BOLETIN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
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Articulo Original / Original Article

Ilex paraguariensis: the effect of genotypes and growth phase on biomass, secondary metabolism and antioxidant activity of *in vitro* cultivated calli

[*Ilex paraguariensis*: el efecto de los genotipos y la fase de crecimiento sobre la biomasa, metabolismo secundario y la actividad antioxidante de los callos cultivados *in vitro*]

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Section
Biological activity

Received: 6 August 2020
Accepted: 24 November 2020
Accepted corrected: 14 December 2020
Published: 30 July 2022

Citation:
Grunennvaldt RL, Degenhardt-Goldbach J, Tomasi JC, Hansel FA, Brasileiro BP, Brooks P, Gomes EN, Rossa UB, Deschamps C. *Ilex paraguariensis*: the effect of genotypes and growth phase on biomass, secondary metabolism and antioxidant activity of *in vitro* cultivated calli **Bol Latinoam Caribe Plant Med Aromat** 21 (4): 548 - 560 (2022). https://doi.org/10.37360/blacpma.22.21.4.33

Abstract: Yerba mate (*Ilex paraguariensis*) produces several secondary metabolites of interest to the pharmaceutical industry, such as chlorogenic acids and methylxanthines. These compounds have been produced *in vitro* by callus culture from different species. However, for *I. paraguariensis*, no studies upon the production of these compounds *in vitro* have been performed to date. In this work, we show that the concentration of secondary metabolites from *I. paraguariensis* callus is possible and highly dependent on the callus growth phase. We observed that the best phase for the production of secondary compounds in calli of yerba mate is the stationary growth phase on both genotypes tested. In this phase, higher levels of phenolic compounds, chlorogenic acid and 3,5-dicaffeoylquinic acid and greater antioxidant activity were observed. Chlorogenic acid and 3,5-dicaffeoylquinic acid presented positive correlation with antioxidant activity. For the first time, secondary compounds were reported in yerba mate calli cultivated *in vitro*.

Keywords: Callus culture; Yerba mate; Methylxanthines; Stationary phase; Phenolic compounds

Resumen: La yerba mate (*Ilex paraguariensis*) produce varios metabolitos secundarios de interés para la industria farmacéutica, como los ácidos clorogénicos y las metilxantinas. Estos compuestos se han producido *in vitro* mediante cultivo de callos de diferentes especies. Sin embargo, para *I. paraguariensis*, hasta la fecha no se han realizado estudios sobre la producción de estos compuestos *in vitro*. En este trabajo, mostramos que la concentración de metabolitos secundarios desde callos de *I. paraguariensis* es posible y altamente dependiente de la fase de crecimiento del callo. Observamos que la mejor fase para la producción de compuestos secundarios en callos de yerba mate es la fase de crecimiento estacionario en ambos genotipos probados. En esta fase se observaron niveles más altos de compuestos fenólicos, ácido clorogénico y ácido 3,5-dicafeoilquínico y una mayor actividad antioxidante. El ácido clorogénico y el ácido 3,5-dicafeoilquínico presentaron correlación positiva con la actividad antioxidante. Por primera vez, se reportaron compuestos secundarios en callos de yerba mate cultivados *in vitro*.

Palabras clave: Cultivo de callos; Yerba mate; Metilxantinas; Fase estacionaria; Compuestos fenólicos

INTRODUCTION

Yerba mate (*Ilex paraguariensis*) is a plant of remarkable potential for uses in both the cosmetic and pharmaceutical industries, due to the production of valuable bioactive secondary metabolites, such as caffeine, theobromine and caffeoylquinic acids (Cardozo Junior & Morand, 2016).

One of the most popular non-alcoholic drinks in South America, yerba mate tea is becoming increasingly popular in Europe and the United States due to its health properties (Mejía *et al.,* 2010), mainly related to antioxidant activities, evidenced both in chemical and biological systems, including clinical trials (Gugliucci, 1996; Matsumoto *et al.,* 2009).

One major issue to the production of commercially important products in yerba mate and other species refers to the effects of seasonality and the environment on the accumulation of secondary metabolites (Esmelindro *et al.,* 2004; Freitas *et al.,* 2018). Due to the need for environmental control, the interest in the development of technologies for the production of phytochemicals *in vitro* has increased, and some companies have been using plant cells grown under controlled conditions for cosmetics manufacturing (PhytoCellTec, 2012; Trehran *et al*., 2017). In addition, several studies have been conducted on producing secondary metabolites from *in vitro* cultured cells and plant organs for health promotion (Verardo *et al.,* 2016). In this sense, callus culture of *I. paraguariensis* may represent a viable approach for the production of medicinally important compounds under controlled environmental conditions.

In the present study, we assessed the antioxidant activity as well as the contents of secondary compounds, such as polyphenols and methylxanthines, in calli from two genotypes of yerba mate (F1 and 6-156-) in different phases of growth, aiming to assess the feasibility of producing important secondary metabolites of yerba mate *in vitro* through callus culture.

MATERIAL AND METHODS

Plant material and growth conditions

Two elite clones, F1 and 6-156-6, from the breeding program developed by EMBRAPA Forestry (Brazilian Agricultural Research Corporation) (Resende *et al.,* 2000) were used as source of plant material for callus induction. The plants were grown under greenhouse conditions in mini-clonal hedges on a semi-hydroponic channel system with sand beds.

The 2nd or 3rd pair of young leaves from parent plants were collected and immersed in antioxidant solution (0.5 $g.L^{-1}$ of ascorbic acid and 0.5 $g.L^{-1}$ of citric acid). Subsequently, the leaves were washed with a neutral detergent in tap water, followed by immersion in Cercobin® fungicide solution (1 $g.L^{-1}$) for 10 min and immersion in mercury chloride (0.05%) for 5 min, followed by rinsing three times with sterilized water. The entire asepsis procedure was performed in a laminar flow hood.

Leaf discs of 2 cm diameter were cut with the aid of a metal punch and placed with the adaxial face in contact with ¼ MS media (Murashige & Skoog, 1964), plus 7 $g.L^{-1}$ agar, 3% sucrose, 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.56 μM zeatin, as described by Stachevski *et al*. (2013). The pH of the culture medium was adjusted to 5.8 ± 0.2, autoclaved and dispensed into Petri dishes (100 x 15 mm). Ten Petri dishes containing five leaf explants each were kept in the dark at 23 ± 2°C throughout the growing period, and the explants were transferred to fresh media every 30 days.

Analysis of calli growth

Calli growth curve was established by plotting fresh weight values of 10 calli measured every 15 days from time 0 to 150 days of culture. The percentage of callus growth was determined according to Santos *et al*. (2008) using the following equation:

$$\% \text{ growth} = [(Pf - Pi)/Pf)] * 100$$

where: *Pi* = initial weight and *Pf* = final callus weight.

Preparation of extracts from yerba mate calli

Leaves (initial condition) and callus at 75, 90, 105, 120, 135 and 150 days of culture were assessed regarding total phenolic compounds, antioxidant activity and the content of secondary metabolites. The experimental unit was composed of 10 Petri dishes with five calli per plate. In each period, three calli were randomly selected for preparation of the extracts. The leaves subjected to biochemical analysis were immediately immersed in liquid nitrogen after

harvest. All samples, leaves and calli, were stored in a freezer at -80°C until the preparation of the extracts. The samples were macerated in liquid nitrogen and then lyophilized for 72 h.

The lyophilized samples (10 $mg.mL^{-1}$ of callus and 5 $mg.mL^{-1}$ of leaves) were extracted with water: ethanol (1:1) solution. The extracts were vortexed for 30 sand subsequently sonication for 30 s. The extraction was performed for 1 h at 60ºC, with rotation at 450 rpm in Thermomixer® equipment. After this period the solution was centrifuged for 40 min at 13000 rpm. The solution was filtered through a 2 micron filter. Part of the solution was used for the biochemical analyses (phenolic compounds and antioxidant activity) while the other part (500 µL) was transferred into a vial, where, subsequently, 2.5 µL of the internal umbelliferone (10 $mg.mL^{-1}$) standard (Sigma®) was added for HPLC analysis.

Identification and quantification of secondary compounds by HPLC-UV

Chromatographic analyzes were conducted on a Shimadzu liquid chromatograph (UFLC), controlled by LC solution Software and equipped with automatic injector and UV detector (SPD-20A). The samples (20 µL) were separated on a Shim-Pack CLC- ODS (M) (250 x 4.6 mm.d.i. 5 µm) column, protected by a Shim-Pack CLC G-ODS (10 x 4.0 mm.d.i. 5 µm) pre column and with water (99%) acetic acid (1%) solution (solvent A) and 100% acetonitrile (solvent B) as the mobile phase. The flow rate was 0.5 $mL.min^{-1}$, with the following gradient mode: 0-15 min (3% B), 15-20 min (3%-20% B), 20-40 min (20% B), 40-45 min (20-30% B), 45-55 min (30%-100% B), 55-75 (100% B), 75-80 min (100-3% B) and 80-95 min (3% B).

The spectra of compounds were recorded at 280 nm, with the temperature at 30ºC. The identification of secondary compounds was carried out based on the retention time of corresponding external standards. The semi-quantification of the compounds was performed by the ratio between the analyte areas and the internal umbelliferone standard.

The sample concentration was expressed in mg of the compound per gram of dry sample ($mg.g^{-1}$). For the identification of the compounds in the extracts, standard solutions (Sigma®) of theobromine, caffeine, chlorogenic acid, and 3,5-dicaffeoylquinic acid were used.

The LC-MS/MS confirmation of the compounds utilized the SCIEX X500R QTOF system with Turbo V™ source and Electrospray Ionization (ESI) was used in positive and negative polarity. IS voltage was set to 5500 V. The extracts (2µL) were separated on LC Synergy Fusion-RP 80A (75 x 4.6 mm, d.i. 4µm) column, protected by LC pre column, using a mobile phase (A- water, acetonitrile, formic acid- 94.9:5:0.1 and B- acetonitrile, formic acid- 99:0.1), flow rate 0.5 $mL.min^{-1}$ in the following gradient mode: 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 min (0% B).

Determination of the total phenol content

The phenol content was determined by the spectrophotometric method of Folin-Ciocalteau (Horžić *et al.*, 2009) with modifications. In a glass test tube, 6.0 mL of water, followed by 0.5 mL of the Folin-Ciocalteau reagent were added to a 0.1 mL aliquot of the calli extract from each experimental unit. The mixture was then agitated and incubated for 5 min at room temperature. Thereafter, 2 mL of aqueous Na_2CO_3 (15%) solution was added to the mixture. The final volume was adjusted to 10 mL and, after 2 h, the samples absorbances were measured at 760 nm in Shimadzu-1800 UV/VIS spectrophotometer. The total phenol content was obtained through an analytical curve plotted with gallic acid in the range of 0.25-10 $mg.L^{-1}$. The results were expressed in milligrams of gallic acid equivalent per gram of dried plant extract ($mgGAE.g^{-1}$).

Determination of antioxidant activity

Leaves and calli extracts were tested for the scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, according to the method of Brand-Willians *et al*. (1995). The extract (1 mL) was added to 3.9 mL of ($6x10^{-5}$ $mol.L^{-1}$) DPPH methanol solution. The mixture was shaken vigorously and incubated for 30 min at room temperature. After this period, the absorbance was determined at 517 nm in a UV-VIS spectrophotometer (Shimadzu-1800).

The antioxidant activity was quantified through analytical curve of Trolox in the range of 0-1000 $mg.L^{-1}$. The results were expressed in µmoles of Trolox equivalents per gram of dried sample ($\mu molTE.g^{-1}$). The DPPH radical scavenging activity (*S%*) was calculated using the following equation:

$$S\% = ((A_{515(C)} - A_{515(A)})/ A_{515(C)}) *100$$

Where, $A_{515(C)}$ is the absorbance of the blank control (containing all reagents except the extract solution) and $A_{515(A)}$ is the absorbance of the test sample, both at 515 nm.

Experimental design and statistical analysis

The experimental design was completely randomized in a split-plot scheme to evaluate callus growth. Ten replicates were used to evaluate the fresh callus weight, with the two clones being the plots and the times in the subplots. The analysis of total phenolic compounds, antioxidant activity and secondary metabolites quantifications, was performed considering a completely randomized design in a 2x6 factorial scheme, with two clones and 6 culture times, with three replications. Variance homogeneity was assessed by the Bartlett test and the data were submitted to analysis of variance (ANOVA). When significant, the means were compared by Tukey's test (p<0.01 and p<0.05) d for the variable of callus fresh weight. Regression analysis (p<0.01 and p<0.05) was performed to the variables total phenolic compounds, antioxidant activity, and the relative percentage of theobromine, caffeine, chlorogenic acid and 3,5-dicaffeoylquinic. The T-test was performed to compare the accumulation of secondary compounds between the two clones. The statistical analyses were performed using the ASSISTAT® software (Silva & Azevedo, 2016).

RESULTS AND DISCUSSION

Calli growth curve

During the growth kinetics, calli of both F1 and 6-156-6 clones had a compact appearance and a light yellow color, which did not change throughout the growth period (Figure No. 1).

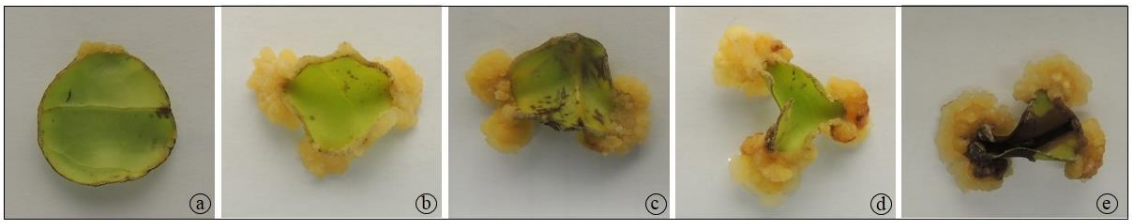

Figure No. 1
Aspects of yerba mate callus growth (6-156-6 clone). Appearance of the calli during the growth phases: a-lag, b- exponential, c- linear, d- deceleration and e-stationary

The F1 clone was more responsive to growth, with higher fresh weight from 45 days to the end of the growth period (p<0.01) (**Table No. 1**).

Table No. 1
Fresh weight of calli from F1 and 6-156-6 yerba mate clones at different growth periods

Clone	Callus fresh weight (mg) by period (days)											
	0	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**	**135**	**150**	**CV%**
F1	45.4 Af	77 Aef	118.1 Ade	138.2 Ad	149.4 Acd	196.9 Ac	328.2 Ab	380.2 Aab	400.2 Aa	410.2 Aa	415.2 Aa	58.36
6-156-6	51.1 Ad	68.3 Ad	78.2 Ad	88.6 Bcd	95 Bcd	143.8 Bc	245.8 Bb	273.3 Bab	293.3 Bab	303.3 Bab	308.3 Ba	18.96

Means followed by the same letter are not statistically different at 0.01 probability level according to Tukey test (uppercase letters for clones and lowercase for the time of growth)

Growth curves plotted with calli fresh weight presented a sigmoid pattern for both yerba mate genotypes (Figure No. 2). The curves show five distinct phases during the analyzed period (0 to 150 days): lag, exponential, linear, deceleration and stationary, as previously described by Smith (1992)

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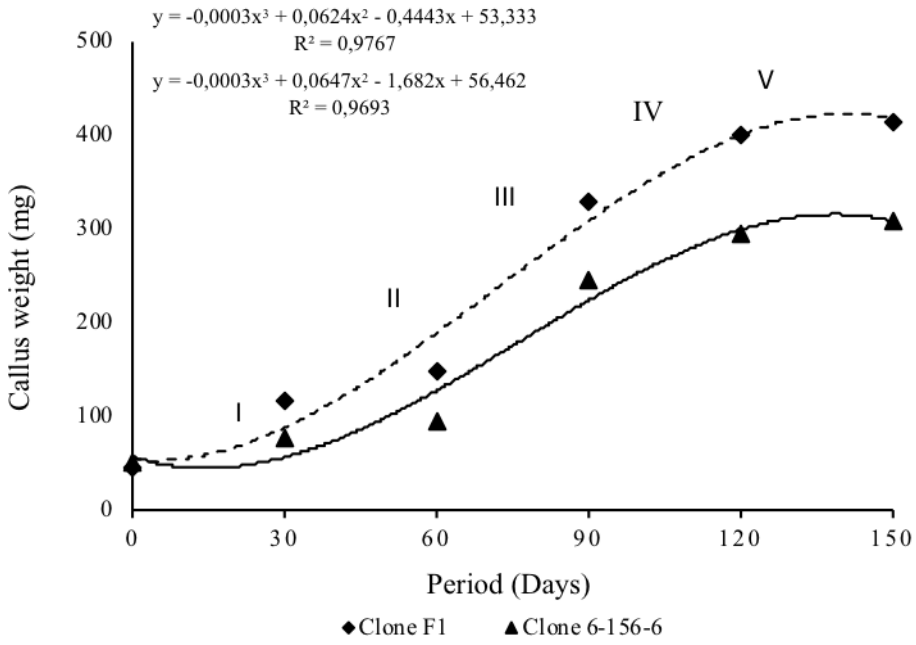


Figure No. 2
Growth curves of calli from two yerba mate clones. Phases: I – lag, II – exponential, III – linear, IV – deceleration, V - stationary

The lag phase (I) is the one in which the explant cells are prepared for cell division, characterized by the small accumulation of fresh weight in the calli. In the present study it was observed from day 0 until the 15th day on culture media, with relatively slow growth (F1: 41.0%, 6-156-6: 25.1%). The duration of the lag phase varies according to the species, taking, for example, up to 42 days in callus of *Coffea arabica* L. cultivar Rubi (Santos *et al.*, 2003) and 15 days for *Jatropha curca* callus (Feitosa *et al.*, 2013).

The exponential growth phase (II), period when the maximum cell division occurs, was observed from the 15th day to the 75th day of culture. A similar period was observed in *Jatropha curcas* callus (10th to 60th day) (Costa *et al.*, 2015). In this period, the largest increase in biomass occurred, 62.5% and 52.5% for F1 and 6-156-6 clones, respectively.

The linear phase (III), period in which there is decrease of the division and increase of the cellular area, was verified between the 75th and 105th days of culture. During this period, intense cellular proliferation was observed, and the increase in biomass was 45.9% and 47.3% for F1 and 6-156-6 clones, respectively. In the same phase, *Tabebuia roseo-alba* calli biomass increased by 57% (Abbade *et al.*, 2010). The deceleration interval (IV) was observed from the 105th to the 120th day, with low biomass increase (F1: 4.9%, 6-156-6: 6.8%).

The stationary phase (V) was observed between the 120th day and 135th day of culture (F1: 2.4%, 6-156-6: 3.2%). In *Jatropha curcas* calli, cultivated for secondary compounds, the stationary phase was observed in a similar period (120 to 130 days) (Costa *et al.*, 2015). After this period, there was no significant increase in the yerba mate callus biomass ($p > 0.01$. F1: 1.2%.6-156-6: 1. 6%).

Previous research suggested that the extraction of secondary metabolites from callus cultures should be carried out during the stationary phase, because in this period the production of the

primary metabolites (important for the cellular development) practically ceases and the secondary metabolites are produced (Smith, 1992).

Polyphenols and methylxanthines

Figure No. 3 shows the characteristic chromatogram of the hydro-alcoholic extracts of yerba mate calli and leaves. Compounds 1 and 3 were identified as theobromine and caffeine, respectively. These two compounds belong to the group of methylxanthines and are representative of yerba mate (Filip *et al.*, 1998).

Peaks 2 5, 6 and 7 were identified as chlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid, respectively. Chlorogenic acids and isomers of dicaffeoylquinic acid have been previously described in yerba mate and have been reported as the major constituents of its leaves (Filip *et al*., 2001; Bravo *et al.*, 2007). Rutin was identified in the leaves of both clones, at concentrations of 3.2 $mg.g^{-1}$ and 4.7 $mg.g^{-1}$, respectively for F1 and 6-156-6. However, in callus, the concentration of this compound was lower than 1 $mg.g^{-1}$.

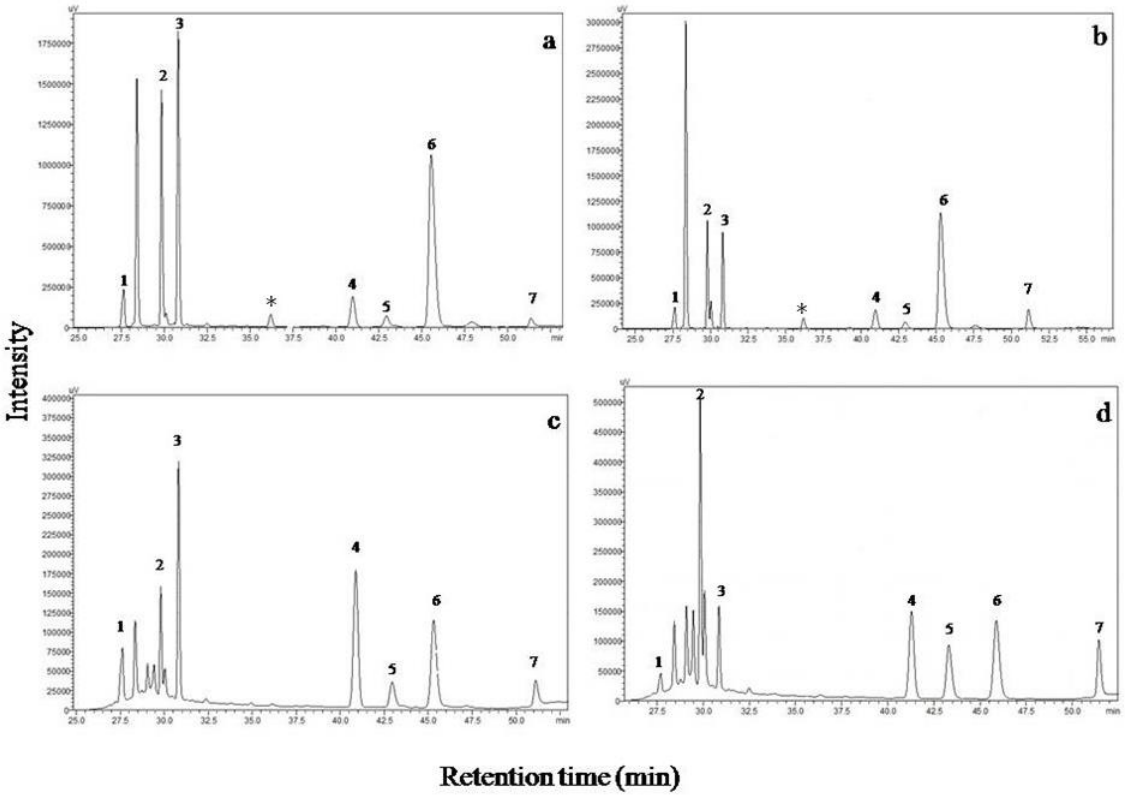


Figure No. 3
Chromatogram of the compounds present in: a) leaves of clone F1, b) leaves of clone 6-156-6, c) calli of clone F1 cultured for 120 days in culture medium, d) calli of clone 6-156-6 cultured for 120 days in culture medium. Retention time (min): 1- theobromine (27.5), 2- chlorogenic acid (29.8), 3- caffeine (30.8), 4-IS, umbelliferone (41.0), 5- 3,4-dicaffeoylquinic acid (42.8), 6- 3,5-dicaffeoylquinic acid (45.5), 7- 4,5-dicaffeoylquinic acid (51.2), * Rutin (36,5) - only detected in leaves

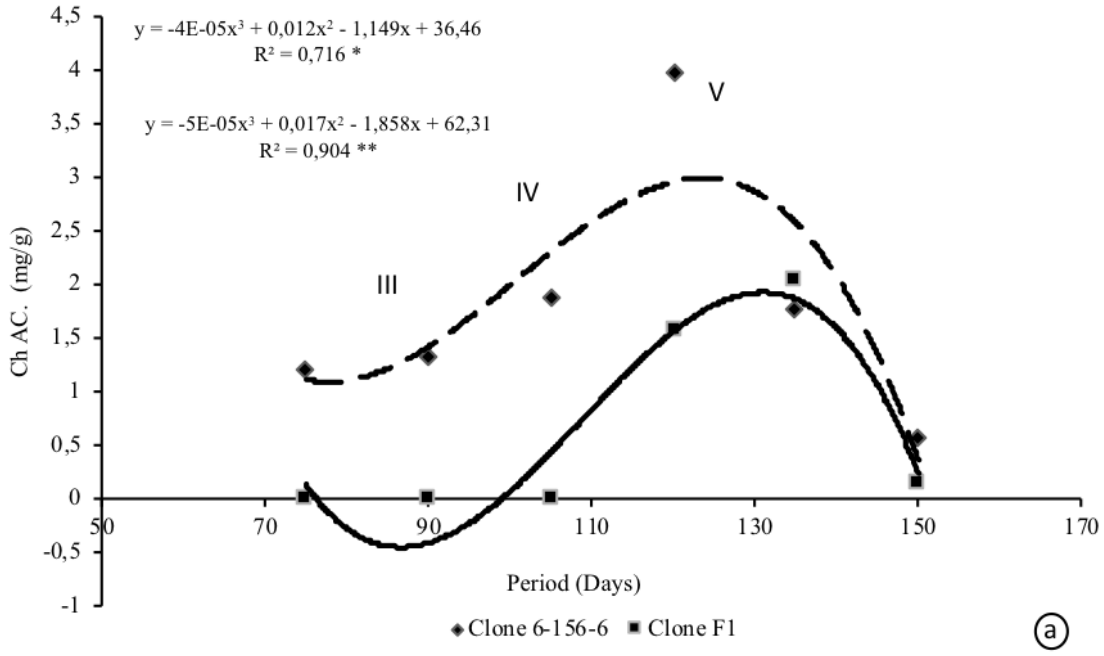
Culture time affected the contents of chlorogenic acid and 3,5-dicaffeoylquinic acid (p<0.05, Figure No. 4) in yerba mate calli as well as caffeine and theobromine, although it was not possible to adjust a significant regression model for these last two compounds. Caffeine was the most abundant secondary compound in calli of 6-156-6 clone, with an average of 9.11 $mg.g^{-1}$ at 135 days (Table No. 1). In calli of F1 clone, the most abundant compound was 3,5-dicaffeoylquinic acid, with 4.79 $mg.g^{-1}$ at 135 days of cultivation.

Table No. 2
Secondary compounds quantitative determination of yerba mate calli from two clones at different growth periods

Clone	Time (days)	Compound			
		Theo (mg.g-1)	3-CQA (mg.g^{-1})	Caf (mg.g^{-1})	3,5-diCQA (mg.g^{-1})
F1	75	1.03 ± 0.30	0.56 ± 0.12	1.44 ± 0.54	2.32 ± 0.28
	90	1.63 ± 0.25	0.33 ± 0.08	2.62 ± 0.15	0.71 ± 016
	105	1.08 ± 0.01	0.034 ± 0.01	1.42 ± 0.04	0.07 ± 0.09
	120	1.20 ± 0.04	1.57 ± 0.34	3.42 ± 0.58	3.89 ± 1.17
	135	1.42 ± 0.23	2.05 ± 0.87	4.25 ± 0.34	4.79 ± 0.06
	150	1.04 ± 0.26	0.15 ± 0.05	1.41 ± 0.31	0.39 ± 0.19
6-156-6	75	0.68 ± 0.04	1.18 ± 0.32	2.78 ± 0.27	4.27 ± 0.71
	90	0.84 ± 0.09	1.32 ± 0.68	3.09 ± 0.28	2.99 ± 1.51
	105	0.55 ± 0.10	1.87 ± 0.19	2.77 ± 0.29	2.79 ± 0.44
	120	0.58 ± 0.05	3.97 ± 1.57	2.10 ± 0.09	3.32 ± 0.77
	135	0.27 ± 0.06	1.76 ± 0.53	9.11 ± 0.60	5.96 ± 1.2
	150	0.51 ± 0.05	0.57 ± 0.27	3.13 ± 0.52	1.73 ± 0.22

Means ± Standard deviation. Theo= theobromine; 3-CQA= chlorogenic acid; Caf=caffeine; 3,5-diCQA = 3,5-dicaffeoylquinic acid

The lowest content of 3,5-dicaffeoylquinic acid in calli of F1 clone occurred at 105 days of culture, during the exponential growth phase. The contents were present in concentrations 68 times lower than those observed at the peak production. These results agree with those observed in callus culture of *Rosmarinus officinalis* during the linear growth phase, which resulted in low levels of rosmarinic acid (Yesil-celiktas *et al.*, 2007).



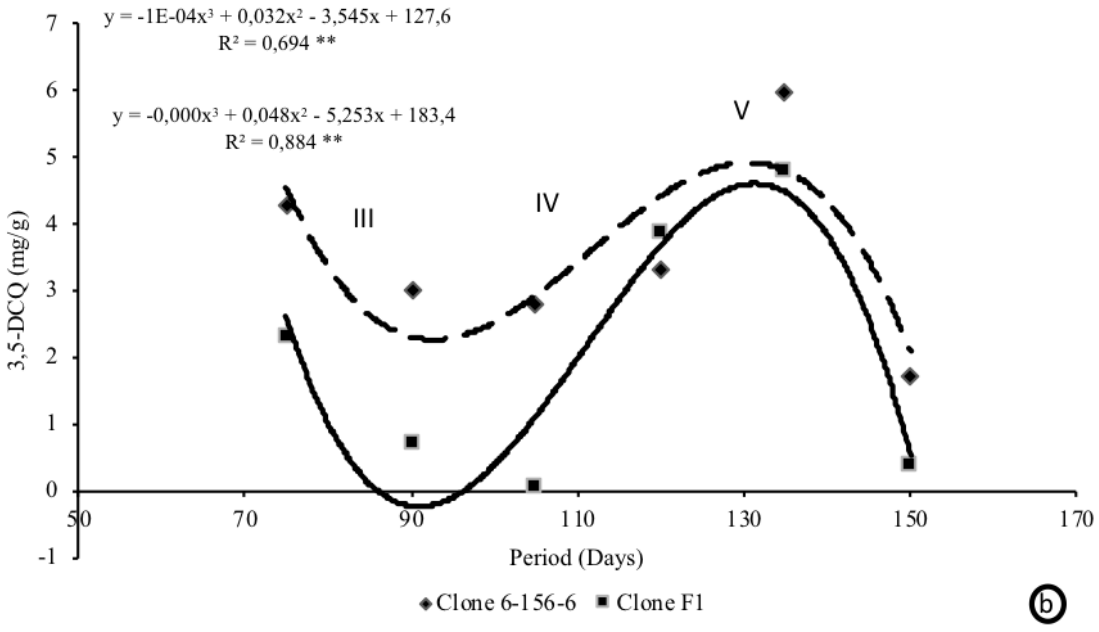


Figure No. 4
Content of chlorogenic acid (a) and 3,5-dicaffeoylquinic acid (b) in different growth periods in yerba mate calli
Phases: III - linear, IV - deceleration, V -stationary

For both chlorogenic and 3,5-dicaffeoylquinic acids in both clones, the highest production occurred during calli stationary growth phase, followed by a pronounced drop at 150 days. Due to the low growth presented it is possible to infer that calli in this period entered in a decline phase, characterized by loss of biomass loss, followed by cell death (Santos *et al.*, 2017). The chromatographic profiles of calli samples were similar, varying only on peak intensity as a function of the culture time (Figure No. 3). Compounds 5 and 7, at significant concentrations in calli of both clones, were identified by UV and LC-MS/MS as 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid, respectively (Grunennvaldt *et al.*, 2020).

Total phenolic compounds

Regardless of callus culture time, a significant difference was observed on phenolic compounds between clones: average of 43.3 mg GAE.g^{-1} for 6-156-6 clone and 39.3 mg GAE.g^{-1} for the F1 clone ($p < 0.01$). However, no significant difference ($p > 0.05$) was observed on total phenolic compounds in leaves of the two clones of yerba mate (average 157.35 mgGAE.g^{-1}).

The lower total phenolic values in calli compared to leaves can be explained by the loss of tissue differentiation during callus formation, along with the brief stationary phase that callus cultures present. Besides this, an inhibition or insufficient distribution of enzymes necessary for the synthesis of secondary compounds in the cells of the cultivated callus may occur (Muhitch & Fletcher, 1985; Amaral & Silva, 2003).

Total phenolics in calli of 6-156-6 clone remained constant until 105 days, followed by increase up to 135 days and a later decrease at 150 days. For calli of F1 clone, total phenolic content decreased 1.2 times at 105 days, when compared to 90 days of growth (Figure No. 5). At 105 days, calli were in the linear phase of growth, characterized by a greater demand of carbon and energy, and, therefore, the biosynthesis of primary metabolites was possibly favored in detriment of secondary compounds (Santos-Gomes, 2003).

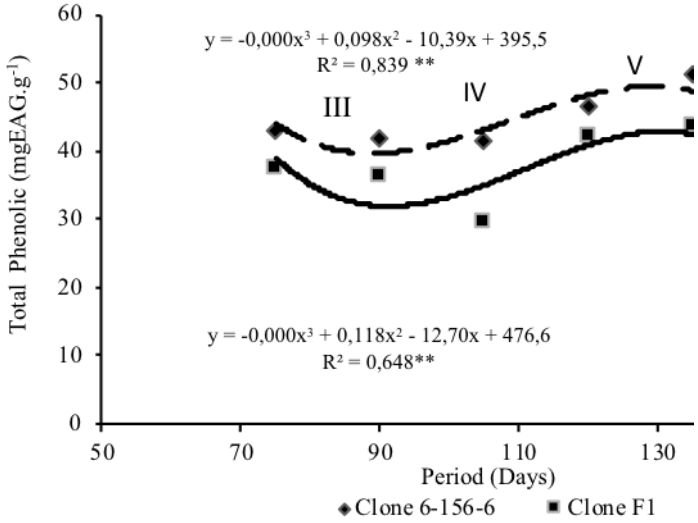


Figure No. 5
Content of total phenolic compounds in different growth periods of yerba mate callus culture. Phases: III - linear, IV - deceleration, V - stationary

In both clones, the highest production of phenolic compounds occurred around 135 days (F1: 43.7 mgGAE.g^{-1}; 6-156-6:51.1 mgGAE.g^{-1}), in the stationary phase. Typically, the peak production of secondary metabolites occurs during the late stationary phase and is associated with inhibition of cell growth and the production of secondary metabolism enzymes (Pradeep *et al.*, 2015; Ochoa-Villarreal *et al.*, 2016).

Antioxidant activity

On average, calli of 6-156-6 clone showed antioxidant activity 1.4 times higher than F1 genotype ($p < 0.01$). The highest antioxidant activity was observed at 120 days of calli growth, for both clones, with an average of 89.4% of DPPH radical scavenging activity. For leaves, no difference ($p > 0.05$) was observed between the 6-156-6 and F1 genotypes (92.2%). Leaves had higher antioxidant activity (216.7 µmolTE.g^{-1}) than 120-day calli (104.6 µmolTE.g^{-1}). A similar result was observed in *Cynara cardunculus* leaves, which showed higher antioxidant activity than calli of the same species (Trajtemberg *et al.*, 2006).

Antioxidant activity across culture time presented different tendecies for the two clones (Figure No. 6). Calli of 6-156-6 clone showed constant activity up to 135 days, with a decline of 1.5 times at 150 days compared to the period of greatest radical scavenging capacity.

Calli of the F1 clone presented a decline in antioxidant activity at 90 days (1.2 times) followed by an abrupt decline at 105 days. Subsequently, an increase in antioxidant activity of almost 8 times was observed, with the greatest DPPH radical scavenging activity at 120 days. In both genotypes, the highest free radical scavenging activity occurred during the calli stationary phase, at which both clones presented similar activity, around 100 µmol.g-1TE.

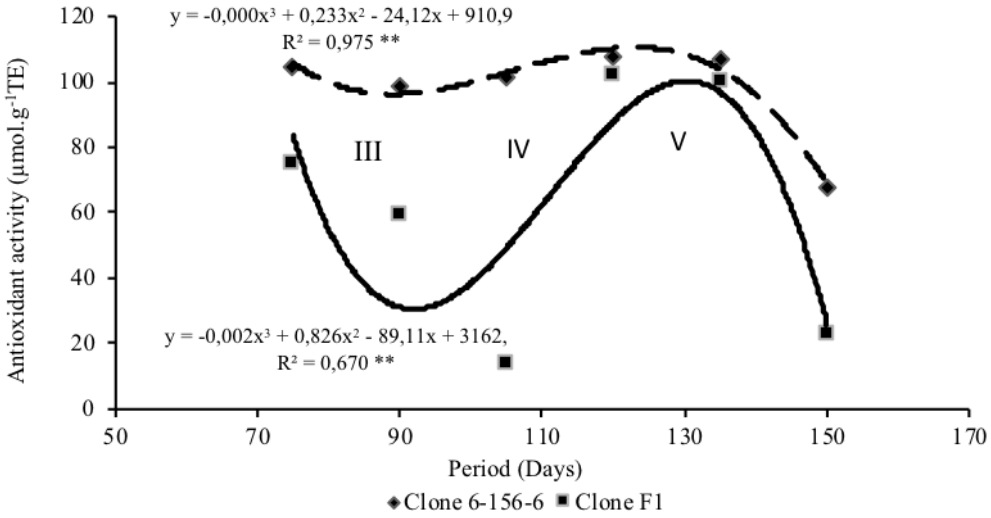


Figure No. 6
DPPH radical scavenging activity in different growth periods of yerba mate calli.
Phases: III - linear, IV - deceleration, V -stationary

Correlation analysis

Table No. 2 shows the Pearson correlation coefficients between the variables assessed in this work. There is an obvious positive correlation between total phenolic compounds and antioxidant activity. The antioxidant properties of polyphenols are attributed to their redox properties, acting as reducing agents, hydrogen donators, metal chelators and single oxygen quenchers (Piluzza *et al.*, 2011). Chlorogenic acid is one of the phenolic compounds observed in callus cultures of yerba mate, which have been previously reported as largely responsible for the antioxidant potential of *I. paraguariensis* extracts (Anesini *et al.*, 2012).

In the present study, however, the positive correlation between 3,5-dicaffeoylquinic acid and antioxidant activity was higher than the correlation observed between chlorogenic acid and free radical scavenging capacity (Table No. 2). This may be due to the fact that dicaffeoylquinic acids have two phenolic rings compared to one in monocaffeoylquinic acids such as chlorogenic acid (Wang *et al.*, 2003).

A positive correlation between 3,5-dicaffeoylquinic acid content and caffeine was also observed. Caffeoylquinic acids are capable of sequestering caffeine in the vacuole and forming complexes with phenylpropanoids (Waldhauser & Baumann, 1996). These phenomena may also explain the observed correlation of caffeine with the total phenolic content.

Table No. 2
Correlation among secondary compounds, total phenolic content and antioxidant activity of *Ilex paraguariensis* calli cultured *in vitro*

	3-CQA ($mg.g^{-1}$)	Caf ($mg.g^{-1}$)	3,5-diCQA ($mg.g^{-1}$)	DPPH ($\mu mol.g^{-1}TE$)	TPC ($mgEAG.g^{-1}$)
Theo ($mg.g^{-1}$)	-0.128	-0.394	-0.386	-0.362	-0.490
3-CQA ($mg.g^{-1}$)		0.077	0.455	0.676	0.680
Caf ($mg.g^{-1}$)			0.745	0.490	0.728
3,5-diCQA ($mg.g^{-1}$)				0.875	0.931
DPPH ($\mu mol.g^{-1}TE$)					0.921

Theo = theobromine; 3-CQA= chlorogenic acid; Caf=caffeine; DPPH= antioxidant activity; 3,5-diCQA = 3,5-dicaffeoylquinic acid; TPC= total phenolic

The interest for natural products is growing due largely to the restrictions on addition of synthetic compounds in drugs, cosmetics and foods (Ochoa-Villarreal *et al.*, 2016). Although there is apparently lower accumulation of secondary compounds in callus compared to yerba mate leaves, the production under *in vitro* conditions can be widely applied in pharmaceutical compositions, with the advantage of having the controlled production of these natural molecules throughout the year.

CONCLUSION

Extracts from yerba mate calli have potential for the production of valuable compounds, such as chlorogenic acid and 3,5-dicaffeoylquinic acid, with high antioxidant activity. Calli stationary growth phase is when the highest content of these compounds occurs.

There is a difference between genotypes for the production of secondary compounds in yerba mate calli. Calli of 6-156-6 clone have a higher accumulation of caffeine, chlorogenic acid, and 3,5-dicaffeoylquinic acid, whereas, calli of F1 clone accumulate more theobromine.

The leaves of clones 6-156-6 and F1 do not differ in the concentration of secondary compounds, although the content of these compounds was higher in the leaves than in the calli. Yerba mate calli are capable of producing secondary compounds even under favorable conditions for growth and future studies focusing on increasing the production of these compounds by elicitation should be carried out.

ACKNOWLEDGEMENTS

The authors would like to thank the Federal University of Parana, the University of the Sunshine Coast, and the Brazilian Agricultural Research Corporation (EMBRAPA – Forestry). We also thank Trong Tran for the support with the LC-MS-MS analyses.

This work was supported by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) (PDSE/proc. Nº 88881.131659/2016-01 and DOC_PLENO/proc. Nº 88881.129327/2016-01).

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