UNIVERSIDADE FEDERAL DO PARANÁ

RENATA LÚCIA GRUNENNVALDT

PRODUCTION OF SECONDARY COMPOUNDS IN *Ilex paraguariensis* A. St. Hill. CALLUS CULTURE

CURITIBA

2018

RENATA LÚCIA GRUNENNVALDT

PRODUCTION OF SECONDARY COMPOUNDS IN *Ilex paraguariensis* A. St. Hill. CALLUS CULTURE

Tese apresentada ao Programa de Pós-Graduação em Agronomia, área de concentração em Produção Vegetal, Departamento de Fitotecnia e Fitossanitarismo, Setor de Ciências Agrárias, Universidade Federal do Paraná, como parte das exigências para obtenção do título de Doutor em Ciências.

Orientador: Dr. Cícero Deschamps
Co-orientadora: Drª. Juliana Degenhardt-Goldbach

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RESUMO

PRODUÇÃO DE COMPOSTOS SECUNDÁRIOS EM CALOS DE *Ilex paraguariensis* A. St. Hill.

A espécie *Ilex paraguariensis* A. St. Hill. (erva-mate) fornece uma ampla variedade de compostos bioativos, e a cultura de calos *in vitro* é uma alternativa para a produção desses compostos em condições otimizadas e controladas. O objetivo deste trabalho foi avaliar a produção de compostos secundários na cultura de calos de erva-mate. Para isso, avaliou-se o potencial de indução da calogênese de dez clones. Posteriormente, o clone mais responsivo foi utilizado para avaliar o efeito de diferentes concentrações de citocininas e sacarose no crescimento. O efeito da sacarose também foi verificado na produção de compostos secundários, compostos fenólicos e atividade antioxidante de calos de erva-mate. Após otimizar as condições de cultura, dois clones foram selecionados para verificar a cinética de crescimento dos calos e o efeito da radiação ultravioleta no acúmulo de fenólicos totais e compostos secundários. Observou-se que o clone 6-156-6 foi mais responsivo à indução de calos (77%). O uso de 4,56 µM de zeatina associada a 4,52 µM de 2,4-D e 3% de sacarose apresentou maiores médias de peso fresco. Maiores rendimentos de fenólicos totais e compostos secundários foram observados em calos crescidos em meio de cultura contendo 3% de sacarose. A atividade antioxidante, os compostos cafeína e ácido 3,5-dicafeoilquínico não foram afetados pela alteração das concentrações de sacarose. Cinco fases de crescimento foram observadas em calos da erva-mate (lag, linear, exponencial, desaceleração e estacionária). O acúmulo de compostos fenólicos, atividade antioxidante e compostos secundários foi maior durante a fase estacionária de crescimento, na qual recomenda-se a extração de tais compostos. Os resultados demonstraram que a resposta à radiação UV-C é genótipo-dependente e o conteúdo de fenólicos totais e compostos secundários depende dos tempos de exposição e incubação. Nas condições testadas, a radiação UV-C não foi eficiente para aumentar a produção de compostos secundários em calos de *Ilex paraguariensis*. Este estudo descreveu pela primeira vez a produção e identificação de compostos secundários de calos de erva-mate cultivados *in vitro*, sendo os principais compostos teobromina, cafeína, ácido clorogênico, ácido criptoclorogênico, ácido neoclorogênico, ácido 3,4-dicafeoilquínico, ácido 3,5-dicafeoilquínico e ácido 4,5-dicafeoilquínico. Em síntese, as condições de crescimento dos calos foram otimizadas, a cinética de crescimento foi entendida e foi possível produzir compostos secundários a partir de calos de erva-mate sob condições controladas. Finalmente, esse procedimento também pode ser usado como uma ferramenta para compreender a via metabólica desses importantes compostos na erva-mate.

Palavras-chave: *Ilex paraguariensis*, genótipo, fenólicos totais, elicitação HPLC, metabólitos secundários.

ABSTRACT

The species *Ilex paraguariensis* A. St. Hill. (yerba mate) provide a wide variety of bioactive compounds, and the *in vitro* callus culture is an alternative for the production of these compounds in an optimized and controlled condition. The objective of this study was to evaluate the production of secondary compounds in yerba mate callus culture. For this, the callogenesis induction potential of ten clones was evaluated. Subsequently, the most responsive clone was used to evaluate the effect of different cytokinins and sucrose concentrations on growth. The effect of sucrose was also verified on the production of secondary compounds, phenolic compounds and antioxidant activity of yerba mate callus. After optimizing the culture conditions, two clones were selected to verify the kinetics of callus growth, and the effect of ultraviolet radiation on the accumulation of total phenolic and secondary compounds. It was observed that clone 6-156-6 was more responsive to callus induction (77%). The use of 4.56 µM zeatin associated with 4.52 µM 2,4-D and 3% sucrose exhibited higher averages of callus fresh weight. Higher yields of total phenolic and secondary compounds occurred in callus grown using 3% sucrose in the culture medium. The antioxidant activity, caffeine, and 3,5-dicaffeoylquinic acid were not affected by the alteration of sucrose concentrations. Five growth stages were identified in yerba mate callus (lag, linear, exponential, deceleration and stationary). The accumulation of phenolic compounds, antioxidant activity, and secondary compounds was higher during the stationary phase of growth, which it is recommended the extraction of such compounds. The results demonstrated that the response to UV-C radiation is genotype dependent and the content of total phenolic and secondary compounds depends on the exposure and incubation times. Under the tested conditions, UV-C radiation was not efficient to increase the production of secondary compounds in *Ilex paraguariensis* callus. This study described for the first time the production and identification of secondary compounds from yerba mate callus *in vitro* cultivated, being the major compounds theobromine, caffeine, chlorogenic, cryptochlorogenic, neochlorogenic, 3,4-dicaffeoylquinic, 3,5-dicaffeoylquinic and 4,5-dicaffeoylquinic acid. In resume, the callus growth conditions were optimized, growth kinetics was understood and it was possible to produce secondary compounds from yerba mate callus under controlled conditions. Finally, such procedure can also be used as a tool to understand the metabolic pathway leading to these important compounds in the yerba mate.

Keywords: *Ilex paraguariensis*, genotype, total phenolics, elicitation, HPLC, secondary metabolites.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Centigrade
µg	Microgram
µm	Micrometre
µmolTE.g^{-1}	µmoles Trolox equivalent per gram
2,4-D	2,4-Dichlorophenoxyacetic acid
2ip	2-isopentenyladenine
3,4-diCQA	3,4-dicaffeoylquinic acid
3,5-diCQA	3,5-dicaffeoylquinic acid
3-CQA	Chlorogenic acid
4,5-diCQA	4,5-dicaffeoylquinic acid
4-CQA	Cryptochlorogenic acid
5-CQA	Neochlorogenic acid
ABTS	2,2`-Azinobis (3-ethylbenzothiazoline 6-sulphonic acid)
CAE	Chlorogenic acid equivalent
CafE	Caffeine equivalent
cm	Centimetre
CQA	Caffeoylquinic acids
d.i.	Diametre
DPPH	2,2-diphenyl-1-picrylhydrazyl
DQA	Dicaffeoylquinic acids
DW	Dried weight
EMBRAPA	National Centre of Forestry Research
FC	Folin-Ciocalteu reagent
FW	Fresh weight
g	Grams
GAE	Gallic acid equivalent
h	Hour
HPLC	High-performance liquid chromatography
L	Litre
LC-MS/MS	Liquid chromatography–Mass spectrometry
mg	Milligrams
min	Minute
mL	Millilitre
mm	Millimetre
MS	Culture medium Murashige and Skoog
Na_2CO_3	Sodium carbonate
nm	Manometre
pH	Potential of hydrogen
rpm	Rotation per minute
Rt	Retention time
s	Second
SD	Standard deviation
SE	Standard error
TDZ	Thidiazuron
TPC	Total phenolic content

UV	Ultraviolet
V	Volt
w/v	Weight per volume
µL	Microlitrer
µM	Micromolar

CONTENTS

1 INTRODUTION

The *Ilex paraguariensis* (yerba mate), a native plant from South America (CARVALHO, 1994), has been consumed in the form of beverages made from the aqueous extracts of the dried leaves and the stem by more than 1 million people worldwide, in South America, and more recently, in North America and Europe as a tea (BRACESCO et al., 2011; ALKHATIB, 2014; LUDKA et al., 2016).

The species has been widely studied due to its beneficial properties (COLPO et al., 2016), being used for its antiobesity action (ARÇARI et al., 2009), antimutagenic activities (MIRANDA et al., 2008), antidepressive, neuroprotective (LUDKA et al., 2016) and antioxidant action (BOAVENTURA et al., 2015).

These properties are related to the content of the secondary compounds of the plant. The species is rich in polyphenols derived from a caffeic acid (chlorogenic acid, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, and 3,5-dicaffeoylquinic acid), as well as caffeine that plays a role in the stimulant activity of the species (BASTOS et al., 2007; HECK & De Mejia 2007; ALKHATIB, 2014).

These compounds can be used as food conservation (FACCIN et al., 2015), pharmaceutical industry, alternative medicine, nutraceutical industry, (HAO et al., 2013); (BOAVENTURA et al., 2015), and on the development of cosmetics (BARROS et al., 2011).

However, obtaining natural compounds is often a challenge due to the slow growth of some species, the low yields found in nature and especially the variable concentration of the compounds (KOLEWE et al., 2008). In this way, callus and plant cell culture technology becomes a promising alternative for the production of interest compounds. It is possible since plant cells are totipotent, and capable of producing the chemical compounds found in the mother plant (ZHOU; WU, 2006).

In order to produce compounds of interest in callus culture, some characteristics in the behaviour of the species must be observed, making it necessary to optimize the culture conditions and determine the ideal growth phase for the extraction of compounds (SMITH, 1992). However, in many species, the production of compounds through callus cultivation is low in comparison to the parent plant. In this case, the use of elicitors that stimulate the production of compounds consists of a possible alternative (ALVERO-BASCOS; UNGSON, 2012).

Bearing this in mind, the general objective of this work was to evaluate the production capacity of secondary compounds from callus culture of *Ilex paraguariensis*.

The present thesis is divided into five chapters, chapter 2 referring to the literature review, chapters 3, 4 and 5 contemplating presentation, results, and discussion of the data obtained in the experiments and the sixth chapter brings the final considerations.

The literature review aims to support the understanding of the work and the discussion of the topics covered in each chapter, highlighting general information about the species studied, secondary metabolism of *Ilex paraguariensis*, cultivation of plant cells for the production of these compounds, as well as their application, and the use of elicitors as a strategy to increase the production of secondary compounds.

Chapter 3 consists of the presentation of the experiments to optimize the induction and growth of callus from yerba mate leaves and to evaluate the productive capacity of secondary compounds of this callus, as well as to identify the present compounds and to quantify them.

Chapter 4 is about the influence of different growth phases of callus on the production of secondary compounds and chapter 5 refer to the influence of UV-C radiation on the accumulation response of secondary compounds in yerba mate callus culture.

The final considerations summarize the main responses obtained with the development of the work and suggest future studies to improve the production of compounds from yerba mate callus.

2 LITERATURE REVIEW

2.1 *Ilex paraguariensis* A. ST. HILL.

The *Ilex paraguariensis* A. St. Hill also known as yerba mate, is a tree belonging to the family Aquifoliaceae, native to South America. It has a variable height, reaching about 3-5 m when cultivated, but in the forest, it can reach 25 m height. The leaves have obovate limbus with 5 to 10 cm long by 3 to 4 cm wide (CARVALHO, 1994).

Yerba mate consumption is associated with a wide range of health effects, and is higher in South American countries (MATEI et al., 2016). The largest producers are Argentina (800 thousand ton/year), followed by Brazil and Paraguay with respectively 600 and 90 thousand ton (FAO, 2014, INYM, 2014). Uruguay has the highest per capita consumption (8-10 kg/hab/year), followed by Argentina (6.5 kg/hab/year) and Brazil (3-5 kg/hab/year) (CARDOZO JUNIOR; MORAND, 2016).

The most popular beverages prepared from yerba mate are chimarrão, tererê dry green leaves prepared with hot or cold water, respectively, and mate tea (toasted leaves infused with hot water) (BRACESCO et al., 2011). Due to its flavour and stimulating properties, the consumption of yerba mate products has expanded to different countries (Spain, Italy, Australia, France, Japan, South Korea and Russia) (CARDOZO JUNIOR; MORAND, 2016). Recently, this plant has been exported in the form of extracts to be used in different functional foods and phytopharmaceutical preparations for the USA, Europe and Asia (MÁRQUEZ et al., 2013; BOAVENTURA et al., 2015).

In addition to the well-known use as a beverage, several applications have already been reported for yerba mate: highlighting the potentiality of food conservation (FACCIN et al., 2015), in the pharmaceutical industry, alternative medicine, nutraceutical industry (HAO et al., 2013; BOAVENTURA et al., 2015) animal supplementation (CELI, 2013) and cosmetics specifically designated for photoprotection (BARROS et al., 2011).

For commercialization, a number of yerba mate products have already been placed and are available on the market, including medicines recommended for

fatigue and weakness (THEODORA, 2017), weight reduction capsules (PHARMATERRA, 2017), cosmetics (SEIVAILEX, 2017) and energy (GUAYAKI, 2017).

Despite the good acceptance of the plant in the market, new studies are looking for more benefits and applications. For example, the reports vary from antifungal activities (FILIP et al., 2010), antibacterial (CARELLI et al., 2011), antiobesity action (ARÇARI et al., 2009); (GAMBERO et al., 2010), antimutagenic (MIRANDA et al., 2008), specifically inhibiting the proliferation of cancer cells in the uterine cervix (MEJÍA et al., 2010), antiflamatory (PUANGPRAPHANT et al., 2011; SOUZA et al., 2015), to antidepressive and neuroprotective activity (LUDKA et al., 2016).

Its medicinal properties are related to the main constituents of the leaves, which are polyphenols, xanthines, saponins, flavonoids (FILIP et al., 2001), amino acids, minerals (P, Fe, and Ca) and vitamins C, B1, and B2 (HECK; MEJIA, DE, 2007). The stimulant properties are due to the presence of methylxanthines, mainly caffeine. The cited organic compounds present in the plant and responsible for the previously mentioned activities belong to the class of secondary compounds.

2.2 SECONDARY COMPOUNDS OF YERBA MATE

The main compounds in yerba mate belong to the class of phenylpropanoids also called phenolic compounds. They consist of one of the most widely distributed groups of secondary compounds in the plants having more than 10,000 different structures, including phenolic acids such as benzoic and hydroxycinnamic, flavonoids such as flavones, stilbenes and lignins (ANDRÉ et al., 2009).

In the plants, they are located in different cellular organelles (AGATI et al., 2012), in which they perform functions of phytoalexins, ultraviolet light (UV) protectors, pigments, signalling molecules and mechanical resistance (cell wall components) (VOGT, 2010). In addition, phenolic compounds act against oxidative damage induced by stress (FINI et al., 2012) and their induction occurs in response to biotic and abiotic stimuli, such as UV, dry, cold, heavy metals, pathogen attack, injury or nutrient deficiency (GRACE, 2005).

Phenolic compounds are produced through the phenylpropanoid pathway (ANDRÉ et al., 2009), which generally generates a huge variety of secondary compounds, having shiquimate pathway as the core unit (VOGT, 2010) (Figure 1). Phenylalanine is the precursor aromatic amino acid of most phenolic compounds, and by the action of the enzyme phenylalanine ammonia-lyase (PAL) it loses one molecule of ammonia, producing cinnamic acid (TAIZ; ZEIGER, 2013). The resulting hydroxycinnamic acids and esters are amplified in various cascades by combining with reductases, transferases and oxygenates, resulting in thousands of compounds, many of which are specific for certain plant species (VOGT, 2010).

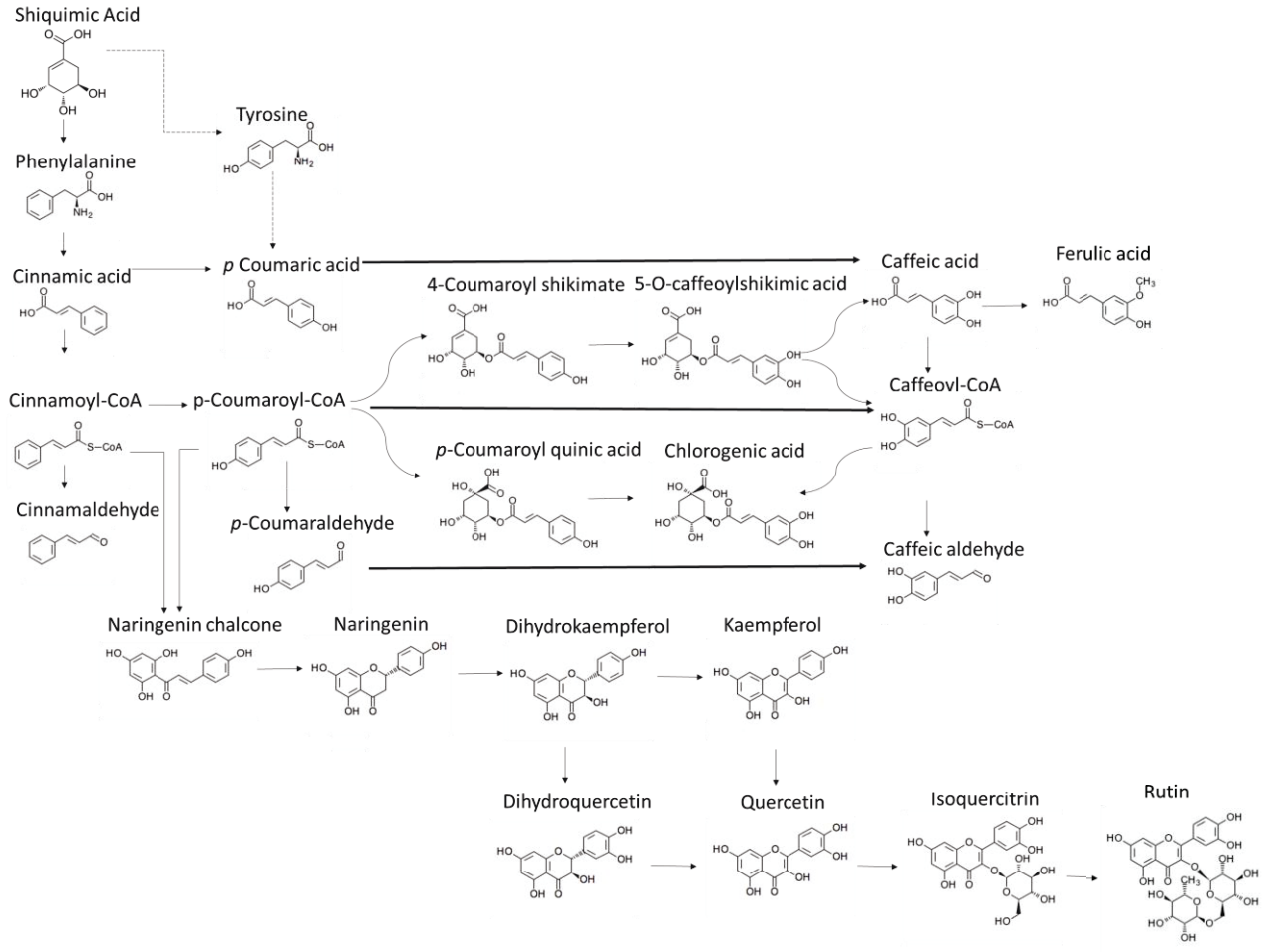

Figure 1 - Metabolic pathway of the formation of phenolic compounds and flavonoids in yerba mate. Source: KEGG (2017) adapted by the author.

In yerba mate the trans-cinnamic acids (caffeic, ferulic and p-coumaric acids) may be in their free forms or in the form of mono or diesters together with quinic acid, such compounds are known as chlorogenic acids, which are the main class of compounds of this species (MARQUES; FARAH, 2009).

The major compounds found in yerba mate leaves are: 3-O-caffeoylquinic acid (chlorogenic acid), 4-O-caffeoylquinic acid (cryptochlorogenic acid), 5-O-caffeoylquinic acid (neochlorogenic acid), 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid (MEJÍA, DE et al., 2010); (FILIP et al., 2001). The chemical structure of these compounds is shown in Figure 2.

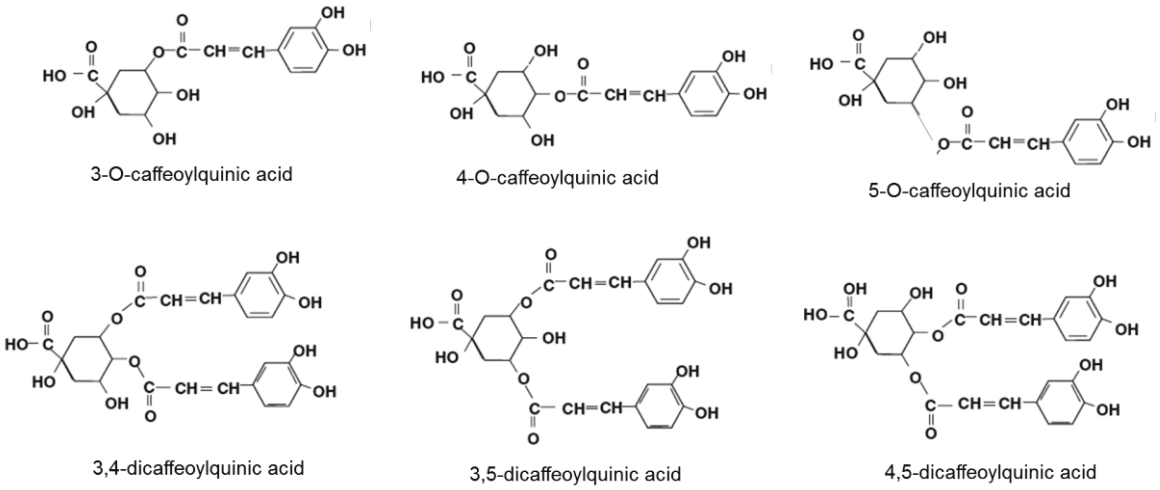

Figure 2 - Chemical structure of caffeoylquinic acids and dicaffeoylquinic acids.

Source: Heck; Mejia, De (2007) adapted by the author.

Due to these compounds, mate beverages are recognized as a rich source of phenolic acids, responsible for *in vivo* and *in vitro* antioxidant activities demonstrated by many studies, the different dicaffeoylquinic acids being a peculiarity, and from which there is a range of products derived from yerba mate (MARKOWICZ BASTOS et al., 2007); (CARDOZO JUNIOR; MORAND, 2016).

Beside these compounds, flavonoids such as quercetin, kaempferol and rutin, were also identified but in a lesser amount (HECK; MEJIA, DE, 2007). Flavonoids belong to a class of multi-functional compounds throughout the plant kingdom (LIU et al., 2015). They are accumulated in all organs and tissues at different stages of development (HICHRI et al., 2011). Protection against UV damage is one of the main functions of flavonoids (ZHAO; DIXON, 2010).

In addition to the aforementioned compounds, the class methylxanthine alkaloids also are found in yerba mate. Being attributed to caffeine and theobromine the stimulant activities of the beverages (SCHUBERT et al., 2006). The alkaloids are a diverse group of low molecular weight, which contains nitrogen in its composition, found in about 20% of plant species. The importance of these compounds is related

to the role they play in protecting plants against herbivores and pathogens (FACCHINI, 2001).

Caffeine and theobromine are purine alkaloids, derivatives of the purine nucleotide. The principal biosynthetic pathway of caffeine is formed from the theobromine. Both are found mainly in younger organs and are located mainly in the vacuole. Caffeine can remain isolated or form complexes with phenolic compounds, especially with chlorogenic acid (ANAYA et al., 2006; ASHIHARA et al., 2008).

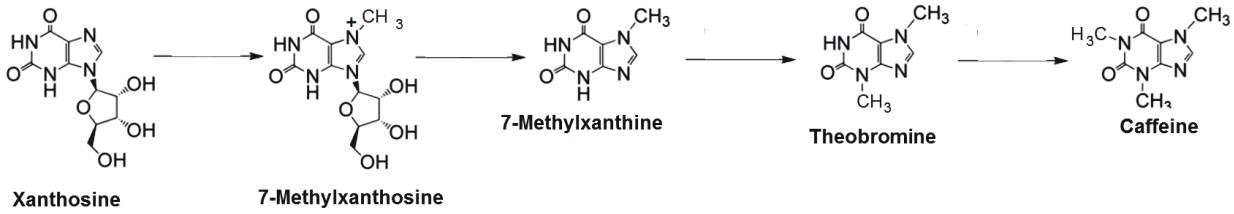

Figure 3 - Metabolic pathway of caffeine and theobromine formation.
Source: ASHIHARA et al., (2008) adapted by the author.

2.3 PLANT CALLUS CULTURE FOR THE PRODUCTION OF SECONDARY COMPOUNDS

In the search for alternatives to the production of medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures have the potential as a complement to traditional agriculture in the industrial production of bioactive plant compounds.

Studies on the production of secondary plant compounds through callus culture and cell suspension culture have been carried out on an increasing scale since the late 1950s (FILOVÁ, 2014). The production of the secondary compounds from cell culture is possible because the cells are totipotent, i.e., each cell in culture retains complete genetic information being able to produce the chemical compounds that are produced by the parent plant (RAO; RAVISHANKAR, 2002).

Secondary compounds can be produced using different biotechnological techniques, such as cell suspension cultures, organ cultures and callus culture (FILOVÁ, 2014). In the callus, the production of secondary compounds pass through two stages: the first one consists of the culture of cells for the growth, multiplication and accumulation of biomass and the second, refers to the synthesis of compounds

from the callus (NIRANJANA et al., 2014). The cells present in the callus are mainly unorganized and undifferentiated and developed from the disordered and disorganized growth of small plant organs or pieces of plant tissue which are called explants (GEORGE, 2008).

All plant tissues might be used as an explant. However, the response efficiency is different depending on the plant species. As a result of the injury, cells on the surface of the explant expand their volume, begin to divide, dedifferentiate and form a mass of cells, which are call the callus (BARBULOVA et al., 2014).

Callus induction occurs in media containing high concentrations of auxin and a combination of auxin and cytokinin under *in vitro* conditions (Figure 4). Callus cultures may be embryogenic or non-embryogenic. Embryogenic callus contain embryogenically competent differentiated cells that can regenerate whole plants through the process called somatic embryogenesis (PTAK et al., 2013).

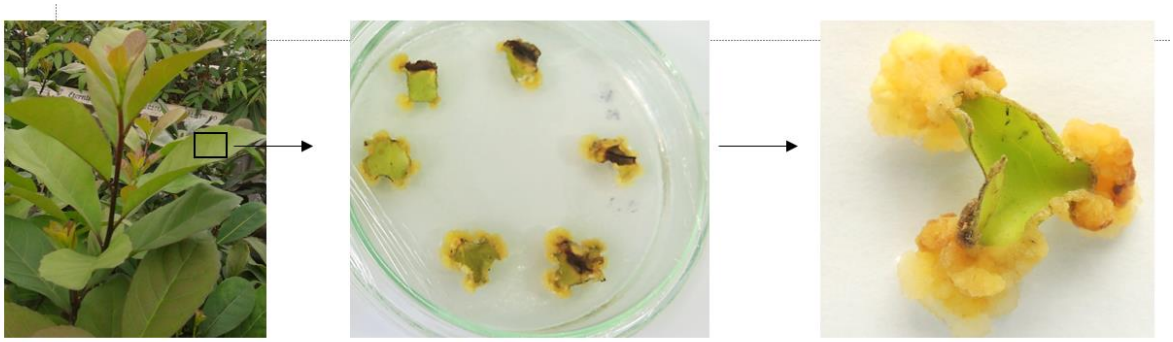

Figure 4 - The process of callus formation from leaf explant.

However, non-embryogenic callus cultures are preferred for the production of secondary compounds in comparison with embryogenic cells, since thehomogeneous clusters of dedifferentiated cells are capable to yield substantial amounts of compounds (IRIAWATI et al., 2014). This behavior have been reported for phenolic compounds, in that higher content of phenolic compounds was observed in non-embryogenic callus than in embryogenic callus in the *Boesenbergia rotunda (*NG et al., 2016) and *Eurycoma longifolia* (IRIAWATI et al., 2014).

Synthesis of phytochemicals by cell masses in contrast to plants is independent of environmental conditions and compound concentration fluctuations (SMETANSKA, 2008). The perspective for using these cell masses is to obtain active compounds for a large number of products. The use of this technique is widespread

in the pharmaceutical industry (CHERMAHINI et al., 2011) and recently has been gaining strength in the cosmetics industry (BARBULOVA et al., 2014).

2.4 APPLICATIONS OF CALLUS CULTURE FOR PRODUCTION OF SECONDARY COMPOUNDS

In the cosmetics industry, some products have already been launched in the market. The Swiss company Mibelle AG Biochemistry, which called the technology of cosmetics production using cell culture of PhytoCellTecTM (PCT) by launching products of the species *Malus Domestica*, *Vitis vinifera* e Alp Rose (PHYTOCELLTEC, 2012); (MORUS et al., 2014).

In addition to this company, L'Oréal has launched products based on undifferentiated rose cells (L'ORÉAL, 2017). The Italian company Instituto di Ricerche Biotecnologiche (IRB) has launched High Tech NatureTM (HTN) technology, providing the innovative bioactive ingredients for nutritional, pharmaceutical, dermo-cosmetic and animal health applications (IRB, 2017).

Also, plant cell cultures represent a potential source of valuable secondary compounds that can be used as food additives and nutraceuticals, since consumers better accept natural food additives as opposed to those that are produced synthetically (SMETANSKA, 2008).

Cases of success for callus and cell culture have been reported for some species. Including *Coptis japonica* for berberine production; whole plants of this species produce 5% berberine in 5 or 6 years of cultivation, while suspended cells produce, respectively, between 5% and 13% of this substance in just three weeks (MATSUBARA et al., 1989). The compound taxol used in a high-value drug called paclitaxel, is also produced from cell cultures. This compound was initially extracted from the bark of *Taxus brevifolia* at age 50 (TABATA, 2006).

These two compounds having extremely high market values and excellent productivity in cell culture are commercially produced. The berberine is manufactured by Mitsui Petrochemical Industries (Japan) and the paclitaxel by Samyang Genex (South Korea) and Phyton Catalytic Company (USA). In addition, shikonin, and ginseng, are marketed using the extracts from cell culture of *Lithospermum*

erythrorhizon and *Ginseng panax*, which were previously extracted from roots of these plants (ZHAO; VERPOORTE, 2007).

However, in general, the production of drugs is low in plant cell culture (LOURENÇO, 2003). An example is the compounds morphine and codeine that despite the pharmacological appeal, the production of these compounds by undifferentiated cells of *Papaver* spp have found little success (DICOSMO; MISAWA, 1995). Another case of failure was reported for the *Atropa belladonna* species where the production of atropine in callus and suspended cell culture was null, whereas only the roots could produce this compound (BHANDARY et al., 1969).

The low production of these bioactive compounds can be explained by the loss of tissue and organ differentiation from cell suspension and callus cultures along with the brief stationary phase that callus cultures present. Also to the insufficient distribution of enzymes required for the synthesis and accumulation of secondary compounds (AMARAL; SILVA, 2003). Because of this, some strategies can be used to intensify the production of compounds in cell cultures, such as optimizing the growth medium and inducing biosynthetic pathways using elicitation (LOURENÇO, 2003).

2.5 METHODS OF ELICITATION IN CALLUS AND CELL CULTURES

Many secondary compounds can be produced in large quantities in response to an external stimulus, an elicitor. The elicitation process is thought to take advantage of the natural defensive reactions of plants. However, the elicitors do not function equally in all species, and the class of elicitors must be selected according to the compounds of final interest. Unfortunately, many elicitors induce a decline in the vitality of an *in vitro* culture, and thus its application is usually lethal to the crop. Because of this, they are often applied when biomass has reached its maximum growth (WEATHERS et al., 2010).

Even so, elicitation is one of the most effective strategies to improve the production of secondary compounds in plant tissue cultures (SMETANSKA, 2008) and is closely related to signal transduction within plant cells (YUE et al., 2016).

The elicitors can be abiotic or biotic, being able to act individually or in combination with other elicitors to regulate the metabolic pathway (WEATHERS et al., 2010). Biotic elicitors are fractions or compounds of plant and microbial cells, and abiotic elicitors include various forms of physical stress, heavy metal ions, osmotic stress, thermal shock, cold and UV radiation (ZHOU; WU, 2006).

2.5.1 Elicitation by ultraviolet radiation

To protect the plants from damage caused by UV radiation, it exhibit physiological responses, including the synthesis of flavonoids, hydroxycinnamic acids and their related compounds (SURJADINATA et al., 2017). Most phenolic compounds play an important role in plant tolerance to UV radiation by reducing UV penetration in plant tissue and by acting as antioxidants, protecting plants from damage caused by reactive oxygen species (JULKUNEN-TIITTO et al., 2005).

The mechanisms by which plants perceive UV radiation and initiate physiological responses are still not fully understood. However, it is known that UV radiation produces reactive oxygen species as the primary signal for the activation of PAL followed by the production and accumulation of phenolic compounds (SURJADINATA et al., 2017).

The ultraviolet (UV) spectrum, although continuous, can be divided into three wavelength bands: UV-A (320-400 nm), UV-B (290-320 nm) and UV-C (200-290 nm). UV-A radiation is less damaging and plays an essential role in the plant photomorphogenesis. The atmospheric ozone does not attenuate it. UV-B radiation is potentially harmful, but it is absorbed to some extent by atmospheric ozone, which reduces its ability to reach the Earth's surface (NIGEL et al., 2012; KATEROVA et al., 2013; YIN et al., 2016).

UV-C radiation is very rare in the environment because it is almost entirely absorbed by the Earth's atmosphere. However, it is the most energetic of the three radiations (YIN et al., 2016). It is very harmful because it interacts directly with DNA, resulting in high lethality against microorganisms, and is especially used for sterilization of food, air and water (BERTOLI et al., 2013). However, UV-C appears to

be the most efficient in increasing the production of secondary compounds with antioxidant properties such as phenolic compounds and flavonoids (CONCONI et al., 1996; ANJUM et al., 2017).

The UV-C irradiation was applied in *Linum usitatissimum* cell culture and was efficient to increase the yield of phenolic compounds and flavonoids (ANJUM et al., 2017). Similarly, the UV-C radiation increases the concentration of caffeoylquinic and dicaffeoylquinic acids in callus of *Cynara cardunculus* L. var. *scolymus* (MENIN et al., 2013). In *Echinacea purpurea* cell cultures the exposure and incubation time after UV-C irradiation showed different answers, and the incubation period 72 hours and exposure time 60 min showed the best caffeic acid derivatives and total phenolic contents (EL-AAL et al., 2016). The UV-C was also efficient to increase the phenolic content, and antioxidant activity of *Moringa oleifera* shoots *in vitro* cultured (PETCHANG, 2014).

2.5.2 Osmotic stress elicitation

Sugars, in addition to the important role on structuring the cell wall, act as signalling molecules that influence the growth, development, and metabolism of cultured cells (PRAVEEN; MURTHY, 2012). Sucrose is one of the essential limiting substrates as a carbon source for the generation of energy that is used in primary and secondary metabolism (FAZAL; ABBASI; AHMAD; ALI; et al., 2016).

In addition to being a significant source of carbon for *in vitro* growth, sucrose can act as an osmotic agent inducing stress when added at high concentrations. This stress may result in the increased production of secondary compounds depending on plant species (CUI et al., 2010). Besides, the osmotic stress, sugars may favour the increase of endogenous abscisic acid concentration in cultured plant cells (MISHRA VK; SINGH RM, 2016), which also acts as a marker for the accumulation of secondary compounds (ZHAO et al., 2005).

The effects of sucrose concentration on the accumulation of secondary compounds in the *in vitro* cultures of several species have been reported. In suspension culture of *Prunella vulgaris,* the sucrose concentration between 2 and 2.5% was efficient to enhance the phenolic, flavonoid content and biomass (FAZAL;

ABBASI; AHMAD; ALI, 2016). The cell cultures of *Hypericum perforatum* increased the amount of total phenols, flavonoids, chlorogenic acid, and total hypericin in the medium supplemented with 5, 7, and 9% sucrose, however, the medium supplemented with 3% sucrose resulted in the maximum biomass accumulation (CUI et al., 2010).

The sucrose concentration of 5% also was effective to the accumulation of phenols and flavonoids in *Echinacea angustifolia*, but the biomass was most increased at 5% and 7% sucrose (WU et al., 2006). In *Satureja hortensis* callus culture the highest rosmarinic acid accumulation was obtained from the medium with 3% sucrose while 5% showed highest biomass yield (TEPE; SOKMEN, 2007).

Due to the growth of the cells and the production of compounds are not correlated, the most appropriate carbohydrate source and ideal concentration should be identified for each species and compound of interest (MISAWA, 1994; DICOSMO and MISAWA, 1995; MURTHY et al., 2014).

Studies related to the production of secondary compounds from yerba mate callus culture may be an alternative for the constant production of secondary compounds with commercial interest. Considering that environmental conditions influence the accumulation of these compounds negatively in the field, the protocol developed in this study may be useful in future, not only as a production system for secondary compounds but also as a tool to understand the metabolic pathway leading to secondary compounds in yerba mate.

3 CULTURE MEDIUM OPTIMIZATION, COMPOUNDS IDENTIFICATION AND ANTIOXIDANT ACTIVITY OF THE *Ilex paraguariensis* CALLUS

ABSTRACT

Ilex paraguariensis (yerba mate) is a native species of South America and is a rich source of bioactive compounds. Despite its potential application, there is a lack of research efforts on cultivation and phytochemicals investigation of callus of this plant. In the present study, an effort has been made to optimize the callus culture conditions and to identify secondary compounds from the callus. Initially, callus were induced from 10 genotypes using leaves as explants on ¼ MS medium supplemented with 4.52 µM 2,4-dichlorophenoxyacetic acid and 4.56 µM zeatin. The best genotype was selected for the following experiments including the effect of cytokininis [zeatin (2.26, 4.56 or 9 µM), 2-isopentenyladenine purine (2.5, 5 or 7.5 µM), thidiazuron (0.125, 0.25 or 0.5 µM) or kinetin (2.5, 5 or 7.5 µM)] on callus induction and biomass. The effect of sucrose concentration (3, 6 and 9%) on callus biomass and secondary compounds accumulation were also investigated. Compounds identification, total phenol content and antioxidant activities were measured from yerba mate callus. Callus initiation was genotype dependent, and 6-156-6 has the best callogenesis response. The medium supplemented with zeatin showed the higher callus induction rates (82%) and the higher biomass accumulation after 120 days (328.2 mg). Maximum levels of biomass and secondary compounds accumulation were observed on callus grown on medium supplemented with 3% of sucrose. The callus antioxidant activity did not change in relation to sucrose concentration. The culture condition allowed the accumulation of eight compounds in yerba mate.

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

3.1 INTRODUCTION

Ilex paraguariensis (Aquifoliaceae) is a South American plant whose leaves are used mainly for the preparation of stimulant beverages and is popularly known as ‘yerba mate’ (FILIP et al., 2001). The first report on the antioxidant activity of yerba mate was brought 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 to cosmetics, foods and mainly to soft drinks (POZEBON et al., 2015).

Several studies concerning the yerba mate potential for medicinal use 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 the main constituents of its leaves. Natural antioxidant defense systems are reported in the *Ilex paraguariensis* species and is attributed, mainly, to the high content of phenolic compounds (BRAVO et al., 2007) In addition to phenolic acids, also are reported in the leaves of this species the methylxanthines, saponins, flavonoids, amino acids, minerals and vitamins (BOAVENTURA et al., 2015).

The main phenolic acids present in yerba mate are the chlorogenic acids (FILIP et al., 2001; HECK; MEJIA, DE, 2007; MARKOWICZ BASTOS et al., 2007). Chlorogenic acid is extensively employed as an additive in food industries to beverages, as well as medicinal substances and cosmetics. This compound is considered within the category of Fine Chemicals showing high added value (BUTIUK et al., 2016).

As an alternative to whole plant cultivation in the production of important secondary compounds, callus cultures are often an effective system 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 used as raw materials for some cosmetics commercial products (PHYTOCELLTEC, 2012; MORUS et al., 2014; L'ORÉAL, 2017) and added as bioactive ingredients for nutritional, pharmaceutical, dermo-cosmetic and animal health applications (IRB, 2017).

The callus and cell cultures follow two steps before secondary compounds production. Initially, the biomass accumulation occurs, when callus and cells grow and multiply and consecutively (step two), ensue the synthesis of compounds from the biomass. Initiation of callus and cell cultures begins with choosing parent plants, since the high-producing callus and cells and accumulation of secondary compounds in plants may be genotype specific (CASTRO et al., 2016).

Several chemical and physical factors have been identified, which can influence biomass accumulation and synthesis of secondary compounds in plant cell cultures (SHEN et al., 2008). Auxins and cytokinins are usually employed to induce callus, as they promote cell growth by stimulating cell division and elongation (CASTRO et al., 2016). Therefore, to obtain maximum yield of callus it is necessary to optimize the type and level of growth regulators in culture medium (WANI et al., 2014). In addition to plant growth regulators, plant cell cultures grown using a carbohydrate source, and supplemental concentration in the medium greatly affected

biomass and compounds production (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 might causes osmotic stress on the tissue (GERTLOWSKI; PETERSEN, 1993). This stress may influence the compounds production, mainly in 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 genotype and to optimize a medium composition to obtain callus culture of *Ilex paraguariensis*, in order to produce secondary compounds production under controlled conditions. The results were also discussed in terms of the antioxidant activity and distribution of major secondary compounds identified in *Ilex paraguariensis* callus culture with respect to the sucrose concentration.

3.2 MATERIAL AND METHODS

3.2.1 Establishment and proliferation of callus culture and effect of genotype on callus induction

Callus tissue were induced from *Ilex paraguariensis* leaves from plants grown in the greenhouse at EMBRAPA Forestry. The 2nd/3rd pair of leaves of elite clones were collected 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 follow: 10 min in a solution of Cercobin® 1% (w/v), 5 min in mercury chloride 0.05% (w/v) and finally the leaves were rinsed three times with sterile distilled water.

Afterward, leaf discs with 2 cm diameter were placed in Petri dishes containing 20 mL autoclaved MS medium (MURASHIGE; SKOOG, 1964) reduced to quarter strength (¼-strength MS) 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 the 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.

3.2.2 Effect of genotype on callus induction

Leaves of ten yerba mate elite clones: A3, A35, A7, F1, F2, M7, 3-65-2, 4-56-2, 4-76-2, 6-156-6 from the EMBRAPA Forestry breeding program (RESENDE et al., 2000) were collected, disinfected and placed in the 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: induction coefficient = (total number of induced callus/number of cultured explants) * 100% for each clone.

3.2.3 Effect of different cytokinins and 2,4-D on callus proliferation

The best callogenesis response genotype was tested on medium previously described varying the concentrations and types of the cytokinins: 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 the percentage of callus induction was evaluated and after 120 days the fresh mass was measured. The callus was transferred every 60 days for fresh medium of the same composition.

3.2.4 Effect of sucrose concentration on proliferation and secondary compounds production in yerba mate callus culture

The best medium for callogenesis were used to study the effect of sucrose concentration on callus growth and secondary compound productions. Callus grown in ¼ MS medium with 0.7% agar, 4.52 µM 2,4-D and 4.56 µM zeatin and 3% sucrose were transferred to the same medium but with different sucrose supplementation (3, 6, and 9%) at 60 days. Callus fresh weight was measured every 15 days up to 120 days of culture. After this period, the callus were stored at -80 ºC for further analysis of secondary compounds.

3.2.5 Extraction of secondary compounds of *Ilex paraguariensis* callus

Callus were frozen in liquid nitrogen and lyophilized for 72 h. Samples (10 mg) were extracted with a hydroalcoholic solution (ethanol: water, 1:1, 1 mL). The extract solution 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 collected and filtered (0.22 µm). Part of the extracts was used for biochemical tests and 500 µL was transferred to the vial (2 mL) which the 25 µg of internal standard umbelliferone was added for quality control (Sigma®).

3.2.6 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 with 0.5 mL of the Folin-Ciocalteu reagent and incubated for 5 min at room temperature (25 ± 2 °C). After incubation, 2 mL of Na_2CO_3 (15%, w/v) was added to above 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 in quantification. The results were expressed as milligrams of gallic acid equivalents ($mgGAE.g^{-1}$) of dried weight (DW).

3.2.7 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). The 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, the 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 an absorbance of 0.7 ± 0.2 at 734 nm in a spectrophotometer (Shimadzu-1800; Japan). Then 30 µL of the extract was add 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 capability 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 ($\mu molTE.g^{-1}$).

3.2.8 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 (0% B), 0.5–25 min (0%–5% B), 25–28 min (5%–40% B), 28–30 min (40%-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.

3.2.9 Statistical analyses

The experimental designs were randomized. For the evaluations of genotype, cytokinin and sucrose effects, 10 Petri dishes with 5 explants each per treatment were used. After 120 days, 10 pieces of callus of each cytokinin treatment were weighed. For sucrose evaluation, after 60 days on culture 10 pieces of callus were weighed each 15 days, until 120 days. The regression analysis ($p<0.05$) was performed to the callus growth.The analyses of total phenolic, antioxidant activity, and HPLC analysis were carried out in quintuplicate. The data were analysed by Barttlet test and ANOVA, followed by a Tukey test ($p<0.05$).

3.4 RESULTS AND DISCUSSION

3.4.1 Effects of genotype on callus induction

The percentage of induction varied according to the genotype (Figure 5), suggesting a strong genotype dependent response. Leaf explants of 6-156-6 clone had the higher induction rates (77%). The lowest induction rates (1% and 0%) were observed in leaf explants of 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 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.

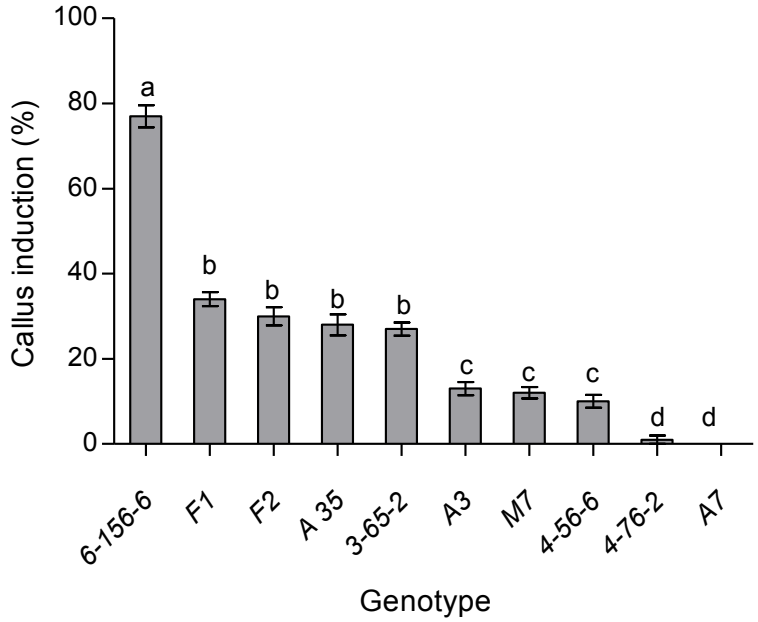

Figure 5 - Callus induction on leaf explants of 10 clones of *I. paraguariensis* after 30 days on culture. Bars followed by the same letter do not show significant differences [P < 0.05 (Tukey)]. The vertical bars represent the standard error of ten replicates.

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

3.4.2 Cytokinins effects

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 (Table 1). However, differences based on cytokinins types and concentrations were observed. Among cytokinins tested, higher zeatin concentrations (4.56 and 9 µM) were effective in stimulating callus induction, together with 0.15 µM and 0.5 µM TDZ, 2.5 µM and 7.5 µM kinetin ($p>0.05$). Regarding the fresh weight of yerba mate callus at 120 days, higher zeatin amounts were also found effective, but 0.5 µM TDZ and 7.5 µM 2iP gave the equal results ($p>0.05$) (Table 1). When both variables (i.e. callus induction and fresh weights) were combined, higher zeatin concentration (4.56 and 9 µM) and 0.5 µM TDZ provided similar results.

However, the callus grown in culture medium with TDZ oxidized after 120 days, whereas those cultivated with zeatin showed better appearance and colour (Figure 6).

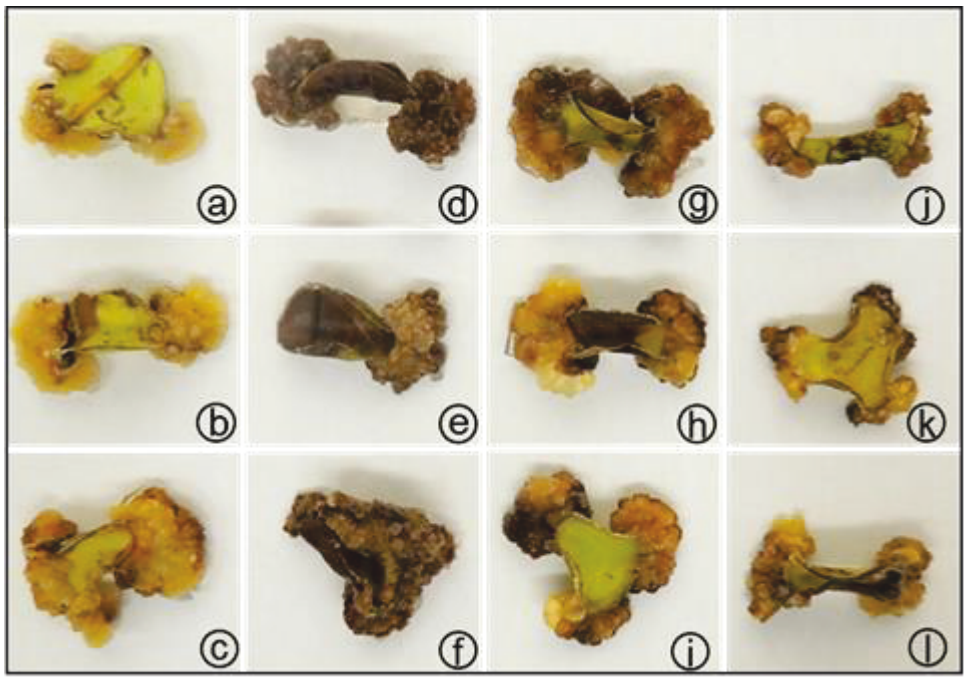

Figure 6: Effect of cytokinins on *I. paraguariensis* callus at 120 days of induction. a) 2.25 µM zeatin, b) 4.56 µM zeatin, c) 9 µM zeatin, d) 0.15 µM TDZ, e) 0.25 µM TDZ, f) 0.5 µM TDZ, g) 2.5 µM 2i P, h) 5 µM 2i P, i) 7.5 µM 2 iP, j) 2.5 µM kinetin, k) 5 µM kinetin, l) 7.5 µM kinetin.

Zeatin was previously effective for the conversion of rudimentary embryos of *Ilex paraguariensis* (SANSBERRO et al., 1998), and the same cytokinin was tested in this work. In experiments with other *Ilex* species: *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 regeneration of plants.

Similarly to zeatin, 2iP also occurs naturally in plants, however, is considered a weak cytokinin when compared with zeatin (SCHUCH et al., 2008). This accertation supports our results, in that callus grown in medium with this regulator showed the lower callus induction percentage and only the highest concentration of 2iP (7.5 µM) showed good results for the yerba mate callus yield.

The opposite trend was observed in callus supplemented with kinetin. The data obtained showed that the callus yield was decreased when the concentrations of kinetin increased. In addition, intermediate concentration of kinetin and TDZ showed lower callus induction, while the extreme concentration showed higher callus induction rate. This was also observed in callus culture of *Centella asiatica* suplemented with 2,4 and 6- Benzylaminopurine (BAP) (MAZIAH et al., 2010).

The manipulation auxin/cytokinin ratio in the culture medium is often a crucial factor in the callus growth increased. The added auxins and cytokinins interact with these other endogenous plant hormones and the concentration and combination of these regulators need to be defined for each species (LOREDO-CARRILLO et al., 2013).

In the other hand, the manipulation of PGR concentrations in the culture medium might influence the accumulation of secondary compounds (RODRIGUES; ALMEIDA, 2010). The PGR manipulation, in the most of the cases, is carry 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).

Due the concentration of 4.56 µM zeatin shown the higher results when combine the variables callus induction and fresh weight, in addition the best callus colour and aspect it was chosen for the following test.

TABLE 1 - EFFECT OF CYTOKININ TYPE AND CONCENTRATIONS ON CALLUS INDUCTION ON LEAF EXPLANTS AND FRESH WEIGHT OF *I. paraguariensis* CLONE 6-156-6 CALLUS CULTURE.

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

3.4.3 Effect of sucrose on callus growth and phenolic total content

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

Figure 7a shows the weight gain over time for callus under the sucrose several concentrations. Medium supplemented with 3% sucrose is superior to the other concentrations after 90 days culture. The concentrations of 6 and 9% sucrose were suboptimal for yerba mate callus growth. Higher concentrations of sucrose may reduce the total fresh mass by increasing the osmotic potential of the medium, hindering the absorption of salts and water (JESUS et al., 2011). As observed in suspension cultures of *Vitis vinifera* (DO; CORMIER, 1990) and *Panax notoginseng* (ZHANG et al., 1996), high concentrations of sucrose repressed cell growth, but in both species, higher concentrations of sucrose favoured the synthesis of secondary compounds and could be used as strategies to improve the accumulating of such compounds in the tissues.

However, in *Ilex paraguariensis* total phenols were not influenced by higher levels of sucrose, since higher values of total phenols were observed in callus supplemented with 3% sucrose (Figure 7b). Similar results were observed in biomass accumulation and phenolic production of *Hypericum perforatum* root cultures (CUI et

al., 2010). In the same way, in cell suspension cultures of *Prunella vulgaris* the maximum levels of phenolics and biomass were observed on cultures treated with low doses of sucrose (2%) (FAZAL et al., 2016).

In contrast, a 5% sucrose supply was shown to be optimal for the production 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 accumulation is not always related to the biomass accumulation.

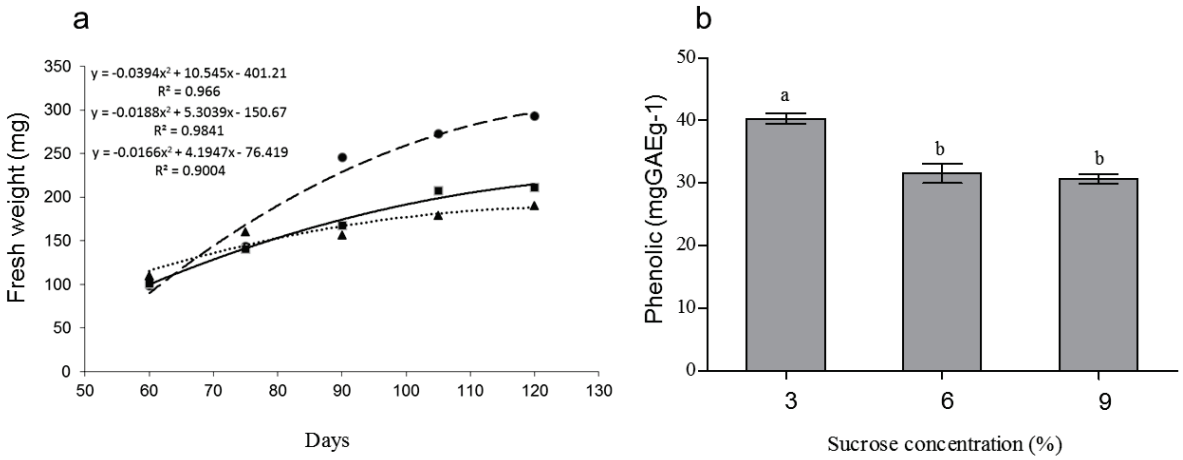

Figure 7 - a) Growth parameters of *I. paraguariensis* callus culture supplemented with increasing sucrose concentrations. Callus growth was expressed as milligrams of fresh weight. b) Total phenolic accumulating after 120 days of culture of *I. paraguariensis.* Data are the mean ± SE (*n* = 10) (●) 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.

3.4.4 Identification of secondary compounds

The six major phenolic constituents and two methylxanthines, present in the yerba mate callus were identified by LC-MS-MS (Table 2). 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 reported as major constituents of mate (BRAVO et al., 2007).

Three dicaffeoylquinic acid isomers, were also identified and corresponded to 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. These compounds identified as the major constituents in yerba mate callus might be

used in different industrial applications, including cosmetics and food preservative. For example, the compounds caffeine and theobromine play a role against physical and mental fatigue (FILIP et al., 2001). Due to higher antioxidant activity, the compounds CQA have a greater range application in the pharmacy and cosmetic industries. For example, the caffeoylquinic acids present digestive and hepatoprotective activities (AZZINI et al., 2017).

The compounds 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 is a potent anti-inflammatory (HONG et al., 2015) and has been reported that this compound inhibited the replication of the human immunodeficiency virus, HIV-1 (ZHU et al., 1999). Following the same direction, 3,4-dicaffeoylquinic acid has been reported as a potent lead compound for anti-influenza activity (KUWATA et al., 2012). The presence of all these compounds in yerba mate callus makes it a potential source of raw-material for many applications.

TABLE 2 - 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)
5.25	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)
6.91	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)
7.14	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).
7.04	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)
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.

3.4.5 Sucrose effect on antioxidant activity and secondary compounds production

The compounds identified by LC-MS/MS were semi-quantified by HPLC-UV. Figure 8 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, 4 and 5. And the peaks 7, 8 and 9 corresponded to three dicaffeoylquinic acid isomers: 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, respectively.

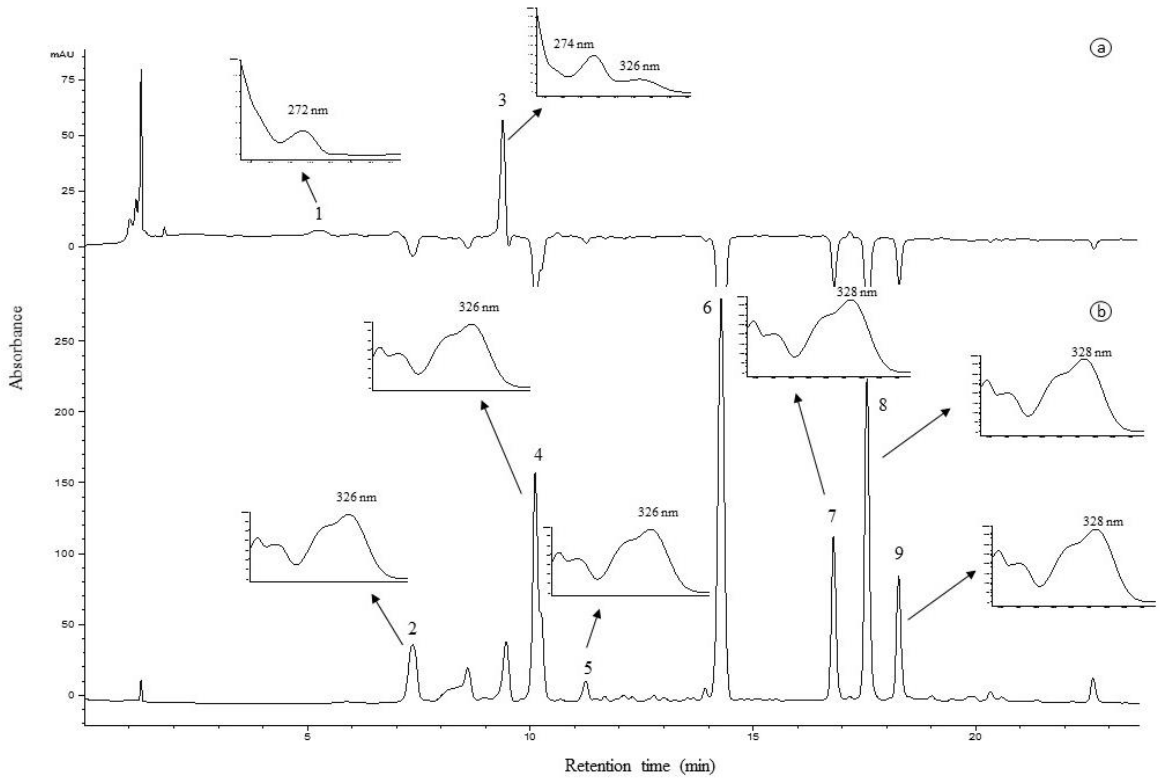

Figure 8 - Typical HPLC chromatogram for *I. paraguariensis* callus extract. a) The UV spectra was 260 nm for caffeine and theobromine and b) 328 nm for caffeic acid derivatives. Peaks: (1) theobromine; (2) neochlorogenic acid; (3) caffeine; (4) chlorogenic acid; (5) cryptochlorogenic acid; (6) Internal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

The data shows that the antioxidant activities and the compounds caffeine and 3,5-dicaffeoylquinic are not significantly affected by changes in sucrose concentration (Figure 9a, c and d). The 3,5-dicaffeoylquinic acid is a compound with a strong antioxidant activity (MENIN et al., 2013; HONG et al., 2015), and its amount in callus culture was higher than other dicaffeoylquinic acids. Witch may justify the non-alteration of the antioxidant activity by the two methods analyzed (Figure 9a, c and d). Both methods for measuring antioxidant potential used in this work (ABTS and DPPH assays) are the easiest to implement and yield the most reproducible results (DUDONNE et al., 2009)

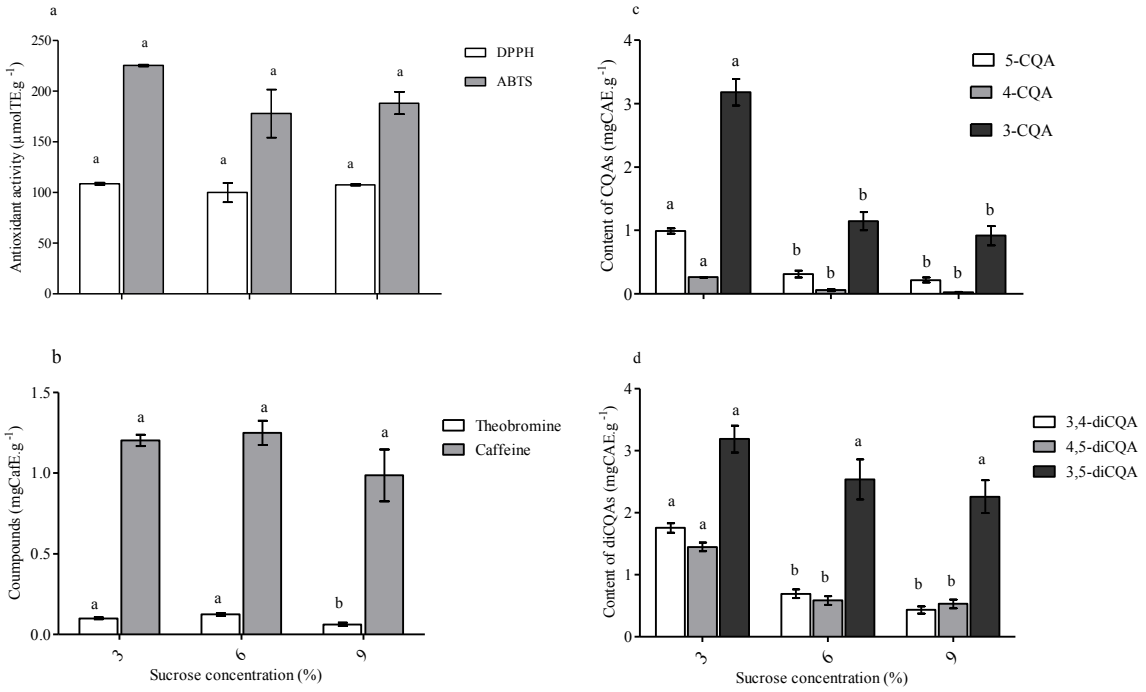

Figure 9 - Effect of sucrose concentration on the production of a) Antioxidant activity b) Theobromine and caffeine c) Chlorogenic acids, g) Dicaffeoylquinic acids, after 120 days of culture of *I. paraguariensis* callus. Means with different letters are significantly different at the 5% level of probability using Tukey's multiple range test. The vertical bars represent the standard error of five replicates.

The accumulation of the chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, 3,4-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid in yerba mate callus was higher in medium supplemented with 3% sucrose. Higher sucrose concentration inhibited the accumulation of these compounds (Figure 9c and d).

An opposite results was stated for *Eryngium planum* callus where the concentrations of 5 and 6% sucrose in the medium led to increasing chlorogenic acid content (KIKOWSKA et al., 2012). For cell suspension culture system of *Gymnema sylvestre* it was found that 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 was found to be associated with elevated levels of sucrose. Higher carbohydrate content in the culture medium might promote stress on the tissue, resulting in changes in cells metabolism, growth tissues and secondary compounds production (CUI et al., 2010). However, in yerba mate callus this was not observed for any compound analysed (Figure 9).

The unexpected results may be related to the class of compounds of which high sucrose concentration can induce or to some negative metabolic alteration caused by osmotic 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 VK; SINGH RM, 2016), which in turn regulates negatively the accumulation of some secondary compounds, 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); 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 applications. In this way, the yerba mate callus might be considered as a source of such compounds in the continuous production through plant callus culture-based technology, but new elicitors should be tested in order to improve the compounds accumulation.

3.5 CONCLUSION

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

The medium supplemented with 3% sucrose resulted in the higher biomass accumulation, and higher accumulation of total phenolic and caffeoylquinic acids.

Two methylxanthines, three caffeoylquinic acids and three dicaffeoylquinic acids were identify as the major compounds in yerba-mate callus.

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4 YERBA MATE (*Ilex paraguariensis*): AN APPROACH TO THE PRODUCTION OF SECONDARY COMPOUNDS IN CALLUS

ABSTRACT

Yerba mate has several secondary compounds of interest to the pharmaceutical industry. The production of these compounds can be performed *in vitro* by callus cultivation. However, for this species, no studies upon the production of these compounds *in vitro* during the growth phase have been performed to date. In this work, we show that the production of secondary compounds is possible in callus culture. We observed that the best phase for the production of secondary compounds in callus is in the stationary growth phase. In this phase, higher levels of phenolic compounds, chlorogenic acid and 3,5-dicaffeoylquinic acid and greater antioxidant activity in callus were observed. Secondary compounds were found in yerba mate callus cultivated *in vitro* and the compounds chlorogenic acid and 3,5-dicaffeoylquinic acid presented high correlation with the antioxidant activity.

Keywords: *in vitro* culture; stationary phase; antioxidant; phenolic.

4.1 INTRODUCTION

Plants produce a diverse array of secondary compounds, which are essential for their development. They are produced mainly under stress conditions or as an adaptation strategy, and present a great chemical diversity (WINK, 1988). The various biochemical properties of the secondary compounds make them suitable for a large number of commercial applications, whether for use in the food, pharmaceutical, cosmetics and even chemical industry (SCHMITZ et al., 2016).

Estimates indicate that the global market of phytonutrients and phytochemicals is expected to grow by 7.2% between 2015 and 2020, in terms of values is expected to reach US $ 4.63 billion. The growth of this sector is driven by the research, development and applications of new products in the market (MARKETS, 2015).

Yerba mate (*Ilex paraguariensis*) is a plant that has potential uses in the pharmaceutical industry, both for the production of cosmetics, due to the presence of antioxidant compounds (BERTÉ et al., 2011) and the production of phytochemicals, due to the presence of stimulant compounds (CARDOZO JUNIOR; MORAND, 2016).

The quantification of phenolic compounds in leaves of *I. paraguariensis* has been reported in other studies (BASTOS et al., 2007; MEJÍA, DE et al., 2010), and

the antioxidant properties of yerba mate have already been studied in chemical and biological systems and proven in humans (GUGLIUCCI, 1996; MATSUMOTO et al., 2009). However, the accumulation of secondary compounds in yerba mate leaves may suffer seasonal fluctuations and vary according to genetic material.

From the industrial and pharmaceutical point of view, the production of secondary compounds by the plant not affected by the season and environmental conditions is interesting, both with respect to composition and quantity of compounds. (JAWAHAR et al., 2014; NAIK; AL-KHAYRI, 2016). The interest in the development of technologies for the production of phytochemicals through the cultivation of plant cells has increased, and some companies have used plant cells grown under controlled conditions for cosmetics manufacturing (PHYTOCELLTEC, 2012); (L'ORÉAL, 2017). In addition, many studies have being conducted on producing secondary compounds from cells and plant organ cultures for health promotion (VERARDO et al., 2016) and *I. paraguariensis* callus culture may act as a source of medicinally important compounds.

Among the different classes of secondary compounds, plant polyphenols constitute the largest group of natural antioxidants (CIEŚLA et al., 2013). Studies aimed at increasing the production of phenolic compounds from plant cell culture have been carried out with the *Habenaria edgeworthii* (GIRI et al., 2012), *Artemisia absinthium* (ALI et al., 2013), *Justicia gendarussa* (AMID et al., 2011) and *Rosmarinus officinalis* species (YESIL-CELIKTAS et al., 2007). However, this potential has not been explored in *I. paraguariensis*, and there are no reports in the literature about the production of high market value bioactive compounds such as caffeoylquinic acids.

This study describes the production of secondary compounds in yerba mate callus culture, aiming to evaluate the antioxidant activity and the production of secondary compounds, such as polyphenols and methylxanthines in callus of two clones of yerba mate. The different stages of callus growing were studied in order to determine the best clone and stage for secondary compounds extraction, as well as to compare with compounds produced in leaves.

4.2 MATERIAL AND METHODS

4.2.1 Plant material and growing conditions

For the accomplishment of this work, the elite clones F1 and 6-156-6 from the genetic breeding program developed by EMBRAPA Forestry were used (RESENDE et al., 2000).

Leaves of yerba mate from miniclonal garden, growing in a semi-hydroponic system in grooves containing sand, kept in a greenhouse were used for the induction of callus. The 2nd/3rd pair of young leaves of the mother 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).

Asepsis was performed by washing the leaves with 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 three washes with sterilized water. The entire asepsis procedure was performed in a laminar flow hood.

Leaf discs of 2 cm in diameter were cut with the aid of a punch and placed with the adaxial face in contact with the ¼ MS (Murashige and 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 5.6 ± 0.2, autoclaved and dispensed into Petri dishes (100 x 15 mm). Ten Petri dishes containing five leaf explants were kept in the dark at 23 ± 2 °C throughout the growing period.

4.2.2 Analysis of growth and production of secondary compounds in callus

The leaf explants of the clones were transferred to a new medium every 30 days. The growth curve of different phases of yerba mate was evaluated by plotting fresh weight values of 10 callus measured every 15 days from time 0 to 150 days. The percentage of callus growth was determined according to Santos et al. (2008) using the equation:

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

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

4.2.3 Preparation of extracts

For the accomplishment of the biochemical analysis, leaves (initial condition) and callus in the periods of 75, 90, 105, 120, 135 and 150 days were used. The experimental unit was composed of 10 Petri dishes with five pieces of callus per plate. In each period, three pieces of callus were randomly selected for preparation of the extracts. The leaves were immediately immersed in liquid nitrogen after harvest. All samples 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 leaf) were extracted with the water: ethanol (1:1) solution. The extracts were mixed for 30 sec and sonicated for 30 sec. The extraction was performed for 1 h at 60 ºC, with rotation of 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 tests while another part (500 µL) was transferred into vial and 2.5 µL of the internal Umbelliferone (10 $mg.mL^{-1}$) standard (Sigma®) was added for analysis on HPLC.

4.2.4 Determination of the total phenol content by the Folin-Ciocalteau method

The phenol content was determined by the spectrophotometric method of Folin-Ciocalteau (HORŽIĆ et al., 2009) with modifications. To a 0.1 mL aliquot of each extract was added 6.0 mL of water, 0.5 mL of the Folin-Ciocalteau reagent, agitated and allowed to react for 5 min. Thereafter, 2 mL of aqueous Na_2CO_3 (15%) solution was added. The final volume was adjusted to 10 mL and after 2 h, the samples absorbance 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 plant extract (mgGAE.g^{-1}) of dried sample.

4.2.5 Determination of antioxidant activity

Leaves and callus extracts were tested for the scavenging effect on DPPH radical 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 515 nm in a UV-VIS spectrophotometer (Shimadzu-1800).

The antioxidant activity was obtained through an analytical curve with Trolox in the range of 0-1000 mg.L^{-1}. The results were expressed in µmoles Trolox equivalents per gram of dry sample (µmolTE.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.

4.2.6 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 pre column Shim-Pack CLC G-ODS (10 x 4.0 mm. d.i. 5 µm) using a mobile phase (A- acetic acid 1%. B- acetonitrile 100%), flow rate 0.5 mL.min^{-1} in an 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 (100-3% B) and 80-95 (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 compound per gram of dry sample ($mg.g^{-1}$). For the identification of the compounds in the extracts, standard solutions (Sigma®) of the theobromine, caffeine, chlorogenic acid, and 3,5-dicaffeoylquinic acid compounds were used.

The LC-MS/MS was used for the structure confirmation of all compound, and it was utilized the SCIEX X500R QTOF system with Turbo V™ source and Electrospray Ionization (ESI) was used in positive (xathines) and negative (caffeoylquinic derivatives) polarity IS voltage was set to 5500 V.

Extracts (2µL) were separated on LC Synergy Fusion-RP 80A (75 x 4.6 mm, d.i. 4 µm) 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 an 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 (0% B).

4.2.7 Experimental design and statistical analysis

The experimental design was randomized in a subdivided plots scheme to evaluate callus growth. Ten replicates were used to evaluate the fresh callus weight, with the two clones being distributed in the plots and the growing times in the subplots. For the analysis of the secondary compounds, a randomized design was used in the factorial scheme (2x6), two clones and 6 culture times, with three replications. For all the data, the homogeneity tests were performed by the Bartlett test and the analysis of variance (ANOVA). The mean comparison by Tukey's test ($p < 0.05$) was performed for the variable of callus fresh weight. The regression analysis ($p < 0.05$) was performed to the variables total phenolic compounds, antioxidant activity, and the compounds: theobromine, caffeine, chlorogenic acid and 3,5-dicaffeoylquinic. The statistical program ASSISTAT® (SILVA; AZEVEDO, 2016) was used.

4.3 RESULTS AND DISCUSSION

4.3.1 Growth curve of yerba mate callus

During the growth kinetics of two yerba mate elite clones, the callus of both clones had a compact appearance and a light yellow color with no changing of the color throughout the growth period (Figure 10). The F1 clone was more responsive to growth, with higher fresh weight from 45 days to the end of the evaluated growth period ($p < 0.01$).

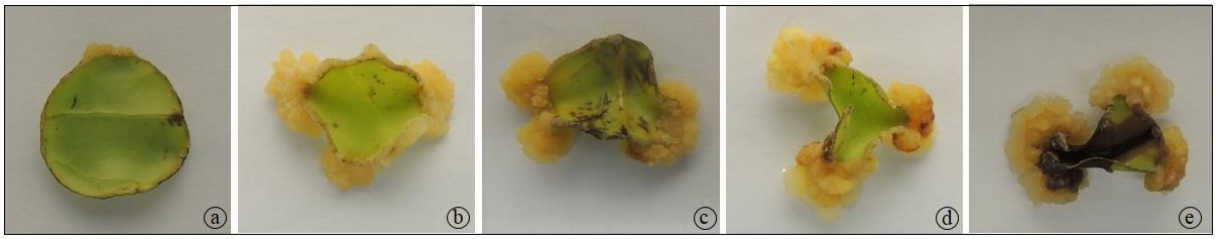

Figure 10 - Growth curve of yerba mate callus. Phases: a-lag, b- exponential, c- linear, d- deceleration, e-stationary.

The growth curve was plotted by measuring the fresh weight of the callus of the two clones, presenting a sigmoid pattern (Figure 11) showing five distinct phases during the analyzed period (0 to 150 days): lag, exponential, linear, deceleration and stationary (SMITH, 1992).

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 of the callus and it was observed from the installation of the experiment until the 15th day with relatively slow growth (F1: 41.0%, 6-156-6: 25.1%). The lag phase varies according to the species studied: 40 days in callus of *Inga vera* (ingazeiro) (STEIN et al. 2010), 30 days in callus of *Tabebuia roseo alba* (ipê-branco) (ABBADE et al., 2010), 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).

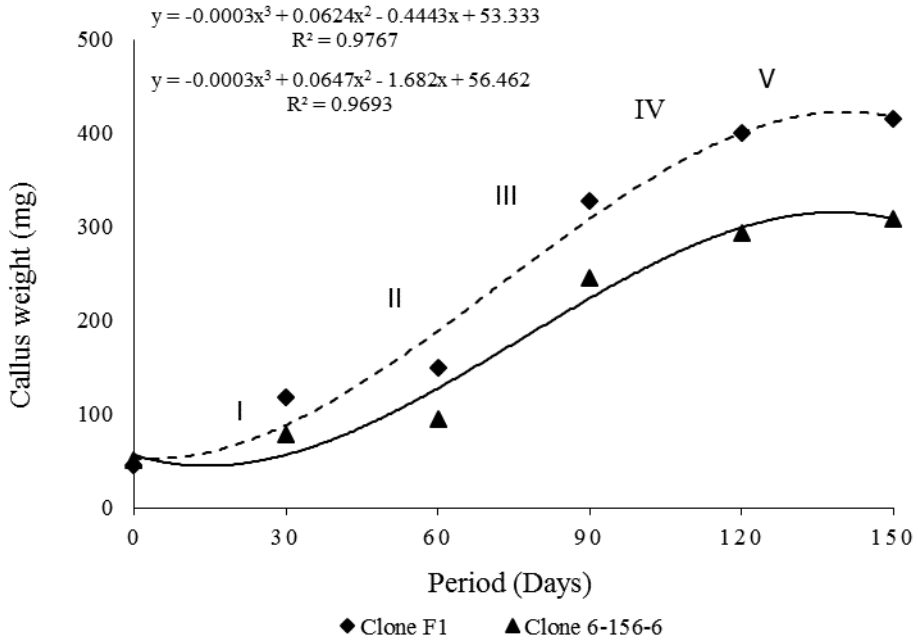

Figure 11 - Growth curve of yerba mate callus. Phases: I – lag, II – exponential, III – linear, IV – deceleration, V - stationary.

The exponential growth phase (II), period which 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 (10 th to 60 th day) (COSTA et al., 2015). In this period, the largest increase in biomass occurred, respectively 62.5% and 52.5% for F1 and 6-156-6 clones.

The linear phase (III), period in which there is decrease of the division and increase of the cellular area, was verified between 75 th to 105 th day. During this period an 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 this phase, *Tabebuia roseo-alba* callus was observed a 57% increase (ABBADE et al., 2010). The deceleration interval (IV) was observed from the 105th to the 120th day with low increase (F1: 4.9%, 6-156-6: 6.8%).

Stationary phase (V) was observed between the 120th day and 135th day (F1: 2.4%, 6-156-6: 3.2%). In *Jatropha curcas* callus, which were cultivated for the purpose of producing 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 ($p > 0.05$. F1: 1.2%. 6-156-6: 1. 6%).

The extraction of the secondary compounds is recommended to occur in the stationary phase, once in this period the production of the primary metabolites (important for the cellular development) practically ceases and the secondary metabolites are produced (SMITH, 1992).

4.3.2 Concentration of total phenolic compounds in yerba mate callus

In this study, significant interaction between clones and callus growth periods in the production of phenolic compounds was observed. Regardless of the callus culture time, a significant difference between clones was observed for the production of phenolic compounds: 43.3 mgGAE.g^{-1} for 6-156-6 clone and 39.3 mgGAE.g^{-1} for F1 clone ($p < 0.01$). No significant differences ($p > 0.05$) were observed between the clones in yerba mate leaves (157.35 mgGAE.g^{-1}).

The low total phenolic values in callus culture 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 of the action of enzymes may occur, usually presented in adult plants or even by the insufficient distribution of these enzymes necessary for the synthesis and accumulation of these compounds in the cells of the cultivated callus (MUHITCH; FLETCHER, 1985; AMARAL; SILVA, 2003).

In relation to the callus growth period, it was observed that in callus of 6-156-6 clone the production remained constant until 105 days, followed by an increase up to 135 days and later a decrease in production at 150 days. On the other hand, phenolic compound production at 105 days in callus of F1 clone decreased 1.2 times when compared to 90 days of growth (Figure 12). In this period (105 days), the callus are in the linear phase of growth, a period that increases the cellular area. This phase, together with the exponential growth phase requires a greater demand of carbon and energy, therefore, the biosynthesis of primary metabolites is favored while the production of secondary compounds is low (SANTOS-GOMES, 2003).

This decline behavior in the production of phenolic compounds in this growth phase was observed in callus of *Theobroma cacau* (JALAL; COLLIN, 1979) and in

isoflavonoids content such as genistein and daidzein in soy suspension culture (GUEVEN; KNORR, 2011).

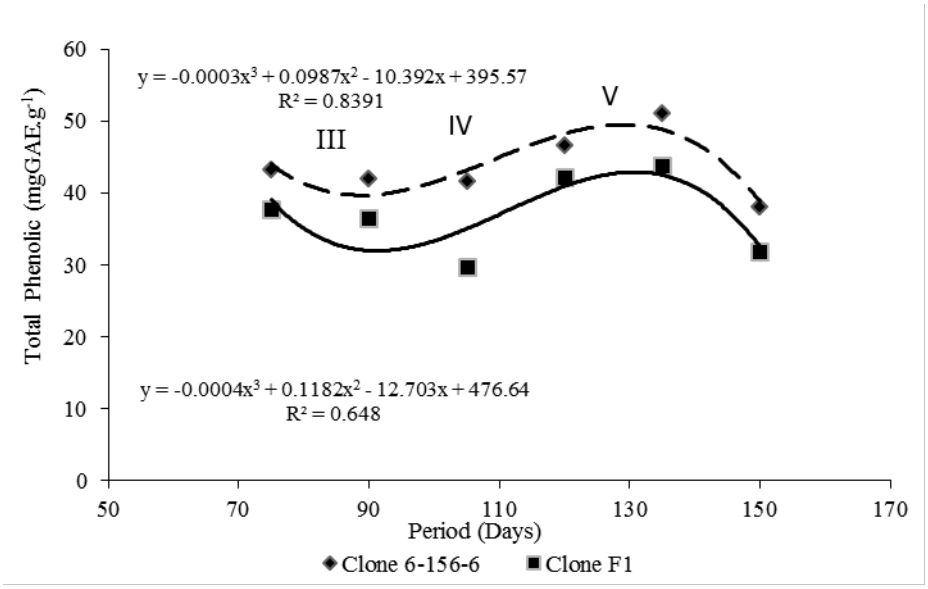

Figure 12 - Production of total phenolic compounds in different growth periods in yerba mate callus. Phases: III - linear, IV - deceleration, V - stationary.

In both clones, the highest production of phenolic compounds occurred near 135 days (F1: 43.7 mgGAE.g^{-1}; 6-156-6: 51.1 mgGAE.g^{-1}), the period in which callus are in the growth stationary phase. Typically, the production of secondary compounds, such as polyphenols, and plant cell growth are inversely correlated. Thus, the production of secondary compounds occurs generally 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).

Our results were consistent with previous reports for the highest levels of phenolic compound production in callus cultures of different medicinal species during the stationary growth stage of callus (GIRI et al., 2012; MANEECHAI et al., 2012; ALI et al., 2013).

4.3.3 Antioxidant activity

In a study comparing yerba mate with 30 other species, the extract of yerba mate had one of the strongest antioxidant activities and one of the highest contents

of phenolic compounds (DUDONNE et al., 2009). In this work, the DPPH free radical scavenging activity of the extracts of yerba mate callus in different periods of *in vitro* growth was evaluated.

Interaction between clones and callus growth periods in DPPH free radical scavenging activity was observed. Callus of 6-156-6 clone showed antioxidant activity 1.4 times higher than the callus of F1 clone ($p < 0.01$) when the average of all the evaluated periods were used.

The highest antioxidant activity was observed at 120 days of callus growth in both clones with an average of 89.4% of DPPH radical scavenging activity. When the leaves were analyzed, no difference ($p > 0.05$) was observed between the 6-156-6 and F1 clones in the DPPH radical scavenging activity (92.2%). However, the antioxidant activity was higher in leaves (216.7 $\mu molTE.g^{-1}$) than in callus with 120 days (104.6 $\mu molTE.g^{-1}$).

A similar result was observed in *Cynara cardunculus* leaves, which showed higher antioxidant activity than callus of the same species (TRAJTEMBERG et al., 2006). On the other hand, in *Stevia rebaudiana* species, the DPPH radical scavenging activity was higher in callus than in leaves (TADHANI et al., 2007).

As for the production of phenolic compounds, the antioxidant activity presented different behavior for the two clones (Figure 13). Callus of 6-156-6 clone showed constant activity up to 135 days, however a decline of 1.5 times was observed at 150 days compared to the period of highest production (120 days).

Callus of the F1 clone presented a decline in antioxidant activity at 90 days (1.2 times) followed by an abrupt decline at 105 days, six times more than the initial period (75 days). Subsequently, an increase in antioxidant activity of almost 8 times was observed, being 120 days the period of greatest DPPH radical scavenging activity.

In both clones the highest free radical scavenging activity occurred during the stationary growth phase of the callus, at which time both clones presented similar activity, around 100 $\mu molTE.g^{-1}$, corroborating with other studies that observed higher antioxidant activity at this stage of growth (ALI et al., 2013).

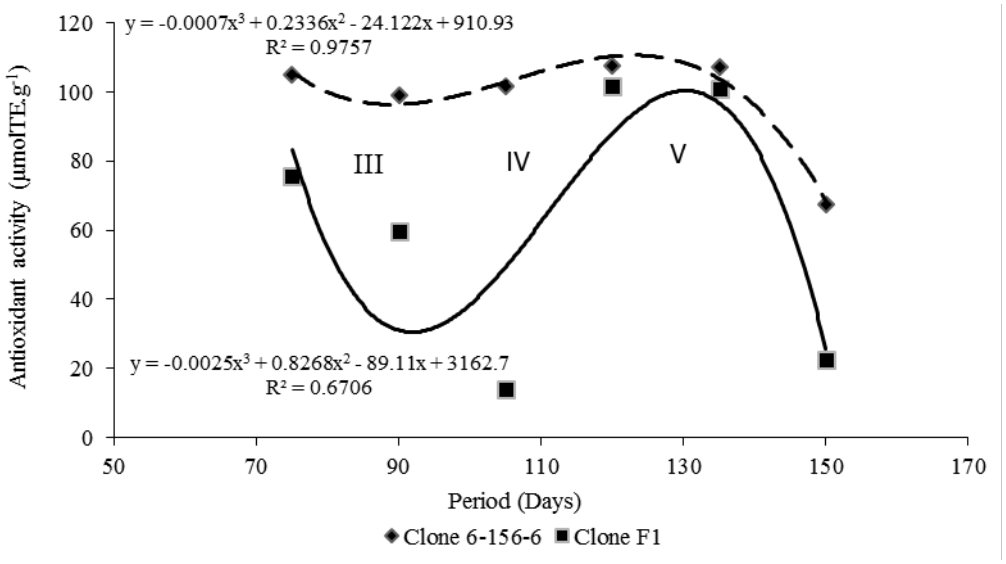

Figure 13 - DPPH radical scavenging activity in different growth periods in yerba mate callus. Phases: III - linear, IV - deceleration, V - stationary.

4.3.4 Characterization and quantification of polyphenols and methylxanthines

The Figure 14 shows the characteristic chromatogram of the hydro-alcoholic extracts of the yerba mate callus. Compounds 1 and 3 were identified as theobromine and caffeine, respectively. These two compounds belong to the group of methylxanthines and are representative in yerba mate (FILIP et al. 1998).

The peaks 2, 5, 6 and 7 were identified respectively as chlorogenic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. Chlorogenic acids and isomers of dicaffeoylquinic acid have been previously described in yerba mate and have been reported as the major constituents of their leaves (FILIP et al., 2001; BRAVO et al., 2007).

Together with the flavonoids class, quercetin and kaempferol are reported in *I. paraguariensis,* but they were neither identified in leaves or callus in this work. This is probably due to the fact that these compounds are found in low concentrations in mate leaves, around 0.06% (FILIP et al., 2001).

In addition to the aforementioned compounds, rutin was identified in the leaves of both clones, with concentrations of 3.2 $mg.g^{-1}$ and 4.7 $mg.g^{-1}$, respectively for clones F1 and 6-156-6. However, in yerba mate callus the concentration of this compound was less than 1 $mg.g^{-1}$. This may be justified, because compared to

cultured plant cells, the intact plant often produces different amounts with different profiles of secondary compounds and these quantitative and qualitative characteristics may change with the culture time (TEPE; SOKMEN, 2007).

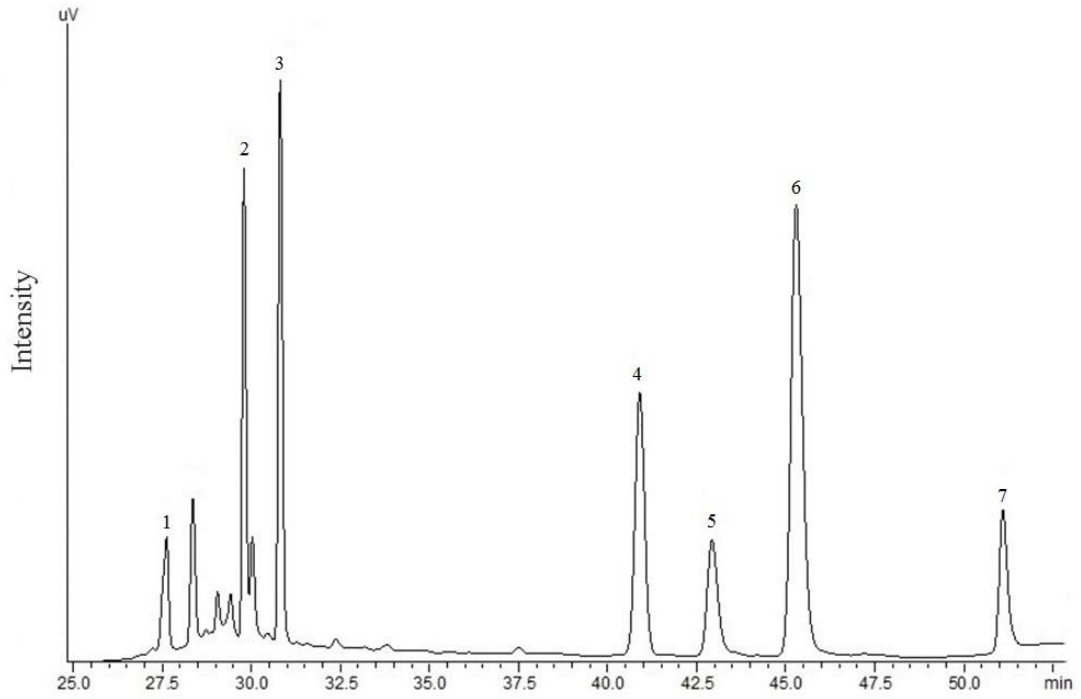

Figure 14 - Chromatogram of the compounds present in callus of F1 clones cultured for 135 days in culture medium. Retention time (min): 27.5 (1- theobromine), 29.8 (2- chlorogenic acid), 30.8 (3- caffeine), 41.0 4- internal standard, umbelliferone), 42.8 (5- 3,4-dicaffeoylquinic acid), 45.5 (6- 3,5-dicaffeoylquinic acid), 51.2 (7- 4,5-dicaffeoylquinic acid), respectively. Signal at 280 nm, chromatographic conditions are specified in the Material and Methods.

This assertion justifies the difference observed in the production of some compounds in yerba mate callus in relation to the cultivation time. The relation between the periods of time evaluated and the production of the compounds showed a significant difference for chlorogenic acid and 3,5-dicaffeoylquinic acid ($p < 0.05$, Figure 15) as well as caffeine and theobromine. For these two compounds it was not possible to adjust a significant regression model. Caffeine was the compound that presented the highest production in callus of 6-156-6 clone, with an average of 9.11 $mg.g^{-1}$ at 135 days.

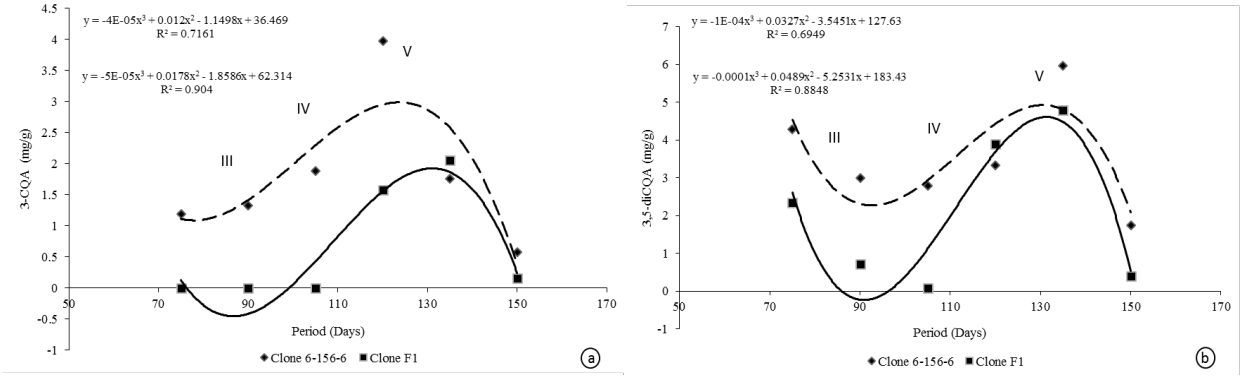

Figure 15 - Production of chlorogenic acid (a) and 3,5-dicaffeoylquinic acid (b) in different growth periods in yerba mate callus. Phases: III - linear, IV - deceleration, V - stationary.

In callus of F1 clone, the most abundant compound was 3,5-dicaffeoylquinic acid with 4.79 $mg.g^{-1}$ at 135 days of cultivation during the stationary phase of growth. The highest production of secondary compounds at this stage were also reported in callus culture of other species (ALI et al., 2013).

On the other hand, the lowest production of 3,5-dicaffeoylquinic acid in callus of F1 clone occurred at 105 days, during the exponential growth phase. This corresponds to 68 times less of this compound at the peak of higher production that occurred during the stationary phase. These results agree with those observed in callus culture of *Rosmarinus officinalis* during the linear growth phase, which resulted in low values of rosmarinic acid (YESIL-CELIKTAS et al., 2007).

Low production during the linear phase may be related to cell and vacuole expansion (SMITH, 1992), the main site of sequestration and storage of secondary metabolites (GROTEWOLD, 2004), as well the main storage site for phenolic compounds such as chlorogenic acid, methylxanthines (BAUMANN; ROHRIG, 1989) and flavonoids (ZHAO; DIXON, 2010).

For both compounds and clones the highest production occurred during the stationary growth phase of the callus, followed by a drop at 150 days. Due to the slow growth, the callus in this period would be entering in the decline phase, which is characterized by mass loss, followed by cell death (SANTOS et al., 2017).

The chromatographic profiles of the analyzed callus samples were similar, varying only the peak intensity as a function of the analyzed period (Figure 14). In addition, the compounds 5 and 7 with significant expression were identified as 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid, respectively by UV and LC-MS/MS (data not show).

4.3.5 Correlation between the content of total phenolic, secondary compounds and antioxidant activity in callus culture

Table 3 shows the correlations between the variables of interest in this work. There is a positive correlation between total phenolic and antioxidant activity in yerba mate callus cultures. Our results are in accordance with previous reports on positive correlations between antioxidant activity and total phenolic content in mate leaves (BRAVO et al., 2007). The antioxidant properties of polyphenols are attributed to the ability to act as reducing agents, which allows them to donate hydrogen, chelate metals and neutralize unique oxygen (PILUZZA et al., 2011).

Among the phenolic compounds, particularly the caffeoyl derivatives have high antioxidant capacity (BRAVO et al., 2007). The chlorogenic acid is reported as largely responsible for the antioxidant potential of *I. paraguariensis* (ANESINI et al., 2012). Chlorogenic acids are described as having antioxidant, hypoglycemic, antiviral and hepatoprotective activities. Similarly, infusion of yerba mate leaves showed higher amounts of chlorogenic acids when compared to other infusions of medicinal plants such as *Camellia sinensis* (MARQUES; FARAH, 2009).

In addition to the chlorogenic acid, the 3,5-dicaffeoylquinic acid presented high correlation, both with total phenolic content and antioxidant activity (Table 3). This compound can be used, mainly in formulations, as potent anti-inflammatory (HONG et al., 2015). The caffeoylquinic acids possess phenolic groups, which determines to a great extent the antioxidant properties. The 3,5-dicaffeoylquinic acid showed a correlation with the antioxidant activity superior to chlorogenic acid. This is due to the fact that dicaffeoylquinic acids have two phenol groups compared to one in monocaffeoylquinic acids such as chlorogenic acid (WANG et al., 2003; MENIN et al., 2013).

A high correlation between 3,5-dicaffeoylquinic acid and chlorogenic acid production with caffeine was also observed. Caffeoylquinic acids are capable of sequestering caffeine in the vacuole and forming complexes with phenylpropanoids (WALDHAUSER; BAUMANN, 1996). This explains the correlation of caffeine with the variable total phenolic compounds.

TABLE 3 - CORRELATION BETWEEN SECONDARY COMPOUNDS AND ANTIOXIDANT ACTIVITY DURING THE PRODUCTION OF COMPOUNDS IN CALLUS OF *I. paraguariensis* CLONES CULTURED *in vitro*.

	3-CQA ($mg.g^{-1}$)	Caf ($mg.g^{-1}$)	3,5-diCQA ($mg.g^{-1}$)	DPPH ($\mu molTE.g^{-1}$)	TPC ($mgGAE.g^{-1}$)
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 ($\mu molTE.g^{-1}$)					0.921

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

Theobromine had no correlation with any other variable analyzed. This compound together with caffeine belongs to the group of methylxanthines and are representative in yerba mate. They have stimulating properties and are used against physical and mental fatigue (FILIP et al., 1998). The administration of extracts of yerba mate, which contain caffeine and theobromine displayed antidepressant effect in rats (LUDKA et al., 2016).

Caffeine is the most widely used psychoactive substance in the world and many studies have proven its effectiveness with numerous benefits. It acts as a neuroprotective in Parkinson's disease (XU et al., 2010) and in combination with theobromine improves mood and cognitive performance (MITCHELL et al., 2011). Besides that the hydroalcoholic extract of *Ilex paraguariensis* showed a differential modulation of learning and short- and long-term memory in rodents (PREDIGER et al., 2008).

The interest for natural products is growing, due largely to the restrictions on addition of synthetic compounds composition of drugs, cosmetics and foods, required by many regulatory agencies (OCHOA-VILLARREAL et al., 2016). Although, there is less accumulation of secondary compounds in callus compared to mate leaves. These compounds produced 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.

4.4 CONCLUSION

The extracts of yerba mate callus had potential for the production of compounds of interest such as chlorogenic acid and 3,5-dicaffeoylquinic acid, with

high antioxidant activity. The stationary growth phase of the callus is the phase at which the highest concentration of these compounds are extracted for use in the pharmaceutical formulation.

There is a difference between the clones for the production of secondary compounds in yerba mate callus. Callus of 6-156-6 clone had a higher accumulation of caffeine, chlorogenic acid, and 3,5-dicaffeoylquinic acid, whereas, callus of F1 clone accumulated more theobromine.

In the leaves no differences were observed between the clones in relation to the concentration of the secondary compounds, although their contents were higher than in the callus. Yerba mate callus are capable of producing secondary compounds even under favorable conditions of their growth and future works focusing on increasing the production of these compounds by elicitation should be carried out.

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5 DOES UV-C RADIATION AFFECT THE ACCUMULATION OF SECONDARY COMPOUNDS IN *Ilex paraguariensis* CALLUS CULTURES?

ABSTRACT

Callus cultivation is a potential tool for the production of secondary compounds, in spite of that the differentiated cells usually do not exhibit the same accumulation of compounds found in the parent plant. Abiotic stresses, such as UV-C irradiation, may promote increased production of secondary compounds in callus culture. In this work, *Ilex paraguariensis* callus were induced from two genotypes using leaves explants on ¼ MS medium supplemented with 4.52 µM 2,4-dichlorophenoxyacetic acid and 4.56 µM zeatin. After 120 days of *in vitro* culture, the callus were subjected to a UV-C radiation for 0, 5, 12.5 and 20 min and incubated for 6, 39 and 72 h at the dark. The secondary compounds accumulation and the total phenolic content (TPC) were analysed. We show that the answer depends on the genotype, exposure time and incubation time after the irradiation. The results demonstrated that the clone F1 is slighty influenced by UV-C irradiation and do not altered the total phenolic content, chlorogenic acid, 3,4-dicaffeoylquinic acid and 4,5-dicaffeoylquinic acid. On the other hand, the clone 6-156-6 decreased the production of dicaffeoylquinic acids, caffeoylquinic acids and TPC depending of exposure and incubation time. The clone 6-156-6 is more suitable than F1 for *in vitro* callus cultivation, since the amount of secondary compounds is higher in the former. *Ilex paraguariensis* callus culture shows to be clone-specific, and under the conditions tested the UV-C is not recommended to increase the secondary compounds in *Ilex paraguariensis* callus.

Keywords: elicitation; yerba mate; secondary metabolites; HPLC.

5.1 INTRODUCTION

Plant natural products, commonly referred to as secondary compounds, have been invaluable sources for drugs generation (KOEHN; CARTER, 2005). The search for novel natural products has declined due to an increased use of synthetic molecules. However, the increasing restrictions by regulatory agencies on the addition of synthetic dyes or flavors in foods associated with limitations in new drug approvals and loss of patent protection for important drugs has amplified the market demand and the interest in new strategies for the production of natural products (LI; VEDERAS, 2009); (OCHOA-VILLARREAL et al., 2016).

Yerba mate (*Ilex paraguariensis*) is a South American plant whose leaves are used mainly for the preparation of stimulant beverages (FILIP et al., 2001) and its potential for medicinal use have already been reported (MIRANDA et al., 2008; ARÇARI et al., 2009; LUDKA et al., 2016). The chlorogenic acids are the major

constituents in this plant and are extensively applied as medicinal, cosmetics and addictive substances with high added values. The production of this valuable plant compounds in controlled conditions without being affected by the season and environmental variations is interesting and match the industrial and pharmaceutical points of view (JAWAHAR et al., 2014; NAIK; AL-KHAYRI, 2016).

The *in vitro* culture has become a viable and reliable technique for the mass production of plant material and a potential tool for the production and accumulation of bioactive compounds (DIAS et al., 2016). Unfortunately, dedifferentiated plant cells are unable to produce the levels of metabolites found in the parent plant (YEOMAN; YEOMAN, 1996) and effective strategies may be used to increase compound yields (ALVERO-BASCOS; UNGSON, 2012).

Among the strategies, abiotic elicitation is one of the methods used to increase the production of secondary compounds in callus culture (CETIN, 2014). This method incorporates a physical stress via the application of heavy metals, osmotic stress, thermal shock, cold or UV radiation to increase the production of compounds of interest (ZHOU; WU, 2006). The UV-C (200-290 nm) radiation is more efficient than UV-A (320-400 nm) and UV-B (290-320 nm) in eliciting physiological responses and to increase the production of secondary compounds with antioxidant properties (CONCONI et al., 1996; ANJUM et al., 2017). The increase in phenolic and flavonoids occurs as a defense mechanism against damage caused by reactive oxygen species, produced in response to UV radiation (JULKUNEN-TIITTO et al., 2005; SURJADINATA et al., 2017).

In a previous work, we have observed that yerba mate callus produce secondary compounds (Chapter III). According to Dias et al., (2016) in most cases, elicitation promotes higher yields of compounds when compared to non-elicited cultures, and this increase in bioactive compounds allows its subsequent application in the pharmaceutical or food industries. Thus, this work aimed to evaluate the production of secondary compounds and total phenolics in the *Ilex paraguariensis* callus culture of two clones in response to UV-C radiation.

5.2 MATERIAL AND METHODS

5.2.1 Plant material and culture conditions

Yerba mate callus were obtained from leaves of two clones (F1 and 6-156-6) from the genetic breeding program of EMBRAPA Forestry, Colombo, Paraná, Brazil. The leaves were collected from greenhouse grown mini-stumps of the clones, disinfected in a solution of Cercobin® 1% (w/v) for 10 min followed by 5 min in mercury chloride 0.05% (w/v) and it was rinsed three times with sterile distilled water. Afterwards, leaf discs were cut and placed on the ¼ MS salts and vitamins (MURASHIGE; SKOOG, 1964), supplemented with 3% sucrose, 4.52 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.56 µM zeatin and 7 $g.L^{-1}$ agar, as described in the Chapter 3.

5.2.2 Exposure to UV-C radiation

To investigate the effect of UV-C radiation on the production of secondary compounds, UV-C lamp (Philips TUV-8 W, wavelength 254 nm) was used as elicitor. Before irradiation, 120-day old callus were placed on Petri dishes with fresh culture medium.

The UV-C light was applied at a distance of 20 cm, lasting 5, 12.5 and 20 min without the lid of the Petri dish in sterile chamber. After irradiation, the Petri dishes were sealed and incubated in the dark at 23 ± 2 °C. At 6, 39 and 72 h after exposure, callus were collected and stored at -80 °C for analysis of the secondary compounds. Callus of the two clones not exposed to UV-C radiation were used as a control. The experiment was carried out in quadruplicate.

5.2.3 Extraction of *Ilex paraguariensis* callus

The callus were extracted as described in the Chapter 3. Briefly, frozen callus (10 mg) were extracted with a *hydroalcoholic* solution (ethanol: water, 1:1, 1 mL) for 1 h at 60 ºC. The extracts were centrifuged and the supernatants collected. Part of the extracts was used for biochemical tests and 500 µL was transferred to the vial (2 mL) which the 25 µg of internal standard umbelliferone was added for quality control (Sigma®).

5.2.4 Identification and quantification of secondary compounds

Chromatographic analyses were conducted on an Agilent 1290 Infinity Liquid Chromatography (HPLC) using a UV detector. Separation and semi-quantification of the compounds were performed as described in the Chapter 3, using a C18 Synergy Fusion-RP 80A (75 x 4.6 mm, d.i. 4 µm) column, with a C18 pre-column. The gradient elution program consisting in the mobile phase 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), using the flow rate at 1 $mL.m^{-1}$, and 20 µL of sample injection.

The identification of the compounds was carried out by LC-MS/MS analysis as described in the Chapter 3, using the same eluents and column as describe above with a slight modification of gradient elution, flow rate and sample injection. The gradient profile was: 0–0.5 min (0% B), 0.5–25 min (0%–5% B), 25–28 min (5%–40% B), 28–30 min (40%-60% B), 30-31 min (60%-0% B), 31-35 (0% B), flow rate at 0.5 $mL.min^{-1}$ and 2 µL of sample injection.

5.2.5 Determination of total phenolic compounds

Total phenol content was determined by the spectrophotometric method. The Folin-Ciocalteu reagent was used according to a method described by (HORŽIĆ et

al., 2009) with modifications as described in the Chapter 3. The absorbance was measured at 725 nm using UV–Visible spectrophotometer (Shimadzu-1800; Japan). The analytical curve of gallic acid (0.25-10 $mg.L^{-1}$) was used as a reference for converting the absorbance measured in equivalent milligrams of gallic acid per gram of sample ($mgGAE.g^{-1}$), of dry weight (DW).

5.2.6 Statistical analyses

The experiment was conducted in a randomized design, in a factorial arrangement with four replicates. The factors analyzed were two clones of yerba mate in 10 exposure/incubation times. Data were tested for homogeneity by the Bartlett test. Analysis of variance was performed using ANOVA, and Scott-Knott mean test at 5% of error probability using ASSISTAT® program (SILVA e AZEVEDO, 2009).

5.3 RESULTS AND DISCUSSION

5.3.1 UV-C irradiation effect on secondary compounds in *I. paraguariensis* callus

The effect of UV-C irradiation for 5, 12.5 or 20 min, after incubation for 6, 39 or 72 h on yerba mate callus secondary compounds accumulation were analysed. The difference between two genotypes was also investigated. Three caffeoylquinic acids (CQA), three dicaffeoylquinic acids (DQA) and two methylxanthines were identified in the callus extract by LC-MS/MS as described in the Chapter 3 and semi-quantification by HPLC-UV. Figure 16 shows a typical chromatogram of the extract obtained from callus of both clones elicited by UV-C.

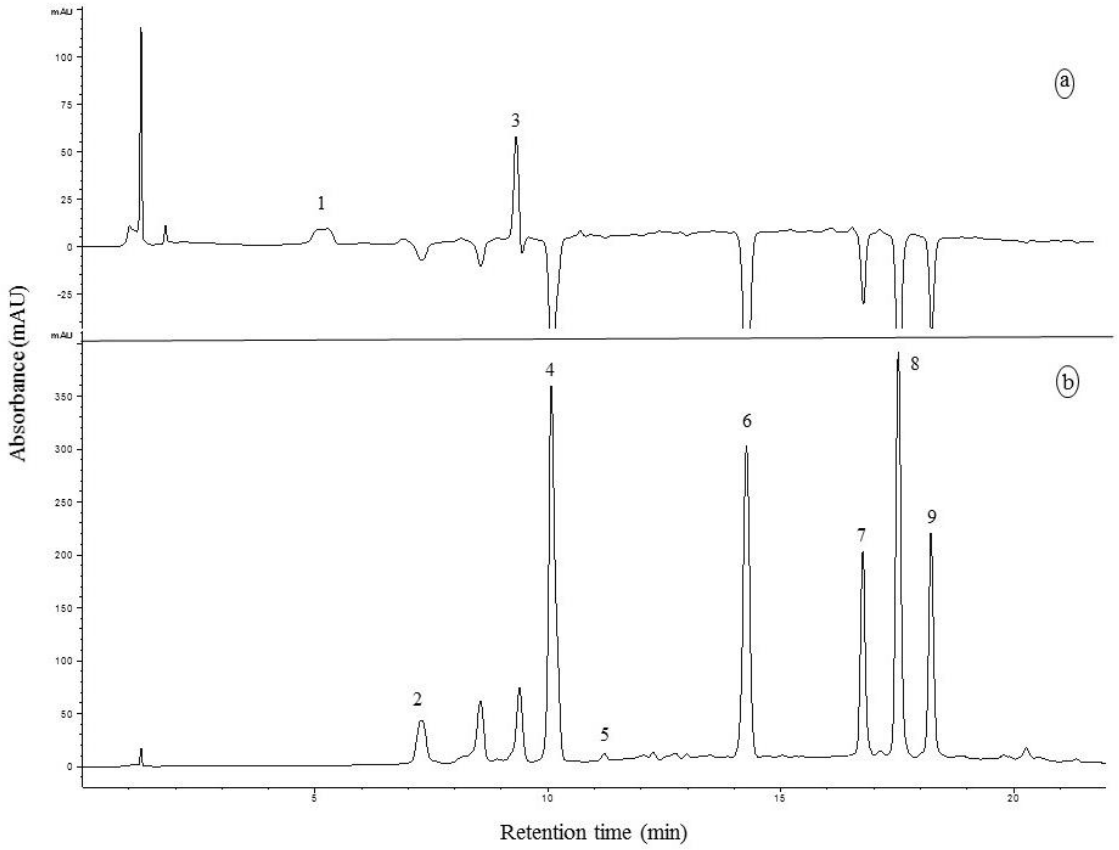

Figure 16 - Typical HPLC chromatogram for *I. paraguariensis* callus extract from clone 6-156-6 elicited by UV-C for 5 min after 72 h. a) detection 260 nm for caffeine and theobromine, b) detection 328 nm for caffeic acid derivatives. Peaks: (1) theobromine; (2) neochlorogenic acid; (3) caffeine; (4) 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.

The results for the CQA accumulation shown in Figure 17, shows differences between callus from the two genotypes in response to different UV-C exposure incubation times. The genetics proved to be determinant in response to the accumulation of CQA in yerba mate callus elicited by UV-C light. The compound cryptochlorogenic acid (peak 5) was detected in callus sample in low concentration (>0.01 $mg.g^{-1}$), and was further excluded from the statistical analysis.

The clone F1 did not change the content of chlorogenic acid ($p > 0.05$), whereas in most of the exposure and incubation times the accumulation of neochlorogenic acid decreased ($p < 0.05$). In callus from clone 6-156-6 the production of both compounds decreased or did not differ from the control, which started with higher compound amounts.

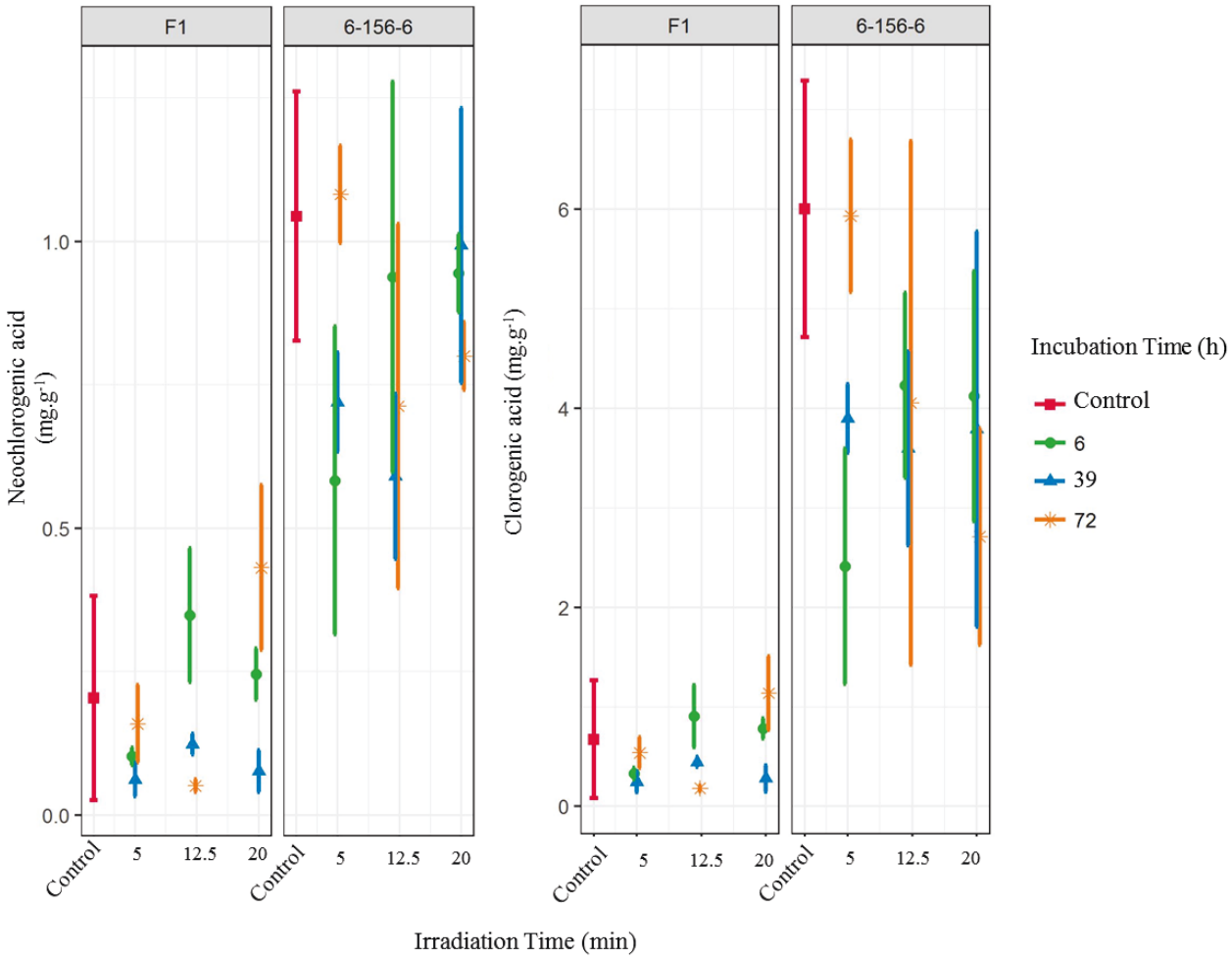

Figure 17 - Effect of UV-C irradiation time and incubation time on the production of caffeoylquinic acids in callus culture of two clones of *I. paraguariensis*. The results are expressed in milligrams per gram of dry weight as mean values ± standard deviation (SD).

Consistent with our results, decrease on the caffeoylquinic acids content was also observed in leaves of *Ilex paraguariensis* subjected to UV-C light (LEWINSKI et al., 2015). In a different way, in callus of *Cynara cardunculus* L. var. *scolymus* (MENIN et al., 2013) and *Solanum lycopersicum* under UV-C elicitation the production of caffeoylquinic acid increased (CLÉ et al., 2008).

We observed that the amount of these compounds were higher in the clone 6-156-6 than in clone F1. For example, the chlorogenic acid was six fold higher in callus of the clone 6-156-6. The same pattern was observed for DQA (Figure 18).

The UV-C radiation did not affect the accumulation of 3,4-dicaffeoylquinic acid (peak 7) and 4,5-dicaffeoylquinic acid (peak 9) for callus of F1 clone ($p > 0.05$) (Figure 18). The 3,5-dicaffeoylquinic acid (peak 8) decreased when the UV-C was applied for 5 min after 6 and 39 h, as well as when the UV-C was applied for 12.5 min

after 72 h ($p < 0.05$). In the other times the amounts of these compounds did not show difference from the control ($p > 0.05$). Similar results were found in yerba mate leaves, being the 3,4-dicaffeoylquinic acid and the 4,5-dicaffeoylquinic acid not influenced by UV-C light (LEWINSKI et al., 2015).

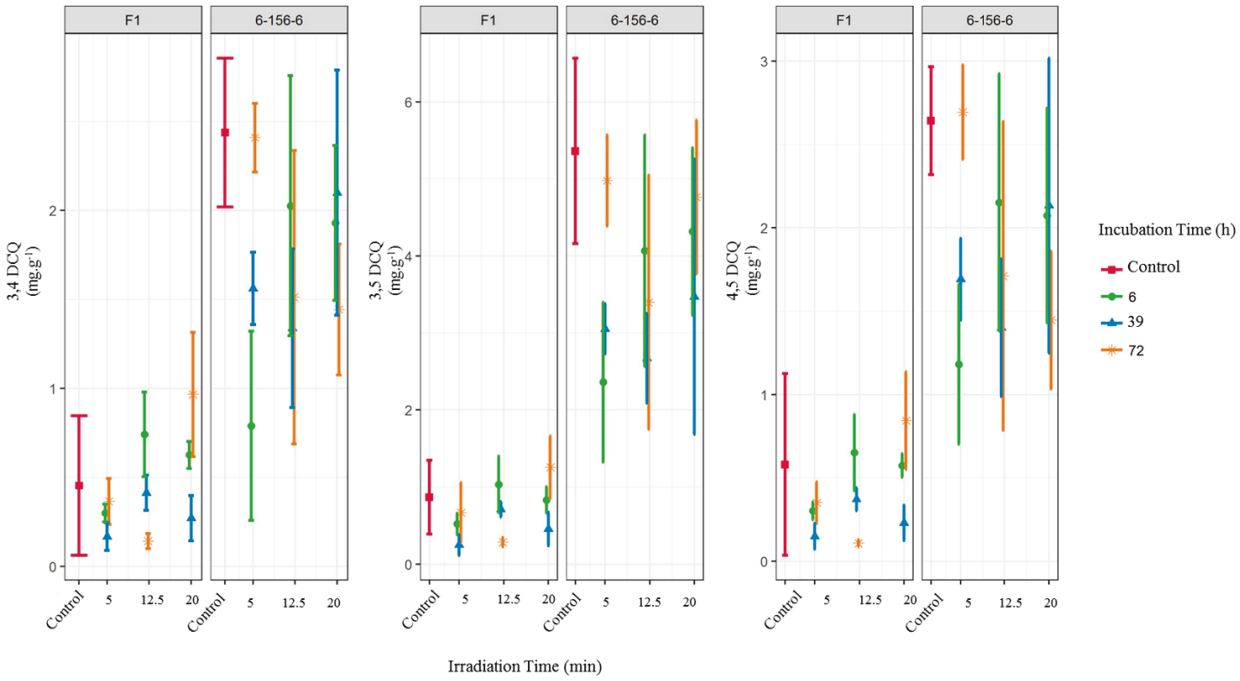

Figure 18 - Effect of UV-C irradiation time and incubation time on the production of dicaffeoylquinic acids in callus culture of two clones of *I. paraguariensis*. The results are expressed in milligrams per gram of dry weight as mean values ± standard deviation (SD).

The clone 6-156-6, started with higher compound contents in the non-irradiated callus and showed a negative response depending of the exposure and incubation times ($p< 0.05$). Following the same pattern, UV-C treatments had no positive effect on *Eurycoma longifolia* callus culture, and shows a decreasing accumulation of isocoumarin (PARIKRAMA; ESYANTI, 2014). In *Vitis vinifera* callus, the accumulation of β and γ-tocopherols also was lower than the control group (CETIN, 2014). Formica-Oliveira et al. (2016) also found reduction in phenolic accumulation in irradiated carrot samples with moderate UV-C dose, compared to non-irradiated samples.

Such decreasing response may be explained by the UV-C photons, which have enough energy to initiate photochemical reactions and cause damages in many cell processes. The damages by UV irradiation activate endogenous sensitisers and the generation of active oxygen species. Active oxygen species signaling process in

plants serve to modify metabolism and gene expression allowing the plant to respond to adverse environmental conditions. The capacity of the antioxidative defense system is often increased at such times, but if the response is not sufficient, radical production will exceed scavenging and ultimately lead to the disruption of metabolism (KOVÁCS; KERESZTES, 2002).

It was possible to observe similar behavior for both clones when the callus was exposed to UV-C for 5 min and incubated for 6 and 39 h the content of CQAs and DQAs decreased ($p < 0.05$). However, after 72 h these compounds increased again reaching the same levels as the control ($p > 0.05$). A possible explanation may be a partial phenylalanine ammonia-lyase (PAL) denaturation by such UV-C dose delaying the stress-enhanced activity of this enzyme. A subsequent PAL reactivation may occur around 72 h, returning to the content observed in control, as was also noted by Formica-Oliveira et al. (2016) in carrot.

Among all the compounds, only theobromine showed the same concentration in callus for both clones ($p > 0.05$). No significative interaction was observed between clones and exposition of UV-C radiation for the compounds theobromine and caffeine (Figure 19).

Theobromine levels (peak 1) did not change in clone F1 ($p > 0.05$), but a small increase was observed in 6-156-6 callus exposed for 5 min after 72 h ($p < 0.05$). The opposite behaviour was observed for caffeine (peak 3), where only the clone F1 showed some alteration in the concentration (Figure 19) ($p < 0.05$). Reports indicating the direct response of caffeine and theobromine to UV elicitation were not found.

However, on works with *Coffea arabica* cell culture, it was observed an increase in the production of caffeine and theobromine as a function of the applied radiation. This effect was attributed to the stress caused by light, which may act as an inducer of methyltransferases, increasing enzymatic activities and causing stress in the primary metabolism. As a result of accumulation in the purine rings that are the precursors of the biosynthesis of alkaloids (FRISCHKNECHT; BAIJMANN, 1985); (KURATA et al., 1997).

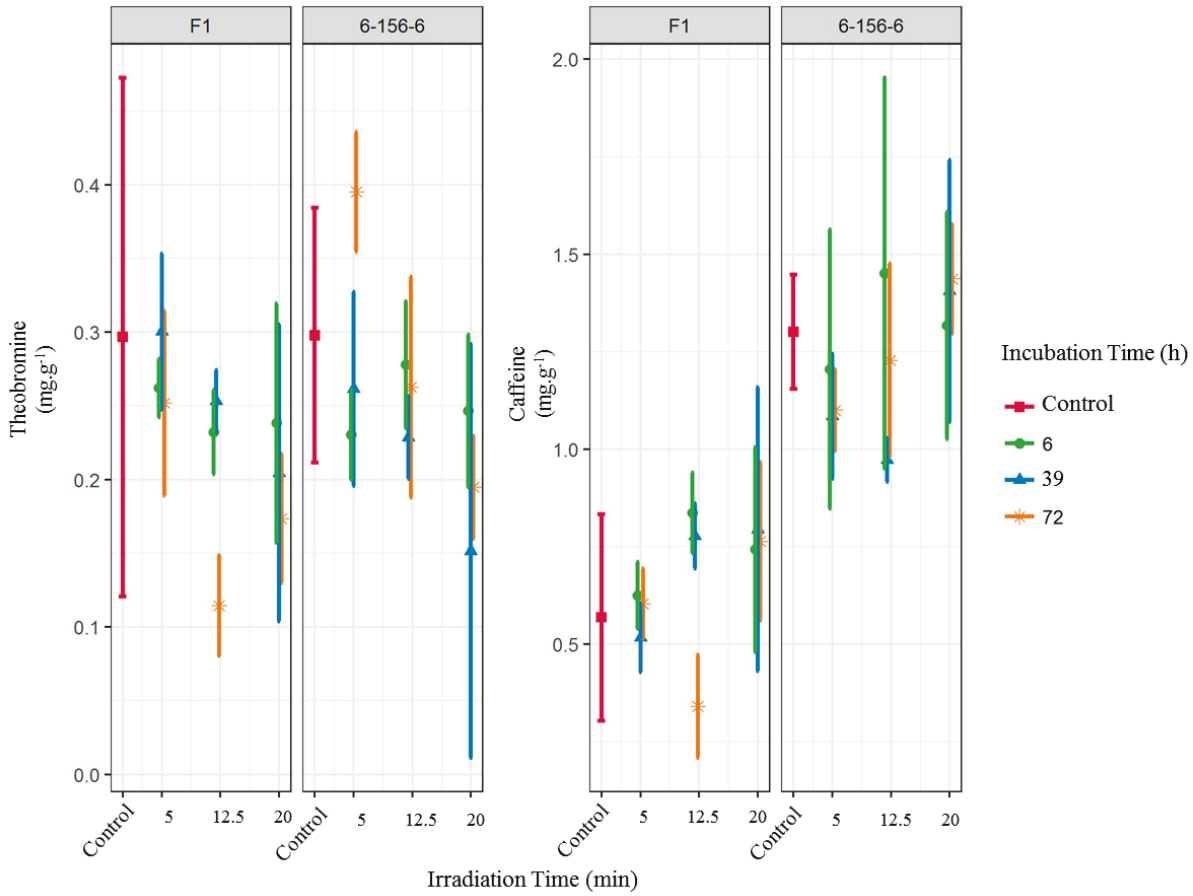

Figure 19 - Effect of UV-C irradiation time and incubation time on the production of theobromine and caffeine in callus culture of two clones of *I. paraguariensis*. The results are expressed in milligrams per gram of dry weight as mean values ± standard deviation (SD).

The results showed that the genotype is a crucial factor in secondary compounds production of *in vitro* yerba mate callus. The clone 6-156-6 showed significant amounts of CQAs, DQAs and caffeine than the clone F1. However, the UV-C promoted a decrease of these compounds in callus of the clone 6-156-6 while did not influence the content of 3,4-dicaffeoylquinic, 4,5-dicaffeoylquinic and chlorogenic acid in callus of clone F1 subjected to UV-C light. This difference suggests that the culture sensitivity to UV-C radiation, might be more clone-specific than species-specific (JULKUNEN-TIITTO et al., 2005).

Besides, compounds elicited by UV light can be genetically determined, and a genotypic dependent elucidation of secondary compounds due to UV irradiation was also observed for grape (LIU et al., 2010) and bean varieties (KACHARAVA et al., 2009). However, for the species *I. paraguariensis* the UV-C elicitation was not efficient for increasing the secondary compounds in any clone tested. The *I.*

paraguariensis is a species that naturally produces higher amounts of secondary compounds (FILIP et al., 2001; BRAVO et al., 2007). Normally plants that under natural conditions already have higher contents of the chemical defences do not increase the compounds production after stress conditions (LATTANZIO, 2013).

The compounds identified in this work could be used in many application as analytical grade reagent, food addictive, bulk product, pharmaceutical active ingredient. The chlorogenic acid and its isomers are considered in the category of Fine Chemicals in the international market (BUTIUK et al., 2016). In addition, some bioactivities have been attributed to CQAs such as digestive, hepatoprotective (AZZINI et al., 2017), anti-thrombotic (FUENTES et al., 2014), analgesic, anti-carcinogenic (MARQUES; FARAH, 2009). The DQAs showed anti-inflamatory (HONG et al., 2015) and anti-HIV activity (HEYMAN et al., 2015). The caffeine acts as a neuroprotective (XU et al., 2010) and in combination with theobromine improves mood and cognitive performance acting as an antidepressant agent (MITCHELL et al., 2011; LUDKA et al., 2016).

5.3.2 Total phenolic content of yerba mate callus subjected to UV-C irradiation

The effects of UV-C treatments on total phenolic content (TPC) was also examined. Phenolic composition of the callus samples changed significantly according to the genotype ($p < 0.05$). Total phenolic contents of the callus samples were estimated with Folin–Ciocalteu colorimetric method and UV-C irradiation do not seems to affect the TPC of the clone F1, but decrease the TPC in callus of the clone 6-156-6 depending on the exposure and incubation times.

It was possible to observe that the TPC is not only represented by the classes of CQAs and DQAs quantified by HPLC (Figure 20 a, b). This happened due to the fact that the Folin-Ciocalteu phenol reagent reacts with other phenols beside the chlorogenic acids which has a reactivity of 0.7 (EVERETTE et al., 2010). Other compounds already identified in yerba-mate leaves such gallic acid and the tannic acid (VIEIRA et al., 2010), have higher reactivity than chlorogenic acid (1 and 0.9) and may also be present in callus, in tiny amount (not detected by HPLC). Besides, other nonphenolic compounds (amino acids) like tryptophan and tyrosine that belong to the primary metabolism also are reactive toward the reagent Folin-Ciocalteu,

explaining the difference between the TPC and the total amount of chlorogenic acids (EVERETTE et al., 2010).

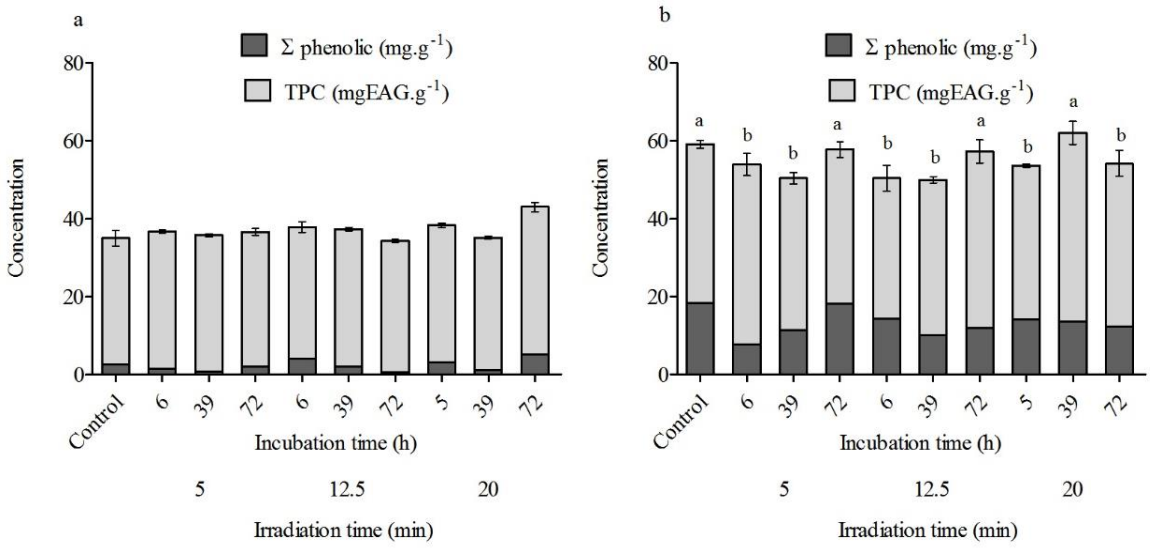

Figure 20 - Effect of UV-C irradiation time and incubation time on the total phenolic content (TPC) and total accumulation of chlorogenic acids (∑ phenolic) of two clones of *I. paraguariensis.* a) Clone F1 and b) clone 6-156-6.

Means followed by the same letter do not differ statistically from one another, uppercase in the column and lowercase in the line by the Scott-Knott test ($p < 0.05$).

The chlorogenic acids and isomers represent around 20% of the total phenolic content in callus of the clone 6-156-6, while for the clone F1 these compounds represent only 10%. This is because, levels of secondary compounds, mainly phenolic compounds in plants are both environmentally induced as well as genetically controlled (LATTANZIO, 2013).

The clone 6-156-6 showed higher amount of TPC and in the total amount of chlorogenic acids than the clone F1. If we observe the control group, the clone 6-156-6 produced seven fold more CQAS and DQAS than the clone F1. However, how was showed in the Chapter 4, the callus of clone F1 had higher growth compared with callus of the clone 6-156-6, and the same behavior was observed for plants in greenhouse conditions (data not shown). In this way, it is possible that the clone F1 dispenses more energy for growth while the clone 6-156-6 uses more energy for the secondary metabolism. These defensive compounds are considered to be costly for plants, reducing the growth rate, as more carbon is diverted from growth toward secondary metabolism (LATTANZIO, 2013).

Due to the higher metabolite production of the clone 6-156-6 compared with the clone F1, the former is more appropriate for *in vitro* secondary compounds production. Although, the clone F1 showed higher growth (Chapter 4), if we calculate the total production of DQAs and CQAs per callus, per cycle of grow (120 days) the production of these compounds in each callus of clone 6-156-6 are five fold higher than the production of the clone F1. This way, the clone 6-156-6 can be used for production of secondary compounds *in vitro* and the elicitation by UV-C is not recommended for this clone.

5.4 CONCLUSION

The results showed that the UV-C radiation effect in the amount of secondary compounds in yerba mate callus is genotype specific. The clone 6-156-6 decreased the content of secondary compounds and TPC depending of the exposure and incubation times. Only the exposure for 5 min followed by incubation for 72 h kept the same level of the control in all the compounds analyzed in this clone.

In callus from clone F1 the content of 3,4-dicaffeoylquinic, 4,5-dicaffeoylquinic acid, chlorogenic acid and TPC were not affected by UV-C radiation in any irradiation and incubation times.

The clone 6-156-6 showed higher contents of CQAs, DQAs, caffeine and TPC than the clone F1, and this clone may be used to exploit *in vitro* production of useful secondary compounds to the industrial and pharmaceutical formulations, being the use of UV-C elicitation not recommended in the tested conditions.

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6 FINAL CONSIDERATIONS

This work report for the first time, the ability that yerba mate callus have to produce secondary compounds with high market value and remarkable applications. Callus growth conditions were optimized, growth kinetics were understood, and elicitation methods were tested.

It was observed that the genetic variation had a strong influence on the callus induction from foliar explants of yerba mate, and the induction responses ranged from 0 to 80% depending on the genotype.

The type and concentration of cytokinin influenced callus induction and growth. When the two variables were combined (induction and fresh weight) high doses of zeatin (4.56 and 9 μM) and 0.5 μM TDZ promoted similar results, allowing both to be used for induction and growth of yerba mate callus.

Sucrose has been shown to influence the accumulation of secondary compounds, but high concentrations suppress callus growth, probably due to osmotic stress in cells. Higher levels of sucrose (6 and 9%) did not alter the antioxidant activity and the compounds caffeine and 3,5-dicaffeoylquinic acid. However, the concentration of 3% sucrose favoured the accumulation of total phenolic compounds and the chlorogenic, neochlorogenic, cryptochlorogenic and 3,4-dicaffeoylquini acids in yerba mate callus. In this way, the use of 3% sucrose can be recommended for the cultivation of callus of this species.

The two clones that had the better response to callus induction (6-156-6 and F1) were used to evaluate the kinetics of growth of yerba mate callus. It was possible to identify five distinct growth phases: lag, linear, exponential, deceleration and stationary.

Callus growth and accumulation of secondary compounds showed to be inversely correlated. The production of secondary compounds, antioxidant activity and the accumulation of total phenolics is lower during the linear phase of growth. In the linear phase, together with the exponential phase, the callus requires more carbon and energy for its growth favouring the biosynthesis of primary metabolites while the production of secondary compounds is smaller.

Higher accumulation of secondary compounds was observed during the stationary phase of growth, at which time the cell growth ceases, favouring the

biosynthesis of the secondary metabolites. This behaviour was observed for the two clones. However, the F1 clone had a higher growth response, whereas clone 6-156-6 had higher concentrations of caffeine, chlorogenic acid and 3,5-dicaffeoylquinic acid.

A positive correlation was observed between phenolic compounds and antioxidant activity, as well as between antioxidant activity and compounds derived from caffeyol, showing that yerba mate callus can be used in pharmaceutical and cosmetic formulations.

In spite of the good results for the production of the compounds in callus, leaf content of secondary compounds was higher than in the former. However, the production of these compounds by callus cultivation is the possibility of production in a controlled manner without the interference of climatic conditions which naturally affect the accumulation of the secondary compounds. Besides, it is possible to produce the compounds of interest in different places where the plant has no natural occurrence.

It was observed that the use of high concentrations of sucrose did not favour the accumulation of secondary compounds in yerba mate callus, in this way, the elicitation by UV-C radiation was used to verify the response in two yerba mate clones. However, under the tested conditions, for both clones, the elicitation by UV-C was not efficient to promote an increase of the secondary compounds and total phenolics.

Clone 6-156-6 had higher total phenolic content production and a 7-fold increase in caffeine and dicaffeoylquinic compounds than clone F1. Considering this production and relating to callus growth per cycle (120 days), the accumulation of these compounds for clone 6-156-6 is five times greater than the clone F1. Therefore, it is recommended to use clone 6-156-6 for the production of CQAS and DQAS compounds, which are considered fine compounds by the international market.

Yerba mate callus can be used as a new raw material for the production of important secondary compounds. The applicability of the compounds produced in yerba mate callus culture, has been reported in several studies, considering the anti-HIV activity of dicaffeyolquinic acids the most notable among the findings. In this way, this work was a pioneer for the production of yerba mate compounds in a controlled manner and is expected to stimulate future studies with bioreactors, new methods of elicitation and even for the future production of these compounds on industrial scale.

In addition, callus culture have been used to study the biosynthesis of economically important secondary compounds, and this such studies may be useful, not only as a production system for secondary compounds but also as a tool to understand the metabolic pathway leading to methylxanthines, CQAs and DQAs.

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