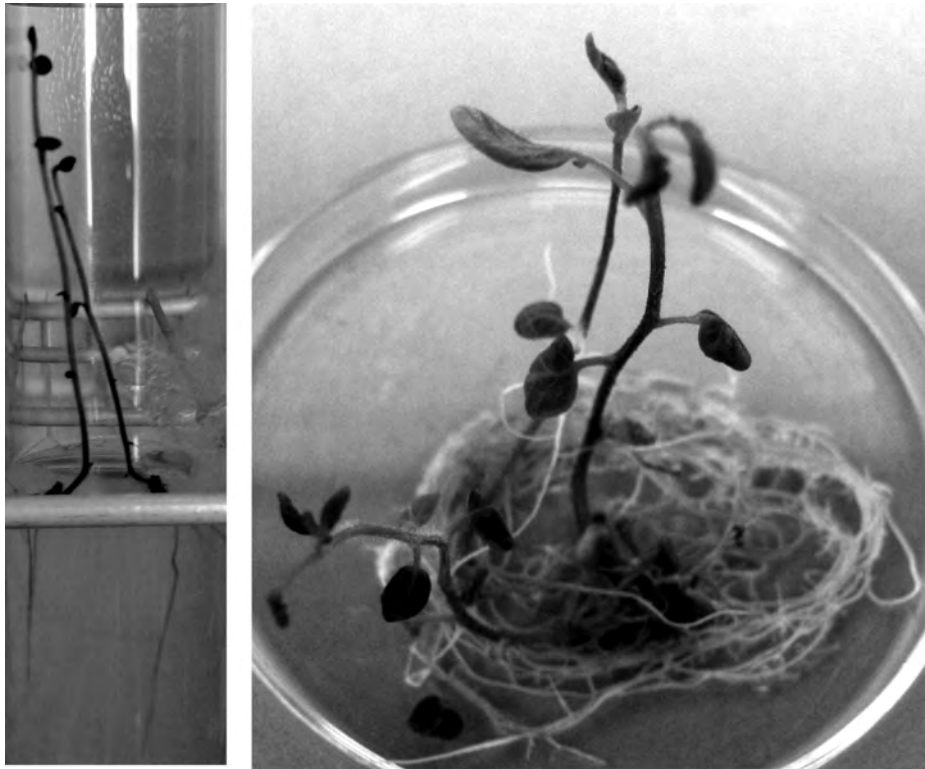


Figure 2. *S. tuberosum* cv. Arbolona negra plantlets after 8 weeks of culture. a) Solid medium. b) Liquid medium.

Discussion

Effect of media consistence on micropropagation

In vitro propagation of potato by the serial culture of axillary shoots on separated nodes has been reported by a number of researchers, and since 1950 has become established as an effective mean of rapidly

multiplying new or existing cultivars in disease-free conditions (Hussey and Stacey, 1984). However, in an attempt to improve the established propagation methods, we compared growth parameters for plantlets growing on semi-solid or liquid medium. In the analysis of our results, we made emphasis in the importance of the foliar area of the plantlets. As we can be sure that there is not another pathogen in the *in vi*



Figure 3. *S. tuberosum* cv. Granola plantlets growing under different silver nitrate concentrations after 8 weeks of culture. Figure shows details of leaf area at each AgNO_3 concentration. a) $0 \text{ mg}\cdot\text{l}^{-1}$; b) $1 \text{ mg}\cdot\text{l}^{-1}$; c) $2 \text{ mg}\cdot\text{l}^{-1}$; d) $5 \text{ mg}\cdot\text{l}^{-1}$.

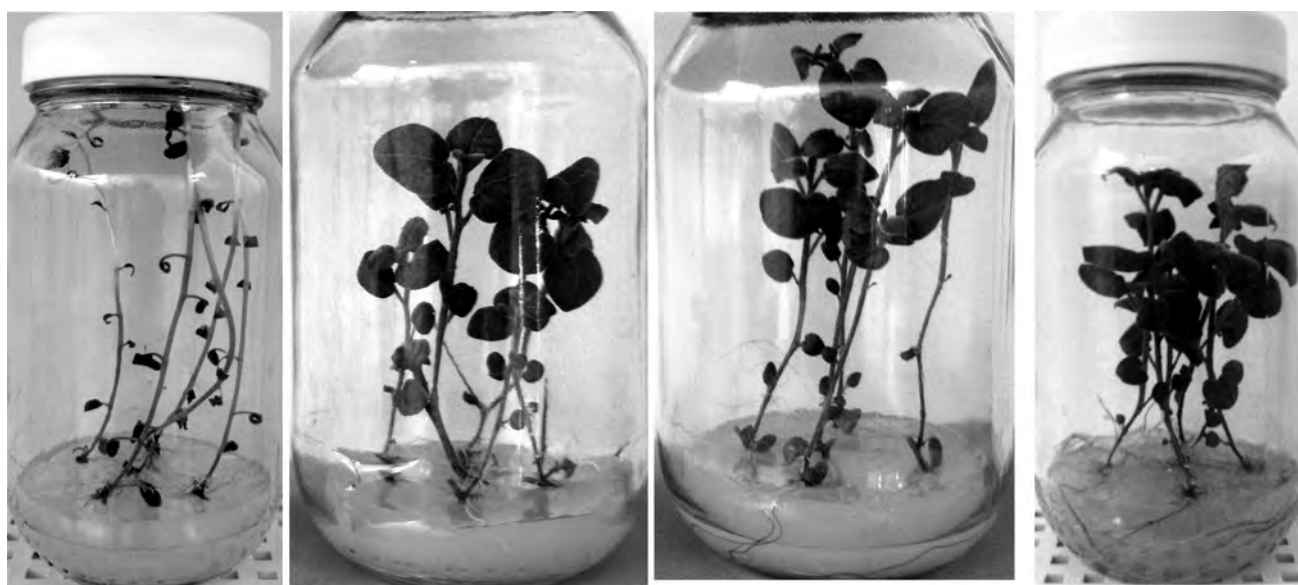


Figure 4. *S. tuberosum* cv. Arbolona negra plantlets growing under different silver nitrate concentrations after 8 weeks of culture. Figure shows details of leaf area at each AgNO_3 concentration. a) $0 \text{ mg}\cdot\text{l}^{-1}$; b) $1 \text{ mg}\cdot\text{l}^{-1}$; c) $2 \text{ mg}\cdot\text{l}^{-1}$; d) $5 \text{ mg}\cdot\text{l}^{-1}$.

in vitro cultured plantlets and the environmental conditions are controlled, these plantlets are the best material for studies of potato-pathogen interaction. However, the foliar area of potato vitroplantlets is not enough for inoculation experiments with bacteria, virus or oomycetes; and this is the main limitation for the use of *in vitro* obtained potato plantlets in these studies. Also, plantlets obtained from *in vitro* culture are semi-autotrophic, with non-developed plastids. An impro-

ved leaf development could guarantee the success of these plantlets during acclimatization phase.

As we saw on table 1, Arbolona negra's response to the micropropagation system established is as good as the response of Granola cultivar to the same culture conditions, either in semi-solid or liquid medium and both cultivars cultured on liquid media showed the highest values for leaf area. Our results shows that leaf area of plantlets developed on liquid medium was 6

to 8 times the leaf area of plantlets cultured on solid media. These differences could be a consequence of the increase in gas exchange in erlenmeyer flasks with liquid medium in constant agitation, which probably originates a diminution of ethylene concentration during the first weeks of culture; however, after 8 weeks of culture, symptoms related with ethylene inhibition of growth were observed in both cultivars, both in semi-solid and liquid medium.

Effect of silver nitrate

A major drawback to the *in vitro* propagation systems is that the potato plants are highly sensitive to ethylene, and ethylene accumulation *in vitro* strongly inhibits the growth and development of shoots. It is known that growth of potato plantlets can be distorted by concentrations of ethylene of 0.1 ml or even less (Jackson *et al.*, 1987; Sung and Huang, 2000). Hussey and Stacey (1981) reported that potato shoots become stoloniferous in tightly-closed culture vessels. Jackson *et al.* (1991) found that shoot height of *Solanum tuberosum* was 64% of that of the control after 14 days of culture in tightly-sealed vessels. They also concluded that accumulated ethylene is responsible for these effects.

To remove ethylene from potato culture vessels, forced ventilation and the use of some chemical compounds have been reported. Among the different chemicals, AgNO₃ has been widely and in most cases the most successfully used one. AgNO₃ was also used in order to reduce the occurrence of hyperhydricity in tissue culture of sunflower (Mayor *et al.*, 2003).

Following the recommendations from Hussey and Stacey (1981), Jackson *et al.* (1987), Sung and Huang (2000), Zhang *et al.* (2006), all our micropropagation experiments were established under diffuse ventilation conditions and we tested the effect of AgNO₃ added to MS (1962) semi-solid medium. In our hands, after eight weeks of culture, Granola and Arbolona negra plantlets growing on MS medium supplemented with 2 mg·l⁻¹ AgNO₃ show an adequate stem length and a highest leaf area; these plants did not show symptoms of ethylene growth inhibition: epinasty or hiperhidricity.

Ours results agree with the results reported by Zabayed *et al.* (2001) who tested different types of ventilation of the culture vessel headspace, each with and without AgNO₃ (0,5 mg·l⁻¹) or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (0.2 mg·l⁻¹) in the culture medium, on the growth of potato cuttings (*Solanum tuberosum* L. "cara"). Growth was substantially enhanced and vitrification was reduced by increasing the efficiency of ventilation. Callus de-

veloped on the stem bases in all sealed (airtight) and diffusive treatments except where AgNO₃ was used. No callus was observed in any treatment where forced ventilation was applied and *in vitro* tuberization (tuber size) was considerably improved by this treatment.

The mode of action of AgNO₃ in potato tissue culture is assumed to be associated with the physiological effects of ethylene, silver ions acting as a competitive inhibitor of ethylene action rather than inhibiting ethylene synthesis per se (Zhang *et al.*, 2006).

Fuentes *et al.* (2000) demonstrated that the addition of AgNO₃ promoted only small modifications of the ionic equilibrium of the medium. This suggests that the effects of this compound are not attributable to any substantial modifications in the levels of available nutrients. However, the mechanism by which AgNO₃ can affect potato plantlets development is difficult to elucidate from an experiment of this type.

Finally, although Arbolona negra takes 9 months to produce tubers in the field and Granola takes 4 to 5 months, Arbolona negra cultured *in vitro* showed the same response than Granola cultivar, in most of the growth parameters and in the same time period. As wild *Solanum* plants could be used to introduce desirable characteristics such as resistance towards certain diseases, insects and stress, into the cultivated potato (Petersen *et al.*, 1993), this result is of great importance for conventional and marker assisted improvement programs.

Conclusions

Our results allow us to recommend the culture of potato microshoots on semi-solid MS (1962) medium supplemented with AgNO₃ 2 mg·l⁻¹ to avoid ethylene produced symptoms and to obtain the best values in most important growth parameters for Granola and Arbolona negra plantlets. The leaves obtained from these plantlets can be used for the establishment of an appropriate system for the study of potato-pathogen interaction.

Acknowledgments

This research was supported by the Scientific and Humanistic Council of the *Universidad Central de Venezuela* PI-03-7281-2008, Caracas, Venezuela.

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