

Full Length Research Paper

Efficiency of the partial substitution of agar with potato starch on the growth and phytochemical parameters of lulo (*Solanum quitoense*) cultured *in vitro*

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The demand for agar, thanks to its different uses, generates the need to look for alternative substances that replace it. This work studied the influence of four different additions of potato starch into agar media (T1 = 50%, T2 = 45%, T3 = 40% and T4 = 0%) on the growth and chemical composition of essential oils of lulo (*Solanum quitoense*) explants cultured *in vitro*. The lengths, fresh and dry biomass weights, and number of nodes of the lulo explants were selected as growth indices and compared against either viscosity of the modified media or extraction percentage and relative concentration of the main components in the essential oils. A higher statistic variability and amount, of the lengths, fresh biomass and number of nodes, were found for the explants cultured in modified agar media compared to those cultured in pure agar. In contrast, minimal variation in metabolite extractions and concentrations were obtained as a function of potato starch addition. Cyclotetradecane, neophytadiene, 1-hexadecene, phytol, oleamide and 3-octadecene were found as the main components of the essential oils by gas chromatography-mass spectrometry (GC-MS). It was found that T1 was the treatment that yielded the best results in the analyzed variables.

Key words: *Solanum tuberosum* starch, *in vitro* culture, micropropagation, partial substitution, *Solanum quitoense in vitro* essential oil, secondary metabolites.

INTRODUCTION

The current increase in the human population has been sustained due to the global expansion in agricultural production, provided mainly from the “green revolution”, which occurred during the second half of the last century, particularly in developing countries. Modern agriculture

requires pathogen-free seeds or propagules of high-quality that must be readily obtained. One of the more efficient and practical techniques presently used to produce seedlings is plant cell/tissue culture (Martin et al., 2013). However, due to the high-cost of the agar used

as gelling agent in the culture medium, this method is expensive and unsuitable for small farmers. The high demand for agar, to use for *in vitro* micropropagation, microbiological techniques and as an additive in various products, has led to the development of less expensive and more readily accessible substitutes (Jain and Babbar, 2002). In this regard, the use of gums and starches of natural origin has been shown to be a promising strategy to replace the commercial agars in culture medium (Ozel et al., 2008; Mohamed et al., 2010).

Starches are natural polysaccharides with many industrial uses (Pacheco and Techeira, 2009) because of their functional properties attributed to the variability of their amylose (linear polymer) and amylopectin (branched polymer) contents, which depend on the species and origin (Salinas et al., 2003). In particular, the outstanding thickening and binding properties of starch make it a suitable gelling agent and agar substitute in plant culture media. Indeed, cassava starch has been successfully used as a gelling agent for the micropropagation of *Celosia* spp. *Solanum lycopersicum* species (Daud et al., 2011a, b), *Musa* spp. (Mbanaso, 2008), *Faidherbia albida* and *Uapaca kirkiana* (Maliro and Lameck, 2004). Likewise, gelled starches from rice (González and Silva, 1999), corn (Zimmerman et al., 1995), sago (Rodríguez and Hechevarría, 2006) and enset (Mengesha et al., 2012) have been used for the *in vitro* propagation of species of *Theobroma cacao* L. and *Malus domestica* Borkh., *Orthosiphon aristatus* (Blume), *Artemisia absinthium* L., and *Vanilla planifolia*, respectively. In addition, some starch mixtures, such as agar/potato/corn (Mohamed et al., 2010) and flour/potato-semolina (Sharifi et al., 2011) have also shown to be good gelling agent in media for micropropagation of *Solanum tuberosum* and *Saintpaulia ionantha*, correspondingly.

Despite the diversity of starches evaluated as alternatives to commercial agar gelling agents, very little is known about the effect of using these types of modified media on the essential oil composition and metabolite concentration of the plants cultured *in vitro*. In this regard, Paek et al. (2005) stated that the most impact of the culture media in plant micropropagation should be observed on its primary and secondary metabolic routes. This was reinforced by the study of Pérez and Jiménez (2011), where the variations in the composition of secondary metabolites of micropropagated species were mainly attributed to the changes in the levels of growing regulators and carbon source and the concentrations of micro- and macronutrients available in the culture media.

In this context the objective of the work was to study the effect of partial agar substitutions with potato starch to culture media, on both the growth and the secondary

metabolites production of *in vitro* micropropagation of lulo (*S. quitoense* L.).

MATERIALS AND METHODS

Location of the study

The research was carried out in the instrumental analysis laboratory (Research Group QUIMOL), and the plant culture laboratory (Research Group BIOPLASMA). Results were acquired from June 2013 to March 2014. In this publication the data of a second subculture were taken, because in an earlier publication, information was published on some variables in the first subculture (Martin et al., 2013).

Culture media

The base medium used for growing lulo nodal explants was MS (Murashige and Skoog, 1962), which was supplemented with 0.5 mg/L of indole-butyric acid and solidified with 7.5 g/L of agar (Difco). Four different agar/potato-starch mixtures (treatments), at ratios of 50/50 (T1), 55/45 (T2), 60/40 (T3) and 100/0 (T4), were prepared as culture media. All culture media were autoclaved at 121°C, 1 kg/cm² for 20 min.

The culture media viscosity was measured at 25°C, 10% torque from 0.3 to 3.0 rpm using a Brookfield LV DV-E viscometer equipped with an S-64 spindle.

Inoculation and incubation

Four nodal segments (approximately 2 cm in length) of lulo (*S. quitoense* L.) were inoculated in 150 ml culture flasks containing 20 ml approximately of MS medium and illuminated at 25 ± 2°C and irradiated with a light intensity of approximately 100 µmol m⁻²s⁻¹. For each of the four tested media, 72 explants were used for the first subculture and 192 for the second (45 days per subculture).

Assessment of the effects of the partial replacement of agar

The number of nodes, seedling length, and fresh and dry biomass weights were selected as measures of plant growth. For each treatment (Tn), a total of 0.63 to 0.79 g of dried plant material and 150 ml of solvent (dichloromethane) were used for Soxhlet-solid-liquid extraction of essential oil for 6 h. The extracts obtained, were concentrated in a rotary evaporator (Buchi, model R-205) using an internal pressure of 732 mbar. GC-MS analysis of the extracts was performed using a gas chromatograph (model 5890 series II, Hewlett Packard), equipped with a mass selective detector (MS 5972) and a Restek RTX-5 (30 m × 0.25 mm id × 0.25 µm) capillary column (crossbond 5% diphenyl/95% dimethyl-polysiloxane). The column temperature ramp was set at 70°C, for 4 min, then increased at 10°C/min up to 125°C, maintained at 125°C for 5 min, then increased at 2°C/min to reach 250°C, which was maintained for a further 12 min. The helium flow was 50 ml/min and the injection temperature was 225°C. The extract samples were diluted in 0.5 mL of chloroform and then an aliquot (0.5 µL) injected into the GC-MS using direct mode. Chemical identification of the extracts was performed by comparison of the GC-MS spectra with the MS

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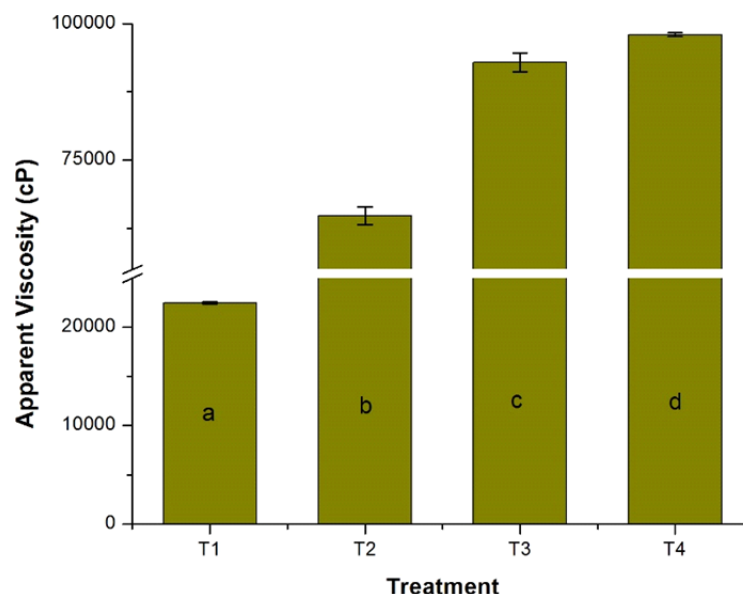


Figure 1. Apparent viscosity of the four agar/potato-starch mixtures in culture media of Letters a and b indicate statistical differences of 5% according to Duncan's test. Values are presented as mean $\pm$ SD (n=3).

spectra stored in Wiley6.1 MS data library of natural products.

Statistical analysis

Data analysis was performed using design experimental complete at random with four treatments (50/50 (T1), 55/45 (T2), 60/40 (T3) and 100/0 (T4) agar/starch ratios) of six replications each. The effects caused by the partial substitution of agar with potato starch were numerically analyzed using an ANOVA single factor treatment along with Duncan's multiple range test, with a significance level of 5% ($p < 0.05$) in SPSS 17 version software. For the variables of explant length, node and leaf numbers, as well as fresh and dry weight, a mean of 32 explants was used as the sampling unit. Three replicates for each culture media treatment were obtained by splitting the plant dry material into three aliquots.

RESULTS

Viscosity media

The progressive partial substitution of agar with potato starch decreased the apparent viscosity of the culture media (T1, T2 and T3) compared to the starch-free culture medium (T4), as shown in Figure 1. However, in all instances, the consistency of the culture media remained stable enough to ensure, on one hand, the vertical position of the explants and, on the other hand, the conditions for the adequate development of seedlings, thus, allowing the plants to grow with both a normal root system as well as a physiologically active appearance (Figure 2).

It was observed that media consistency increased at

the end of the subculture, but it was neither measured nor analyzed.

Growth indices

Seedling length

As depicted in Figure 3, the seedling lengths ranged from 0.10 to 10.60 cm, corresponding to a significant difference in the ANOVA analysis ($p = 0.000$). As also shown in Figure 3, the longer explant lengths were consistently derived from the agar culture media enriched with potato starch (T1 to T3).

Number of nodes

As also shown in Figure 3, all explants grown in potato starch culture media (T1 to T3) had fewer nodes (1 to 9) compared to those grown in starch-free media (T4) ($p = 0.09$).

Fresh and dry weights of biomass

The fresh and dry biomass weights ranged from 4.66 to 8.47 g ($p = 0.036$) and 0.27 to 0.46 g ($p = 0.268$) respectively. However, the lowest values were always obtained from the explants cultured in the medium free of potato starch (T4) (Figure 4).

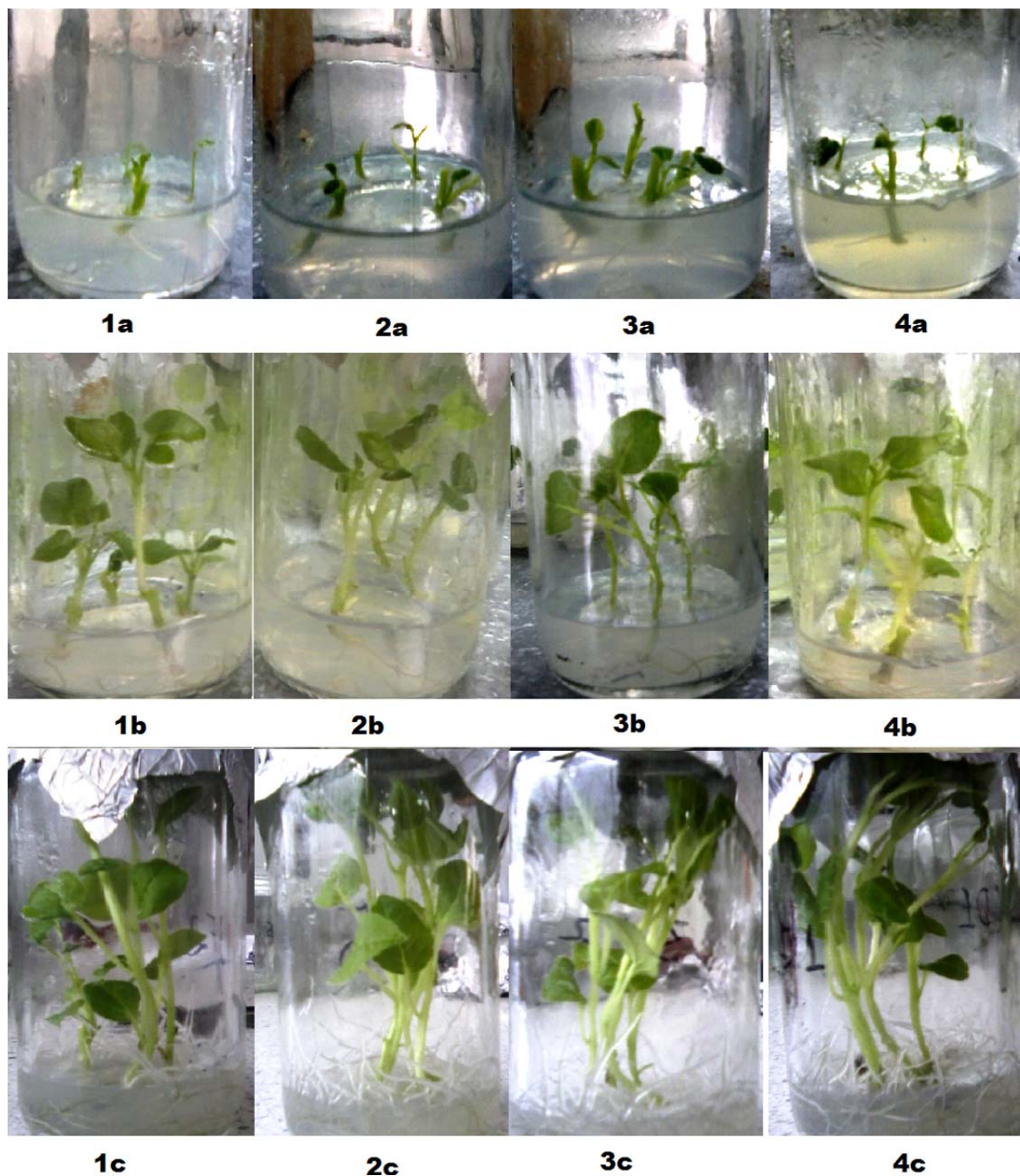


Figure 2. Physical appearance of the lulo (*S. quitoense*) explants in four treatments (T1 to T4) after 12 (a), 24 (b) and 40 (c) culture days.

Essential oils

The percentages of essential oil extracted from the explants, ranged between 9.7 and 19.0% (dry biomass basis), as shown in Figure 5. Significantly less essential oil was produced by the explants grown in the culture media containing potato starch ($p = 0.970$).

GC-MS analysis of the essential oils

Table 1 lists the main molecular components in the essential oils extracted from the lulo explants cultured in modified potato-starch media *in vitro*, as detected by GC-MS. A comparison of the relative concentration means of the major components in the essential oils derived from

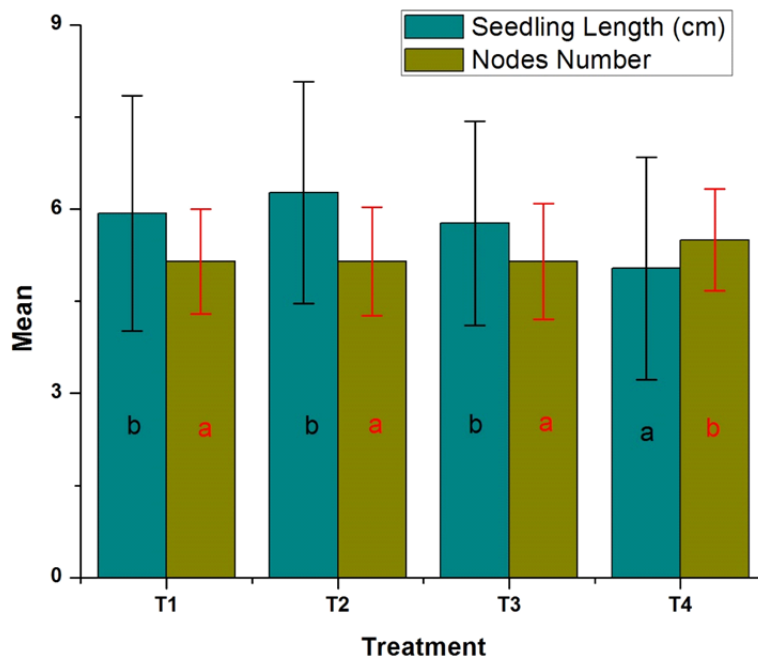


Figure 3. Mean seedling length and number of nodes for explants. Letters a and b indicate statistical differences of 5% according to Duncan's test. Values are presented as mean $\pm$ SD (n=6).

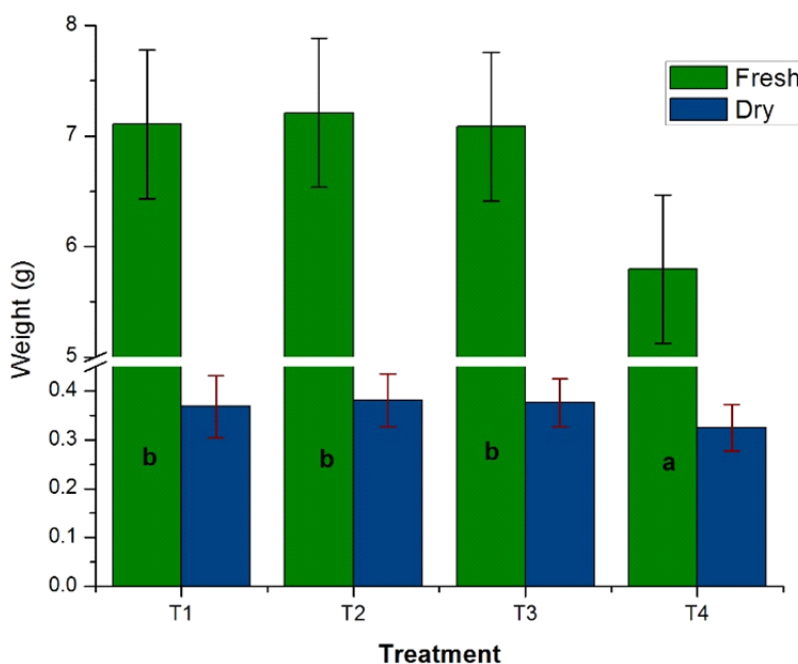


Figure 4. Biomass produced after 45 days of culture treatment. Letters a and b indicate statistical differences of 5% according to Duncan's test. Values are presented as mean $\pm$ SD (n=6).

the explants presented no significant difference, irrespective of the culture media: Dodecanol ($p = 0.423$),

cyclotetradecane ($p = 0.768$), neophytadiene ($p = 0.470$), 1-hexadecene ($p = 0.442$), phytol ($p = 0.596$), palmitic

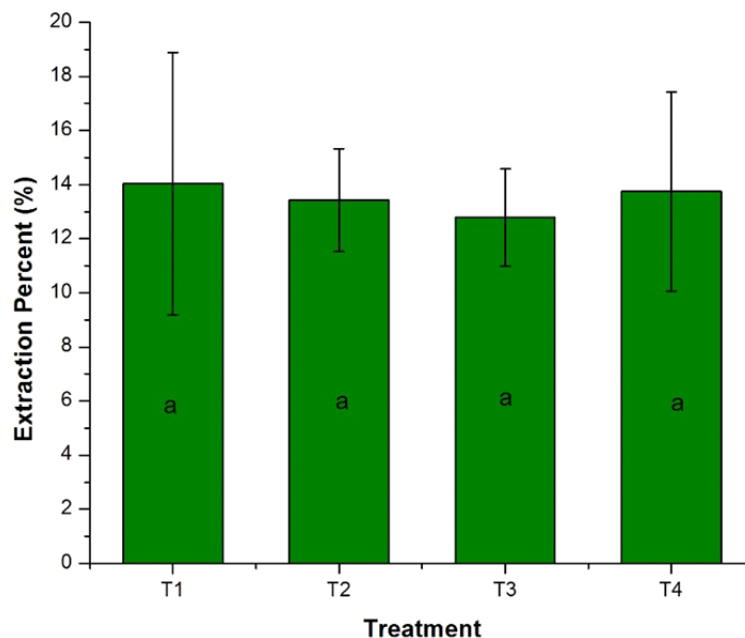


Figure 5. Essential oil percentages extracted as a function of culture media modified with potato starch. Letter a not indicate statistical differences of 5% according to Duncan's test. Values are presented as mean $\pm$ SD (n=3).

Table 1. Main molecular components in the essential oils of the lulo (*S. quitoense*) explants cultured in modified agar/potato-starch media. Values are presented as mean $\pm$ SD (n=3).

Molecule	T1	T2	T3	T4
Dodecanol	8.26 $\pm$ 7.09	17.90 $\pm$ 5.08	12.21 $\pm$ 10.30	14.36 $\pm$ 2.15
Cyclotetradecane	6.17 $\pm$ 6.85	5.45 $\pm$ 4.88	5.87 $\pm$ 5.14	6.55 $\pm$ 0.83
Neophytadiene	1.13 $\pm$ 1.10	2.35 $\pm$ 3.26	11.20 $\pm$ 15.86	3.91 $\pm$ 1.55
1-Hexadecene	0.84 $\pm$ 1.19	2.87 $\pm$ 2.72	2.55 $\pm$ 2.25	3.37 $\pm$ 0.71
Phytol	0.51 $\pm$ 0.44	0.78 $\pm$ 0.71	0.51 $\pm$ 0.43	0.49 $\pm$ 0.15
Palmitic acid	1.89 $\pm$ 3.23	12.31 $\pm$ 3.36	0.03 $\pm$ 0.01	9.11 $\pm$ 15.75
Oleamide	7.28 $\pm$ 9.67	9.72 $\pm$ 8.57	13.81 $\pm$ 3.07	8.34 $\pm$ 14.40
3-Octadecene	2.78 $\pm$ 0.82	1.32 $\pm$ 1.15	1.52 $\pm$ 0.25	1.38 $\pm$ 1.30

acid ($p = 0.273$), 3-octadecene ($p = 0.283$) and oleamide ($p = 0.855$). This indicated a negligible effect of the partial substitution of agar medium with potato starch in the formation and relative concentration of secondary metabolites of the lulo explants cultured *in vitro*. Other molecules, such as limonene, cresol, 1-octadecene, heneicosane and hexatriacontane were also detected in the essential oils of lulo explants but at lower amounts.

DISCUSSION

The lower viscosity of the culture media containing potato starch can be attributed to the weaker gelling effect of the

starch compared to agar (González and Silva, 1999; Martin et al., 2012). The difference arises from the stronger interaction that the sulfate and pyruvate groups of agar form with water molecules, resulting in higher viscosity and consistency of the media, compared to the interaction between the hydroxyl moieties of starch and water molecules. A decrease in media consistency and solidification was also reported by Jain and Babbar (2011) and by Lucyszyn et al. (2006) in the partial substitution of agar with guar gum, isubgol or xanthan, and with galactomannans, respectively.

However, that lowers viscosity of the culture media showed positive effects on the plantlets growth. Thus the longer lengths of lulo explants cultured in enriched potato

starch media, can be associated with a higher absorption of nutrients (Sharifi et al., 2011), and the availability of specific nutrients, like maltose, saccharose and glucose, formed by hydrolysis of starch molecules during autoclaving (Maliro and Lameck, 2004). Explants with longer lengths have also been reported in cell and tissue culture e.g. in somatic embryogenesis of cultured in media enriched with isubgol (Ozel et al., 2008), micropropagation of cultured in gelling media containing mixtures of isubgol/agar and guar gum/agar (Jain and Babbar, 2011), micropropagation of *Dioscorea alata* and *D. trifida* using phytigel as cultured media (Chacón et al., 2000), and propagation of culture media gelled with cassava starch (Maliro and Lameck, 2004). A decrease in the length of explants cultured in modified agar media has been reported by Lucyszyn et al. (2005) for *in vitro* propagation of apple burgeon (*Malus prunifolia* Borkh.) using guar gum/agar and cassia gum/agar mixtures as gelling media at 50/50 ratio.

Similar to the present results, a decrease in the number of nodes has been also observed for other plant species cultured in modified media using natural gelling agents. Rodríguez and Hechavarría (2006) reported fewer leaves and nodes in *O. aristatus* and *A. absinthium* explants micropropagated using solidified media with either full or partial replacement of agar with sago flour and Aloe vera gel. As well, Mohamed et al. (2010) and Mengesha et al. (2012) found fewer nodes in explants of *S. tuberosum* and *V. planifolia* cultured in media enriched with mixtures of agar/potato starch (40-60%) and agar/enset starch (0.2%/6%), respectively, compared to starch-free media. In addition, both previous authors observed statistical differences among the various starch/agar ratios used, which is due not only to the particular nutritional requirements and metabolic behavior of each species cultured *in vitro* but also to stem elongations, which decrease the number of nodes.

In the present study, the increase in fresh biomass obtained from the lulo explants cultured in agar media partially substituted with potato starch compared to agar alone, reflects a higher absorption of micro- and/or macronutrients and water, increasing the cellular metabolism (Sharifi et al., 2011). As aforementioned, the availability of these nutrients is favored by the lower viscosity and consistency of media partially substituted with potato starch (Mbanaso, 2008). Mohamed et al. (2010) and Mengesha et al. (2012) also reported an increase in fresh biomass in potato nodal segments and *V. planifolia* explant micropropagated by culture media modified with mixtures of agar and corn, potato or enset starch, respectively. In contrast, Romay et al. (2006) showed no statistical differences in fresh material weight of cassava explants by replacing phytigel with cassava starch as culture media. The low variability in biomass weights contrasts with the length and number of nodes of the explants observed among the various media treatments, suggesting a minimal dependence of this

variable on the agar/potato starch media.

To date, the chemical composition of *S. quitoense* essential oils has been reported for the fruit (Silva et al., 1990; Acosta et al., 2009) and seeds (Jurado and Muñoz, 2009). However, no published reports are found regarding the composition of essential oil from this plant cultured *in vitro*. According to Silva et al. (1990), the fruit aroma of lulo *S. quitoense* is attributed mainly to the presence of methyl butyrate, ethyl acetate, 2-(E)-methyl butyrate, linalool, γ -hexalactone, α -terpineol and acetic acid. Whereas the essential oil of lulo fruit are consisted of methyl benzoate, methyl hexanoate, 4-hydroxyl-4-methyl-2-pentanone, butyric acid, methyl 3-hydroxyhexanoate, 2-methyl-2-pentenal and 2-(E)-methyl butanoate (Acosta et al., 2009). Conversely, Jurado and Muñoz (2009) reported high concentrations of palmitic, oleic and linoleic acids in essential oil extracted from seeds of *S. quitoense* variety.

Several of the compounds presently found in the essential oils (Table 1) have been also identified in other species belonging to the same genre. Such as for instance, 6.09% of 1-dodecanol detected in the essential oil of *S. sessiliflorum* fruit (Marx et al., 1998) and neophytadiene, phytol, 1-hexadecene and 1-octadecene in essential oils extracted from *S. subinerme* (Ordaz et al., 2011).

The low variability in the relative concentrations of the main compounds in the essential oil of lulo explants regarding culture media treatment indicates a negligible influence on the secondary metabolism of the plant for all ratios of potato starch added into the agar media. Therefore, variations in the relative concentrations of the main essential oil components for each treatment (Table 1) should be associated with specific physiologic or metabolic factors rather than absorption of potato starch by the plantlet. This type of association was inferred previously by Pérez and Jiménez (2011), who observed significant variations in the concentration of some secondary metabolites by the action of the culture media which should mostly originate from changes in growth regulators and the carbon source. In this context, Paek et al. (2005) corroborated that modifying the amounts of micro- and macronutrients could induce selective formation of metabolites in plants cultured *in vitro*.

Conclusion

Significant differences in each of the analyzed variables show that the partial substitution of agar by potato starch produced positive effects in the growth and development indexes analyzed, probably because the decrease in the viscosity of these media allows a better diffusion of nutrients. In addition, the percentages of essential oil and the relative concentration of the major secondary metabolites were not significantly modified, allowing to recommend potato starch as an efficient partial substitute

as a solidifying agent for the culture media used for *in vitro* micropropagation of lulo (*S. quitoense*). It was found that T1 was the treatment that yielded the best results in the analyzed variables.

Conflict of Interests

The authors have not declared any conflict of interests.

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