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AGRARIAN SCIENCES

Callus culture as a new approach for the production of high added value compounds in *Ilex paraguariensis*: genotype influence, medium optimization and compounds identification

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Abstract: *Ilex paraguariensis* (yerba mate) is a native species from South America and is a rich source of bioactive compounds. There is a lack of research efforts on the phytochemical investigation of callus culture from this species. In the present study, an effort was made to optimize callus culture conditions and to identify secondary compounds. Calli were induced from 10 genotypes using leaf explants and the best genotype was selected to evaluate the effects of cytokinin types and concentrations on callus induction and biomass accumulation. The best genotype and cytokinin treatment were used to conduct one last experiment with sucrose concentrations in culture media and its effects on calli biomass, antioxidant activity and secondary compounds accumulation. Callus initiation was genotype dependent, and the 6-156-6 line had the best response. Zeatin supplemented medium showed higher callus induction rate (82%) and higher biomass accumulation after 120 days (328.2 mg). Higher biomass and secondary compounds accumulation were observed for calli on 3% sucrose medium. Antioxidant activity was not affected by sucrose concentrations. Yerba mate callus culture allowed the accumulation of chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, theobromine and caffeine.

Key words: antioxidant activity, biomass accumulation, plant growth regulators, sucrose, yerba mate.

INTRODUCTION

Ilex paraguariensis (Aquifoliaceae), popularly known as 'yerba mate', is a traditional plant species from South America, whose leaves are used mainly for the preparation of stimulant beverages (Filip et al. 2001). The first report on the antioxidant activity of yerba mate was brought to public only in the late 1990s, and since then the interest in this plant is gradually increasing (Markowicz Bastos et al. 2007). Nowadays, the leaves of this species are exported to several countries around the world for addition in cosmetics, foods and mainly to soft drinks (Pozebon et al. 2015).

Several studies concerning yerba mate potential for medicinal uses were carried out and some properties of this plant have already been reported, such as antiobesity action (Arçari et al. 2009), antimutagenic (Miranda et al. 2008), anti-depressive and neuroprotective activities (Ludka et al. 2016).

The medicinal properties of this species are related to its leaves main constituents. Natural antioxidant defense systems are reported in *Ilex paraguariensis* and are attributed, mainly, to the high content of phenolic compounds (Bravo et al. 2007). In addition to phenolic acids, methylxanthines, saponins, flavonoids, amino acids, minerals and vitamins are also reported as significant bioactive compounds in the leaves of this species (Boaventura et al. 2015).

The main phenolic acids present in yerba mate are known as chlorogenic acids (Filip et al. 2001, Heck & de Mejia 2007, Markowicz Bastos et al. 2007). Chlorogenic acids are extensively employed as additives in food industries for preparation of beverages, as well as medicinal substances and cosmetics. This class of compounds is considered as part of the Fine Chemicals category, products characterized by high added value (Butiuk et al. 2016).

Callus cultures are often used as alternative systems to whole plant cultivation, and can represent an efficient approach for producing natural compounds for pharmaceutical, fragrances, flavours, food additives, colouring agents, and agrochemicals applications (Kikowska et al. 2012, Wang et al. 2017). Plant cell cultures have been widely used as raw materials for cosmetics commercial products and added as bioactive ingredients for nutritional, pharmaceutical, dermo-cosmetic and animal health applications (PhytoCellTec 2012, Morus et al. 2014, L'Oréal 2017).

Callus and cell cultures follow two steps before secondary compounds production. Initially, biomass accumulation occurs when callus and cells grow and multiply (step one) and, subsequently, the biosynthesis of compounds from the biomass ensues (step two). Initiation of callus and cell cultures begins with choosing adequate parent plants, since the high-producing callus and cells as well as the accumulation of secondary compounds in plants may be genotype specific (Castro et al. 2016).

Several chemical and physical factors have been identified as influencing biomass accumulation and secondary compounds biosynthesis in plant cell cultures (Shen et al. 2008). Auxins and cytokinins are usually employed to induce callus formation, as they promote cell growth by stimulating cell division and elongation (Castro et al. 2016). Therefore, optimizing the type and level of growth regulators in culture medium is necessary to obtain maximum calli yield (Wani et al. 2014). In addition to plant growth regulators, plant cell cultures grow as a function of a carbohydrate source. For that reason, improvement of carbohydrates supplemental concentration in the medium can greatly affect biomass and compounds production in callus cultures (Castro et al. 2016). Sucrose, for example, is used as a vital carbon and energy source at concentrations of 3%. Higher concentrations of this carbohydrate in the culture medium, however, might cause osmotic stress on callus tissues (Gertlowski & Petersen 1993). This type of stress may influence secondary metabolites biosynthesis, mainly the phenylpropanoid pathway, that are associated to phenolic acids yields (Kikowska et al. 2012).

With this in mind, the present study aimed to select a suitable genotype to callus induction and subsequently optimize culture medium composition to obtain an efficient callus culture of *Ilex paraguariensis*, in order to produce highly valued secondary compounds under controlled conditions. The results are also discussed in terms of the antioxidant activity and distribution of major secondary compounds identified in *Ilex paraguariensis* callus culture according to changes in sucrose concentration.

MATERIALS AND METHODS

Establishment and proliferation of callus culture

Callus tissues were induced from leaves of *Ilex paraguariensis* plants grown in greenhouse at EMBRAPA Forestry (Brazilian Agricultural Research Corporation). The 2nd/3rd pairs of elite clones leaves were collected and immediately placed in antioxidant solution (0.5% ascorbic acid and 0.5% citric acid, w/v). The leaves were washed with neutral detergent in tap water and disinfested as follows: 10 min in Cercobin® 1% solution (w/v), 5 min in 0.05% mercury chloride (w/v) and finally rinsing three times with sterile distilled water.

Afterwards, leaf discs with 2 cm diameter were placed in Petri dishes containing 20 mL autoclaved MS medium (Murashige and Skoog 1964) reduced to quarter strength (¼-strength MS) and supplemented with 4.52 µM 2,4-dichlorophenoxyacetic acid (2.4-D). The concentrations of cytokinin and sucrose were modified according to the experiment. The pH was adjusted to 5.8 prior to addition of 0.7% agar. The Petri dishes containing the explants were incubated in the dark at 23 ± 2 ºC, and the explants were subcultured to freshly medium every 60 days.

Selection of clones for callus induction

Leaves from ten yerba mate elite clones (A3, A35, A7, F1, F2, M7, 3-65-2, 4-56-2, 4-76-2 and 6-156-6) from EMBRAPA Forestry breeding program (Resende et al. 2000) were collected, disinfected and placed in culture medium ¼ MS, containing 3% sucrose, 4.52 µM 2,4-D, 4.56 µM zeatin and 0.7% agar for callus induction. After 30 days, the callus culture was evaluated according to the induction coefficient for each clone: *induction coefficient = (total number of induced callus/ number of cultured explants) * 100*.

Cytokinins and callus proliferation

The best callogenesis response genotype (6-156-6) was tested on medium previously described varying the concentrations and types of cytokinins as follows: zeatin (2.26, 4.56 or 9 µM), 2-isopentenyladenine (2iP) (2.5, 5 or 7.5 µM), thidiazuron (TDZ) (0.125, 0.25 or 0.5 µM) or kinetin (2.5, 5 or 7.5 µM). After 30 days, callus induction percentage was evaluated and after 120 days, calli fresh mass was measured. The calli were transferred every 60 days for fresh media of the same composition.

Effects of sucrose concentration on callus proliferation and secondary metabolites

The best yerba mate genotype and cytokinin supplemented medium were used to study the effects of sucrose concentrations on callus growth and secondary compounds production. Calli were grown for 60 days in ¼ MS medium with 0.7% agar, 4.52 µM 2,4-D, 4.56 µM zeatin and 3% sucrose and then transferred to the same medium but with different sucrose supplementation (3, 6, and 9%). Calli fresh weight was measured every 15 days up to 120 days of culture. After this period, calli were stored at -80 ºC for further analysis of secondary compounds.

Extraction of secondary compounds from *Ilex paraguariensis* calli

Calli were frozen in liquid nitrogen and lyophilized for 72 h. Samples (10 mg) were extracted with hydroalcoholic solution (ethanol: water, 1:1, 1 mL). The extract solutions were mixed for 30 s, sonicated for 30 s, and kept on a rotatory shaker (450 rpm) for 1 h at 60 ºC. The extracts were centrifuged (13000 rpm) for 40 min, and the supernatants were collected and filtered (0.22 µm). Part of the extracts was used for biochemical analysis (phenolic content and antioxidant activity) and 500 µL of each were transferred to vials (2 mL) containing 25 µg of

the internal standard umbelliferone, added for quality control (Sigma®).

Determination of total phenolic content

Total phenolic content (TPC) was determined using Folin-Ciocalteu (FC) reagent according to the method reported by Horžić et al. (2009) with modifications. Briefly, 0.1 mL of the extracted sample was mixed with 6.0 mL of deionized water and 0.5 mL of the Folin-Ciocalteu reagent and subsequently incubated for 5 min at room temperature (25 ± 2 °C). After incubation, 2 mL of Na_2CO_3 (15%, w/v) was added to the mixture and the final volume was adjusted to 10 mL. The absorbance was measured at 760 nm using UV–Visible spectrophotometer (Shimadzu-1800, Japan) after 2 h. Gallic acid (0.25-10 $mg.L^{-1}$) was used to create the standard curve for quantification. The results were expressed as milligrams of gallic acid equivalents per gram of dried weight (DW) ($mgGAE.g^{-1}$).

Determination of antioxidant activity by DPPH and ABTS assay

The effect of yerba-mate callus extracts on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was estimated using the method described by Brand-Willians et al. (1995). Each yerba mate extract (0.1 mL) was added to 3.9 mL of DPPH in methanol ($6x10^{-5}$ $mol.L^{-1}$). The mixture was shaken vigorously and incubated for 30 min at room temperature. After this period, absorbance was determined at 515 nm in a UV-Vis spectrophotometer (Shimadzu-1800, Japan).

Total antioxidant activity was measured by 2,2`-Azinobis (3-ethylbenzothiazoline 6-sulphonic acid) radical scavenging (ABTS) method. The ABTS cation radical was produced by the reaction between 7 mM ABTS and 140 mM potassium persulfate. This mixture was stored in the dark at room temperature for 16 h. Before it was used, the ABTS solution was diluted until reaching an absorbance of 0.7 ± 0.2 at 734 nm in a spectrophotometer (Shimadzu-1800, Japan). Subsequently, 30 µL of the yerba mate callus extract was added to 3 mL of ABTS solution and the reduction was determined after 2 h in a spectrophotometer at 734 nm (Re et al. 1999, Yim et al. 2013).

The scavenging capacity of DPPH radicals and ABTS was calculated using the equation obtained from the standard Trolox analytical curve at range of 0-1000 $mg.L^{-1}$ and 0-2500 $mg.L^{-1}$, respectively. The results were expressed in µmoles Trolox equivalent per gram of DW ($\mu molTE.g^{-1}$).

Identification and quantification of secondary compounds

Chromatographic analyses were conducted on an Agilent 1290 Infinity Liquid Chromatograph (HPLC), using a UV detector and a C18 Synergy Fusion-RP 80A (75 x 4.6 mm, d.i. 4 µm) column, with a C18 pre column. The UV spectra were recorded in 208, 260, 280 and 328 nm. The mobile phases consisted of a gradient elution of acetonitrile, water and formic acid (5:94.9:0.1, v/v/v, solvent A), and acetonitrile and formic acid (99.9:0.1, v/v, solvent B). The gradient profile was: 0–3 min (0% B), 3–23 min (0%–3% B), 23–28 min (30%–100% B), 28–30 min (100% B), 30-31 min (100%-0% B), 31-35 (0% B) at 1 $mL.min^{-1}$. The injected volume was 20 µL.

The semi-quantification of the compounds was calculated by 5 point external analytical curves, the caffeine and theobromine were performed using a caffeine curve at range of 0 to 0.5 $mg.mL^{-1}$. The caffeoylquinic derivatives were semi-quantified using a chlorogenic acid curve at range of 0 to1 $mg.mL^{-1}$. The secondary compounds amounts were expressed in mg compound per gram of DW of caffeine equivalent ($mgCafE.g^{-1}$) or chlorogenic acid equivalent ($mgCAE.g^{-1}$).

The identification of the compounds was carried out by LC-MS/MS analysis using the SCIEX X500R QTOF system with Turbo V™ source and Electrospray Ionization (ESI). The positive mode was used for caffeine and theobromine and a negative mode was used for caffeoylquinic derivatives. The IS voltage was set to 5500 V. The mobile phases consisted of a gradient elution of acetonitrile and water and formic acid (5:94.9:0.1, v/v/v) (solvent A), and acetonitrile and formic acid (99.9:0.1, v/v/v) (solvent B). The gradient profile was: 0–0.5 min (5% B), 0.5–25 min (5%–40% B), 25–28 min (40%–60% B), 28–30 min (60% B), 30-31 min (60%-0% B), 31-35 (0% B) at 0.5 $mL.min^{-1}$. The injected volume was 2 µL of sample injection.

Statistical analyses

Completely randomized experimental designs were used for all experiments. For the evaluations of genotype, cytokinin and sucrose effects, 10 Petri dishes (replications) with 5 explants each (experimental unit) per treatment were used. In the cytokinin experiment, after 120 days, 10 calli of each treatment were weighed to determine callus fresh mass. Data of the abovementioned experiments were analysed by Barttlet test and ANOVA, followed by a Tukey test ($p<0.05$). For evaluation of sucrose effects on different times, after 60 days on culture, 10 calli were weighed each 15 days, until 120 days. Regression analysis ($p <0.05$) was performed to the variable of callus growth. The analyses of total phenolics, antioxidant activity, and HPLC analysis were carried out in quintuplicate.

RESULTS AND DISCUSSION

Effects of genotype on callus induction

The percentage of callus induction varied according to the genotype (Figure 1), suggesting a strong genotype dependent response. Leaf

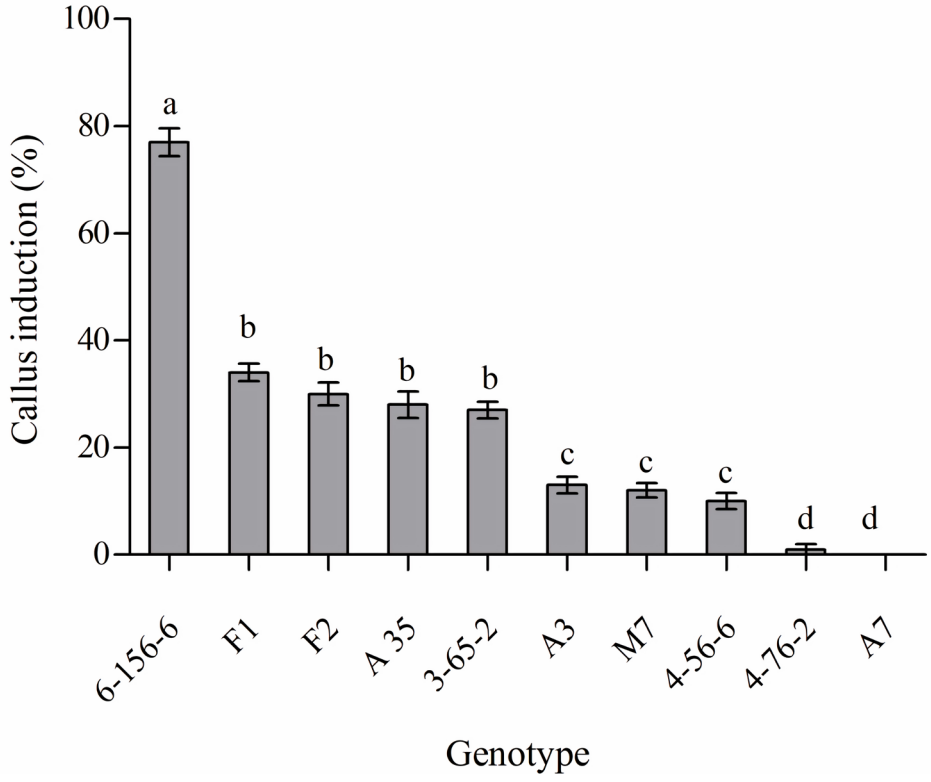

Figure 1. Callus induction on leaf explants of 10 clones of *I. paraguariensis* after 30 days on culture. Bars followed by the same letter do not show significant differences [P < 0.05 (Tukey)].

explants of 6-156-6 clone had the higher induction rates (77%). The lowest induction rates (1% and 0%) were observed in leaf explants from 4-76-2 and A7 clones.

Genotypic differences in callus forming ability *in vitro* culture have been observed in a wide range of species. Some genotypes can exhibit high capacity, while others are recalcitrant for callus induction (Atak & Çelik 2009, Głowacka et al. 2010, Liu et al. 2010). Kandasamy et al. (2001) reported that specific genes, capable of responding rapidly to auxin and other plant growth regulators, are required for the growth and proliferation of tissues in cultures and its low expression might inhibit callus formation.

The callus appearance was similar for all clones: yellowish with compact texture. As Clone 6-156-6 had the highest percentage of callus induction, it was selected as the explants source for the following experiments.

Cytokinins effects on callus induction and growth

The type, concentration, and combination of plant growth regulators (PGR) in media may also affect callus induction. The present study showed that cytokinin type and concentration have significant effects on the induction and growth of yerba mate callus. The 2,4-D has been shown to be the most effective auxin for callus induction from leaf explant of a variety of species (Santos et al. 2008, Vasconcelos et al. 2012). Previous studies showed the necessity of using 2,4-D to induce callus in yerba mate leaves, being the amount of 4.52 µM giving the higher callus induction, thus such concentration was fixed in this experiments.

The results indicated that all treatments induced callus from yerba mate leaves (Table I). However, differences based on cytokinins types and concentrations were observed. Among the cytokinins tested, higher zeatin concentrations

Table I. Effects of cytokinin type and concentrations on callus induction and calli fresh weight in callus culture from *I. paraguariensis* (clone 6-156-6) leaf explants.

Cytokinins (µM)		Callus induction (%)	Fresh weight (mg) (120 days)
Zeatin	2.25	36 ± 4 ^{b}	175.5 ± 9.5 ^{b}
	4.56	82 ± 5.5 ^{a}	328.2 ± 23.4 ^{a}
	9	56 ± 6.5 ^{ab}	299.2 ± 15.7 ^{a}
TDZ	0.15	60 ± 7.8 ^{ab}	123.0 ± 7.6 ^{b}
	0.25	44 ±5.8 ^{b}	166.1 ± 4.6 ^{b}
	0.5	58 ± 5.3 ^{ab}	295.6 ± 23.5 ^{a}
2 iP	2.5	46 ± 4.2 ^{b}	177.3 ± 14.4 ^{b}
	5	48 ± 8.5 ^{b}	194.7 ± 8.6 ^{b}
	7.5	42 ± 5.4 ^{b}	295.6 ± 23.5 ^{a}
Kinetin	2.5	58 ± 6.9 ^{ab}	191.2 ± 23.8 ^{b}
	5	44 ± 7.7 ^{b}	150.6 ± 7.9 ^{b}
	7.5	60 ± 7.3 ^{ab}	143.8 ± 12.4 ^{b}

Means followed by the same letter in each column are not statistically different at 0.05 probability level according to Tukey test. Callus induction was evaluated after 30 days and fresh weight at 120 days in dark conditions, cultured on the ¼ MS medium supplemented with 4.52 µM 2,4-D and different cytokinins (mean ± SE, *n* = 10).

(4.56 and 9 µM) were the most effective in stimulating callus induction, not differing statistically from 0.15 µM and 0.5 µM TDZ, 2.5 µM and 7.5 µM kinetin ($p>0.05$). Regarding calli fresh weight at 120 days, higher zeatin amounts were also found the most effective, together with 0.5 µM TDZ and 7.5 µM 2iP ($p>0.05$) (Table I). When both variables (i.e. callus induction and fresh weights) were taken together, higher zeatin concentration (4.56 and 9 µM) and 0.5 µM TDZ provided similar results. However, calli grown in culture medium with TDZ oxidized after 120 days, whereas those cultivated with zeatin showed better appearance and colour (Figure 2).

Zeatin was previously described as effective for conversion of rudimentary embryos of *Ilex paraguariensis* (Sansberro et al. 1998). In previous experiments with other *Ilex* species such as *I. crenata* (Yang et al. 2015), *Ilex brasiliensis*, *I. pseudoboxus* and *I. theezans* (Dolce et al. 2015), zeatin also shown to be the best cytokinin for plants shoot regeneration.

According to Schuch et al. (2008), 2iP also occurs naturally in plants, but it is considered a weaker cytokinin when compared to zeatin. This fact is in agreement with our results, since calli grown in medium with this plant growth regulator showed the lower callus induction percentage and only the highest concentration of 2iP (7.5 µM) showed good results for yerba mate calli yield.

The opposite trend was observed in callus supplemented with kinetin. Data obtained showed that calli biomass was decreased when the concentrations of kinetin increased. At the same time, intermediate concentrations of kinetin and TDZ showed lower callus induction, while the extreme concentrations showed higher callus induction rate. This was also observed in *Centella asiatica* callus culture suplemented with 2,4-D and 6- Benzylaminopurine (BAP).

Manipulation of the auxin/cytokinin ratio in culture media is often a crucial factor for increasing calli growth. The exogenous supplemented auxins and cytokinins act by

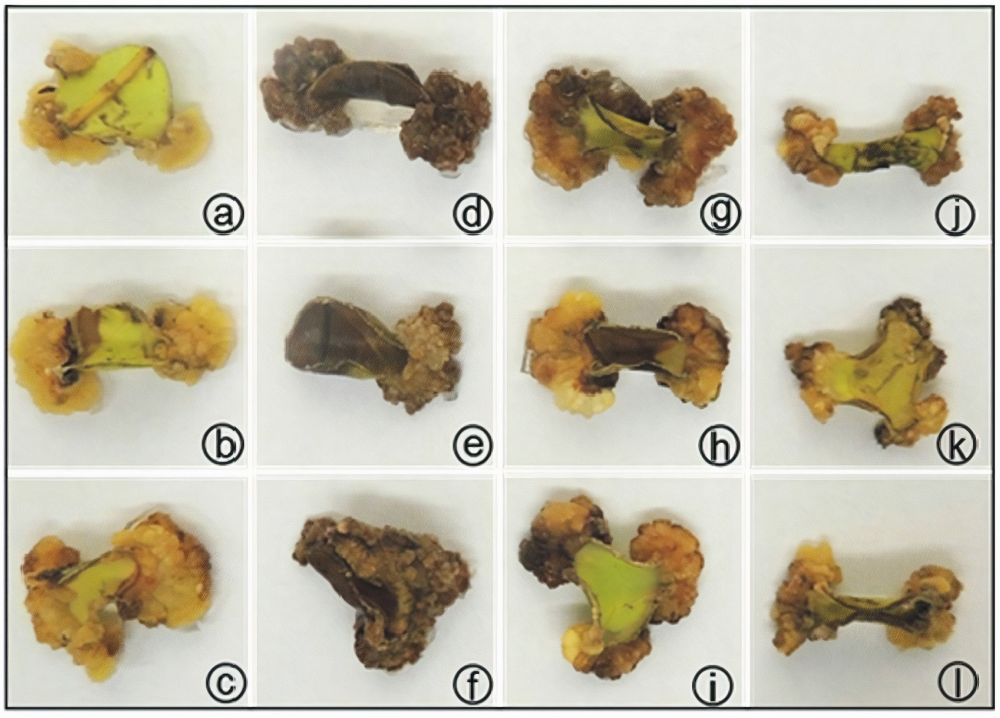

Figure 2. Effect of cytokinins on *I. paraguariensis* calli at 120 days of induction. a) 2.25 µM zeatin, b) 4.56 µM zeatin, c) 9 µM zeatin, d) 0.15 µM TDZ, e) 0.25 µM TDZ, f) 0.5 µM TDZ, g) 2.5 µM 2iP, h) 5 µM 2iP, i) 7.5 µM 2iP, j) 2.5 µM kinetin, k) 5 µM kinetin, l) 7.5 µM kinetin.

interaction with endogenous plant hormones and consequently, the concentration and combination of these regulators need to be defined for each species (Loredo-Carrillo et al. 2013).

Additionally, the manipulation of PGR concentrations in the culture media might also influence secondary compounds accumulation (Rodrigues & Almeida 2010). PGR manipulation, in most of the cases, is carried out in the first step of metabolite production, by altering the factors which could improve the callus growth during the biomass accumulation phase (Castro et al. 2016).

Taking together the results for callus induction and calli fresh weight, the concentration of 4.56 µM zeatin showed the better results and, in addition, promoted a better colour and aspect in yerba mate calli. Therefore, this cytokinin type and concentration were chosen to perform the experiment with variable sucrose concentrations.

Effects of sucrose on callus growth and total phenolics content

The effect of sucrose on *I. paraguariensis* callus induction was investigated by varying sucrose concentration (from 3 to 9%) after 60 days on culture. At this time, calli weights were, on average, 100 mg of fresh weight.

Figure 3a shows the biomass accumulation over the time for calli under different sucrose concentrations. The medium supplemented with 3% sucrose was superior to the others after 90 days culture. Concentrations of 6 and 9% sucrose were suboptimal for yerba mate calli growth. Higher concentrations of sucrose may reduce calli fresh mass by increasing the medium osmotic potential, hindering the absorption of salts and water (Jesus et al. 2011). As observed in *Vitis vinifera* (Do & Cormier 1990) and *Panax notoginseng* (Zhang et al. 1996) cell suspension cultures, high concentrations of sucrose repressed cell growth, but, in both species, favoured the synthesis of secondary compounds and could be used as strategies to

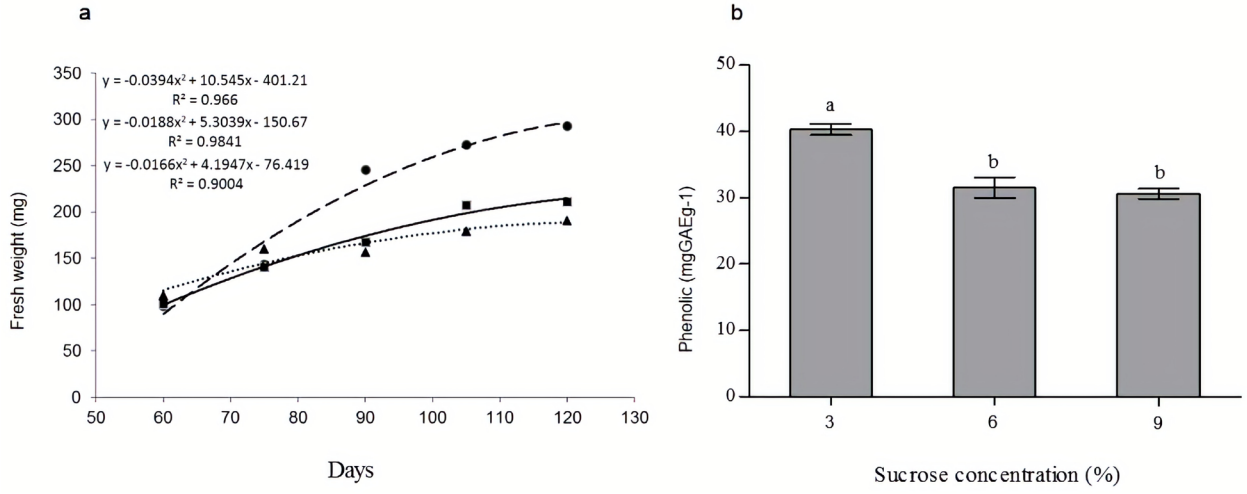

Figure 3. a) Growth parameters of *I. paraguariensis* callus culture supplemented with increasing sucrose concentrations. Callus growth was expressed as milligrams of fresh weight. b) Total phenolic accumulating after 120 days of culture of *I. paraguariensis*. Data are the mean ± SE (*n* = 10) ( ) 3% sucrose; ( ) 6% sucrose; ( ) 9% sucrose. Means with different letters are significantly different at the 5% level of probability using Tukey's multiple range test. The vertical bars represent the standard error of five replicates.

improve the accumulation of such compounds in the culture.

In *Ilex paraguariensis,* however, total phenols were not positively influenced by higher levels of sucrose, since the higher content was observed in calli supplemented with 3% sucrose (Figure 3b). Similar results were observed for biomass and phenolic compounds accumulation in *Hypericum perforatum* root cultures (Cui et al. 2010) and for *Prunella vulgaris* cell suspension cultures (Fazal et al. 2016), with maximum yields when treated with low sucrose concentrations.

In contrast, a 5% sucrose supply was shown to be optimal for enhancement of *Morinda citrifolia* root growth, but the maximum production of phenolics was achieved at 1% sucrose-treated culture (Baque et al. 2012). The results indicate that the optimal sucrose concentration for phenolic compounds accumulation is not always related to the biomass accumulation, which justifies species-specific studies to improve secondary metabolites yield.

Identification of secondary compounds from yerba mate callus culture

Six major phenolic constituents and two methylxanthines present in yerba mate callus cultures were identified by LC-MS-MS (Table II). Three caffeoylquinic acids were identified as 3-O-caffeoylquinic acid (chlorogenic acid), 4-O-caffeoylquinic acid (crypto-chlorogenic acid) and 5-O-caffeoylquinic acid (neochlorogenic acid). These compounds have been previously reported as major constituents of mate (Bravo et al. 2007).

Three dicaffeoylquinic acid isomers were also identified, corresponding to 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. The compounds identified as the major constituents in yerba mate callus culture in the present study have a significant potential for use in different industrial applications, such as pharmaceutical drugs, cosmetics and food preservatives. Caffeine and theobromine, for example, play an important role against physical and mental fatigue (Filip et al. 2001). Due to their higher antioxidant activity, the caffeoylquinic acids have a great range of applications in the pharmacy and cosmetic industries. In addition, caffeoylquinic acids have been recently reported to present digestive and hepatoprotective activities (Azzini et al. 2007).

Dicaffeoylquinic acids also show strong antioxidant activity and several potential uses. The 4,5-dicaffeoylquinic acid has been reported as an inhibitor of pigmentation and can be used to treat pigmentation disorders (Tabassum et al. 2016). The 3,5-dicaffeoylquinic acid, in turn, is a potent anti-inflammatory (Hong et al. 2015) and has been reported to inhibit the replication of the human immunodeficiency virus, HIV-1 (Zhu et al. 1999). In the same context, 3,4-dicaffeoylquinic acid has been reported as a potent lead compound for anti-influenza activity (Takemura et al. 2012). The presence of all these compounds in yerba mate callus culture makes it a potential source of raw-material for many industrial applications.

Sucrose effects on antioxidant activity and secondary compounds production

The compounds identified by LC-MS/MS were semi-quantified by HPLC-UV. Figure 4 shows a typical chromatogram of the extract obtained from callus, as well as the corresponding on line UV spectra. The compounds from peaks 1 and 3, were identified as theobromine and caffeine, respectively, which belong to the class of methylxanthines.

Three caffeoylquinic acids were identified as 5-O-caffeoylquinic acid (neochlorogenic acid), 3-O-caffeoylquinic acid (chlorogenic acid) and 4-O-caffeoylquinic acid (crypto-chlorogenic acid), respectively peaks 2,

Table II. Ion MS fragmentation data of secondary compounds in *I. paraguariensis* callus.

RT (min)	Compound	Formula	Found mass	MS–MS	Reference
4.39	Theobromine	$C_7H_8N_4O_2$	[M+1] 181.0723	67.02, 138.06, 163.06, 181.07	(Choi et al. 2013)
4.96	5-O-caffeoylquinic acid - **neochlorogenic acid**	$C_{16}H_{18}O_9$	[M-1] 353.0878	191, 135.04, 179.03, 134.03	Standard, (Carini et al. 1998, Dartora et al. 2011)
6.63	**4-O-Caffeoylquinic acid** -Cryptochlorogenic acid	$C_{16}H_{18}O_9$	[M-1] 355.1030	173, 179	Standard, (Carini et al. 1998, Dartora et al. 2011)
6.76	Chlorogenic acid (3-O-Caffeoylquinic acid)	$C_{16}H_{18}O_9$	[M-1] 353.0878	191, 135.04, 173.04	Standard, (Dartora et al. 2011)
6.85	Caffeine	$C_8H_{10}N_4O_2$	[M+1] 195.0878	138.06, 110.07, 83.06, 123.04, 195	Standard, (Choi et al. 2013).
13.19	3,4-*O*-[*E*]-dicaffeoylquinic acid (3,4-diCQA)-	$C_{25}H_{24}O_{12}$	[M-1] 515.1195	173.04 , 179.03, 191.05, 353.08, 135.04, 335.07, 160, 155.03, 137.02	(Dartora et al. 2011, Aboy et al. 2012)
13.92	3,5-*O*-[*E*]-dicaffeoylquinic acid (3,5-diCQA)	$C_{25}H_{24}O_{12}$	[M-1] 515.1195	191.05, 179.03, 353.08, 135.04	(Dartora et al. 2011, Aboy et al. 2012)
14.78	4,5-*O*-[*E*]-dicaffeoylquinic acid (4,5-diCQA)	$C_{25}H_{24}O_{12}$	[M-1] 515.1195	173.04, 179.03, 353.08, 191.05, 135.04	(Dartora et al. 2011, Aboy et al. 2012)

Samples analysed by HPLC-MS-MS in positive ion mode was used for theobromine and caffeine and negative mode for the other compounds.

4 and 5. Peaks 7, 8 and 9 correspond to the three dicaffeoylquinic acid isomers: 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, respectively.

Data show that antioxidant activities and the compounds caffeine and 3,5-dicaffeoylquinic are not significantly affected by changes in sucrose concentration (Figure 5a, c and d). 3,5-dicaffeoylquinic acid is a compound with strong antioxidant activity (Hong et al. 2015, Menin et al. 2013), and its amount in yerba mate callus culture was higher than other dicaffeoylquinic acids. This fact may justify the stability of the antioxidant activity as a function of sucrose concentration, regardless of the analytical method (Figure 5a, c and d). Both methods for measuring antioxidant potential used in this work (ABTS and DPPH assays) are known to be the easiest to implement and to yield the most reproducible results (Dudonne et al. 2009).

The accumulation of chlorogenic acid, neochlorogenic acid, crypto-chlorogenic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid in yerba mate callus was higher in the medium supplemented with 3% sucrose. Higher sucrose concentrations

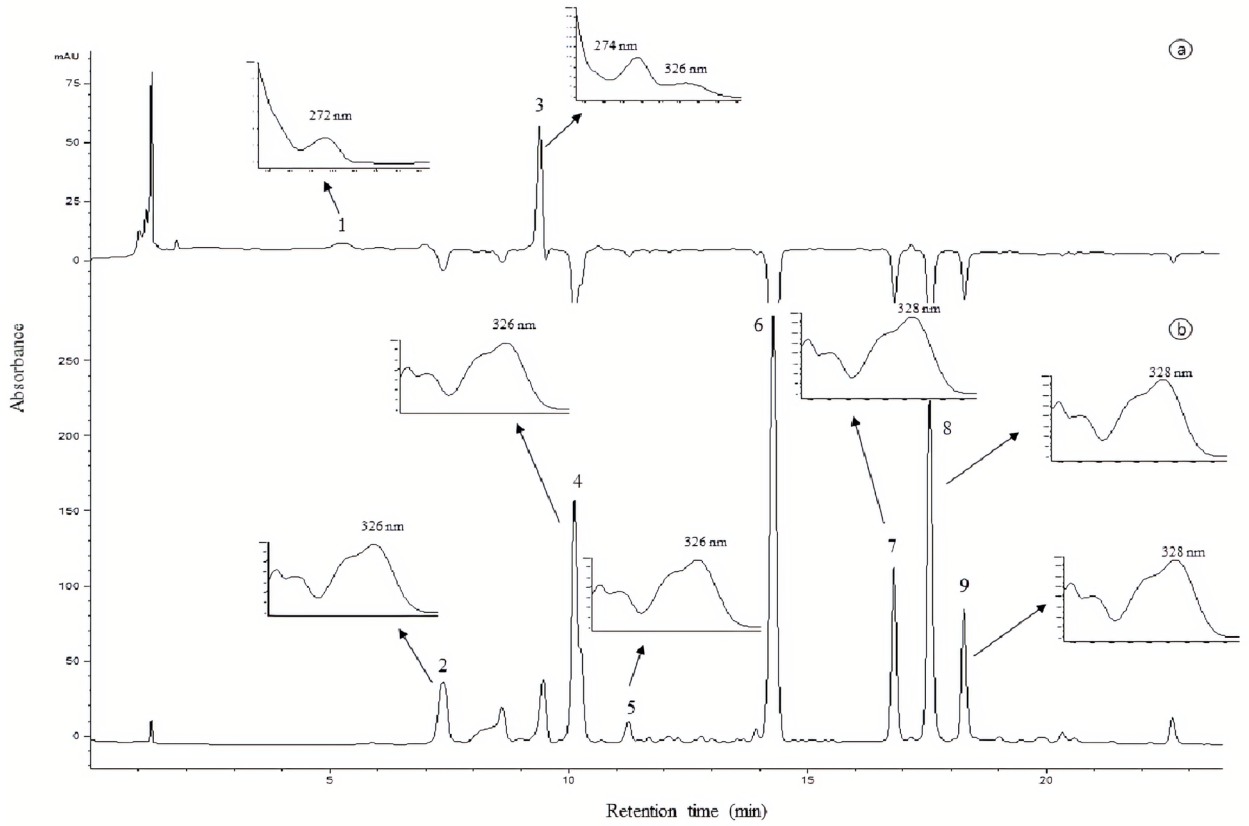

Figure 4. Typical HPLC chromatogram for I. paraguariensis callus extract. a) The UV spectra was 260 nm for caffeine and theobromine and b) 328 nm for caffeic acid derivatives. Peaks: (1) theobromine; (2) neochlorogenic acid; (3) caffeine; (4) chlorogenic acid; (5) cryptochlorogenic acid; (6) Internal standard umbelliferone; (7) 3,4-dicaffeoylquinic acid; (8) 3,5-dicaffeoylquinic acid; and (9) 4,5-dicaffeoylquinic acid.

inhibited the accumulation of these compounds (Figure 5c and d).

An opposite result was reported for *Eryngium planum* callus culture, where concentrations of 5 and 6% sucrose in the medium led to increasing chlorogenic acid contents (Kikowska et al. 2012). For *Gymnema sylvestre* cell suspension culture system, 3% sucrose favoured the accumulation of biomass, whereas the highest amount of gymnemic acid was accumulated at 4% sucrose (Nagella et al. 2011). In *Solanum aviculare* hairy root cultures, the optimum alkaloid content was obtained in medium supplemented with 6% sucrose (Yu et al. 1996).

In most of the cases, the enhanced induction of metabolites production is found to be associated with elevated levels of sucrose in culture media. A higher carbohydrate content in the medium might promote stress on plant cells and tissues, changing cellular metabolism, tissues growth and secondary compounds production (Cui et al. 2010). In yerba mate callus culture, however, this behaviour was not observed for any compound analysed (Figure 5).

These unusual results may be related to the class of compounds that higher sucrose concentrations can induce or hinder through metabolic alterations caused by osmotic or carbonyl stress. For example, the osmotic stress caused by sugars may favour the increase of the concentration of endogenous abscisic acid in cultured plant cells (Mishra & Singh 2016), which in turn regulates negatively the accumulation of some secondary compounds,

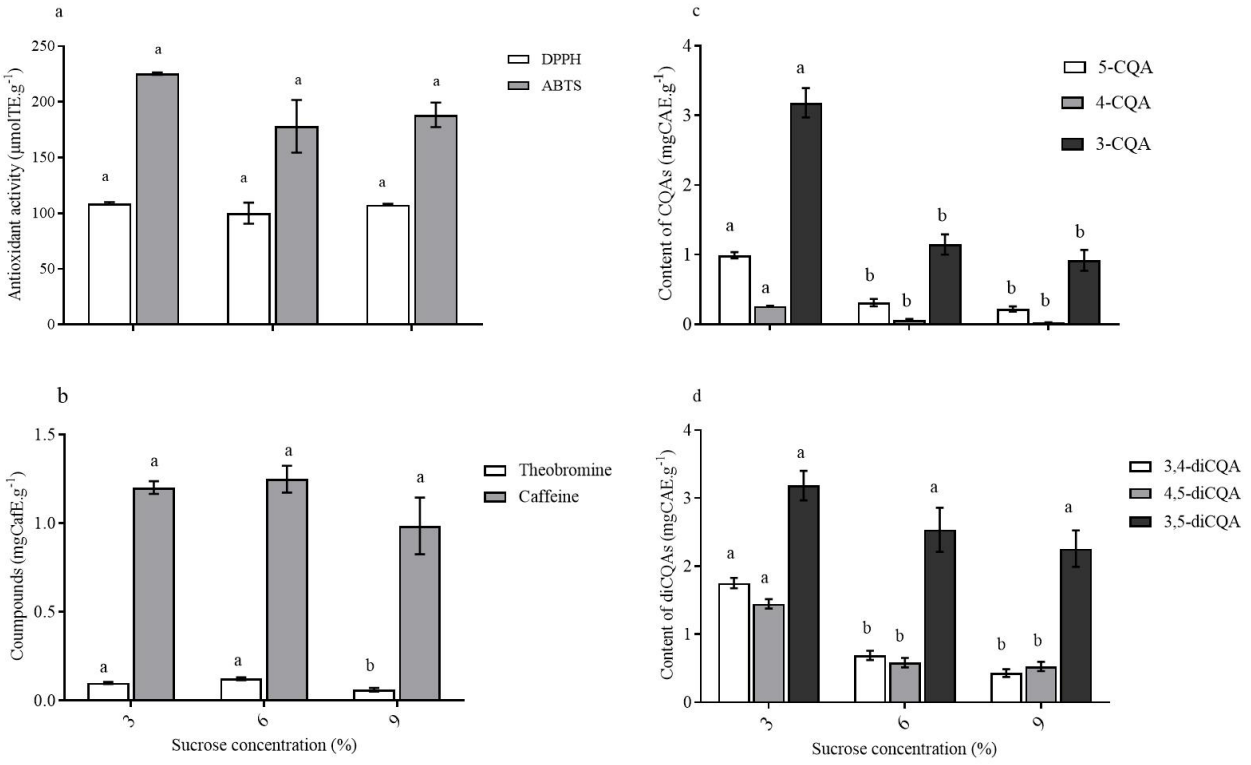

Figure 5. Effect of sucrose concentration on the: a) Antioxidant activity, b) Theobromine and caffeine, c) Chlorogenic acids and d) Dicaffeoylquinic acids of *I. paraguariensis* callus culture after 120 days. Means with different letters are significantly different at the 5% probability level according to the Tukey's multiple range test. The vertical bars represent the standard error of five replicates.

like phenylpropanoids (Graham & Graham 1996). In addition, the most suitable carbohydrate source and its optimal concentration should be identified for the production of secondary compounds in cell and callus cultures. These factors depend on plant species and compounds of interest. Therefore it is necessary to optimize the carbon sources in each case as suggested by Misawa (1994) and Murthy et al. (2014).

Sucrose at 3% concentration was efficient for the production of compounds with high added value (i.e. caffeoylquinic and dicaffeoylquinic acids), which may be used in different industrial segments. In this way, yerba mate callus culture might be considered as an alternative source of such compounds, allowing the continuous production through plant callus culture-based technology. New elicitors should be evaluated in order to improve the compounds accumulation and calli biomass.

CONCLUSIONS

The clone 6-156-6 is the most responsive genotype among the tested ones for callus induction. Medium supplemented with 4.56 μM zeatin and 4.52 μM 2,4-D is efficient for callus induction and biomass accumulation.

Medium supplementation with 3% sucrose results in higher calli biomass accumulation, and higher total phenolics and caffeoylquinic acids content.

Two methylxanthines, three caffeoylquinic acids and three dicaffeoylquinic acids were

identify as the major compounds in yerba-mate callus culture.

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