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Original Paper

Effects of different factors on friable callus induction and establishment of cell suspension culture of *Hovenia dulcis* (Rhamnaceae)

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Abstract

Medicinal plants are an important therapeutic option for a large share of the world's population. To establish an *in vitro* culture system for the production of secondary metabolites from *Hovenia dulcis*, we studied the effect of auxins, cytokinins, absence of light, and silver nitrate on the development of friable callus. Callus cultures were established for the first time and used to obtain cell suspension cultures. Supplementation with KIN (Kinetin) produced calli with both compact and friable areas, while the addition of TDZ (Thidiazuron) only produced compact callus. The maintenance of cultures in the dark induced a slight enhancement on friability when the auxin PIC (Picloram) was present in the culture medium. The addition of silver nitrate promoted the formation of friable calli. Dry weight analysis showed no significant differences in biomass growth, and, therefore, 2.0 $mg.L^{-1}$ was considered the most suitable treatment. The presence of silver nitrate was not required for the establishment of cell suspension cultures. Dry weight analysis of cell suspensions showed higher biomass production in the absence of silver nitrate. PIC promoted 100% of cell suspension culture formation in the absence of silver nitrate, and higher biomass production was observed with the lowest concentration (0.625 $mg.L^{-1}$). No morphological differences were observed among the different concentrations of PIC. Phytochemical screening showed the presence of saponins, flavonoids, flavonols and catechins in the extracts obtained from *H. dulcis* calli. These results show that the cell cultures herein established are potential sources for the production of *H. dulcis* secondary metabolites of medicinal interest.

Key words: cytokinins, medicinal plant, plant tissue culture, saponins, silver nitrate.

Resumo

Plantas medicinais são uma importante opção terapêutica para uma grande parte da população mundial. A fim de se estabelecer um sistema *in vitro* para a produção de metabólitos secundários de *Hovenia dulcis*, foram estudados os efeitos de auxinas, citocininas, ausência de luz e nitrato de prata no desenvolvimento de calos friáveis. Culturas de calos friáveis foram estabelecidas pela primeira vez para a espécie e usadas na obtenção de culturas de células em suspensão. A suplementação com KIN (Cinetina) produziu calos com regiões compactas e friáveis, enquanto a adição do TDZ (Thidiazuron) produziu somente calos compactos. A manutenção das culturas no escuro induziu o aumento da friabilidade quando a auxina PIC (picloram) estava presente no meio de cultura. A adição do nitrato de prata promoveu a formação de calos friáveis. A análise do peso fresco não demonstrou diferença significativa na produção de biomassa e por essa razão 2.0 $mg.L^{-1}$ foi considerada a concentração mais apropriada. A presença do nitrato de prata não foi necessária para o estabelecimento de culturas de células em suspensão, e a análise do peso seco das culturas demonstrou maior acúmulo de biomassa na ausência dessa substância. O uso de PIC (picloram) na menor concentração (0.625 mg.L-1) promoveu 100% de formação de culturas de células em suspensão na ausência de nitrato de prata com

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a maior produção de biomassa. Não foram observadas diferenças morfológicas nas diferentes concentrações de PIC. A análise fitoquímica demonstrou a presença de saponinas, flavonoides, flavonóis e catequinas nos extratos obtidos a partir de calos de *H. dulcis*. Estes resultados demonstram que as culturas de células aqui estabelecidas são potenciais fontes de produção de metabólitos secundários de *H. dulcis*.
Palavras-chave: citocininas, planta medicinal, cultura de tecidos vegetais, saponinas, nitrato de prata.

Introduction

For a large share of the world's population, plants represent the foundation of primary health care. Unsustainable harvesting of medicinal plants severely depletes these resources (Cordell 2011), endangering biodiversity. It is estimated that over 1,300 medicinal plants are used in Europe, of which 90 % are harvested from natural resources. Worldwide, between 50,000 and 80,000 flowering plant species are used for medicinal purposes, and some 15,000 of these are threatened with extinction from over harvesting and habitat destruction (Chen *et al.* 2016). In Brazil, the majority of the native medicinal plants of commercial importance are harvested from natural populations (Melo *et al.* 2009). This type of harvesting of medicinal plants not only endangers biodiversity, but also risks potential variation in plant quality and, occasionally, improper plant identification (Petronilho *et al.* 2012). In general, the production of plant secondary metabolites in nature is linked to a particular growth or developmental stage, or it can be influenced by seasonal or stress conditions. This calls for the development of alternative methods of production (Baque *et al.* 2013). Also, the amount of secondary metabolites obtained from plant tissues is about 1%, often less. For example, the content of paclitaxel in the bark of *Taxus* species is only 0.01% of dry weight (Georgiev *et al*. 2009).

Plant tissue culture methods are a useful technology for the production of secondary metabolites. *In vitro* systems may improve the accumulation of compounds when compared to the wild plant (Cai *et al*. 2012), and plant cell cultures can be continuously carried out without seasonal constraints. According to Ribeiro *et al.* (2015a), the use of biotransformation on plant cell culture systems has immense potential for production of compounds with commercial interest, especially considering the biochemical capability for production of secondary metabolites. So, biotransformation is also gaining considerable attention as a step towards green chemistry by reducing the usage of hazardous chemicals. In this sense, plant cell cultures exhibit a vast biochemical potential for production of specific secondary metabolites. Once established, *in vitro* cultures can lead to the production of plants and metabolites indefinitely and are available on demand (Doran 2009). The most impactful case of commercial use of plant cell cultures is related to the antineoplastic drug paclitaxel. The U.S. Food and Drug Administration (FDA) approved the use of paclitaxel for the treatment of breast, lung, and ovarian cancers, as well as AIDS-related Kaposi's sarcoma. Initially extracted from harvested bark of *Taxus brevifolia*, it faced problems related to slow growth and availability of the tree and low product yield. In response, alternative methods, including total synthesis, semi-synthesis, and plant cell culture, were developed. In 2002, Bristol-Myers Squibb switched from semi-synthesis to plant cell culture production methods for Taxol® (Vongpaseuth & Roberts 2007; Wilson & Roberts 2012; Cusido *et al.* 2014; Wilson *et al.* 2018).

Plant cell and tissue cultures allow the increase of biomass and secondary metabolite production through the application of several techniques. These methods include the exploitation of exudation to the liquid medium, bioreactor scale-up, elicitation, addition of metabolic precursors, genetic engineering and metabolic farming, among others (Smetanska 2008; Bernabé-Antonio *et al.* 2010; Sabater-Jara *et al.* 2010; Cai *et al.* 2012; Peng & He 2013). The effectiveness of such methods can be exemplified by the high-yield production in cultures of *Perilla frutescens*, *Morinda citrifolia* and *Salvia officinalis* (Smetanska 2018). Among the tissue culture techniques, callus and cell suspension cultures represent important strategies for the production of metabolites.

Callus is the term frequently used to name the disorganized masses, including cells in various degrees of differentiation (Ikeuchi *et al.* 2013). Traditionally, it was thought that the balance between two classes of plant hormones, auxins and cytokinins, determined the state of differentiation and dedifferentiation (Skoog & Miller 1957), but until recently, little was known about the molecular mechanism(s) leading to

callus formation. However, it seems that callus development occurs as a response to various physiological and environmental stimuli (Ikeuchi *et al.* 2013).

Cell suspension cultures consist of single cells or small cell groups dispersed and growing in liquid medium under agitation. A plant cell suspension culture is usually initiated by placing friable callus fragments into a suitable sterile liquid medium (Dixon 1985). Plant cell suspension cultures offer advantages for large-scale production of chemicals in bioreactors and for the study of cellular and molecular processes, since such cell suspension cultures are a simplified model system for the study of plants. Cell suspension cultures contain a relatively homogeneous cell population, allowing rapid and uniform access to nutrition, precursors, growth hormones and signal compounds for cells (Mustafa *et al.* 2011). Thus, callus and cell suspension cultures offer suitable conditions for the production of natural products. These methods are of great value since they allow controlled cultivation, providing a continuous and homogeneous synthesis of raw material, regardless of environmental and seasonal factors. However, callus consistency is an important condition for the establishment of cell suspensions, and friable calli are indicated for the establishment of these systems.

Auxins are involved in developmental processes including stem and internode elongation, tropisms, apical dominance, abscission and rooting. In plant tissue cultures, auxins have been used to induce cell division, cytodifferentiation as well as organogenic and embryogenic differentiation. At low concentration, auxins tend to favor root initiation, whereas at higher concentration they induce callus formation. Cytokinins act on cell division, apical dominance and shoot differentiation. When present in culture media they are capable of triggering cell division, promoting callus formation, inducing differentiation of adventitious shoots and promoting shoot proliferation. Ethylene is also recognized as a plant hormone which influences growth and development of plants. *In vitro* studies have shown that ethylene can affect callus growth, shoot regeneration and somatic embryogenesis. Silver nitrate ($AgNO_3$) has been shown to inhibit ethylene action and therefore it can be used with this purpose in tissue culture studies (Bhojwani & Dantu 2013; Ikeuchi *et al.* 2013; Beyer 1976; Kumar *et al.* 2009).

Hovenia dulcis Thunb. is commonly known as Japanese raisin tree (alternatively named Japanese cherry tree or Chinese raisin tree), and it has been used in Asia for more than a millennium as a medicinal plant. *H. dulcis* belongs to the Rhamnaceae family, and its natural occurrence ranges from Japan, Korea, and East China to the Himalayas up to altitudes of 2,000 m (Hyun *et al.* 2010). Its medicinal properties were reviewed by Hyun and co-workers (2010). Among these properties, antineoplastic (Park & Chang 2007), and hepatoprotective (Fang *et al.* 2007; You *et al.* 2009) effects have been of particular interest. But pharmacological effects, such as antifatigue, antidiabetic and antigiardia have been achieved too (Na *et al.* 2013; Yang *et al.* 2019; Gadelha *et al.* 2005). It was proposed that dihydromyricetin, a flavonoid found in *H. dulcis* (Yoshikawa *et al.* 1997), could be used as a drug to treat alcohol dependence (Shen *et al.* 2012). Aqueous extract of *H. dulcis* induced osteogenic differentiation of calvarial osteoblasts and increased femoral bone mass in mice. Moreover, methyl vanillate, which is present in this extract, activated the Wnt/β-catenin pathway and induced osteoblast differentiation *in vitro*, suggesting that *H. dulcis* extracts are potential agents for treating osteoporosis (Cha *et al.* 2014).

So far, the methods of plant tissue culture developed for *H. dulcis* aim at the establishment of protocols for plant propagation, including phytochemical analysis of *in vitro* plants (Yang *et al.* 2013; Echeverrigaray *et al.* 1998; Eom *et al.* 2002; Jeong *et al.* 2009; Park *et al.* 2012; Li *et al.* 2006; Ribeiro *et al.* 2010). Friable calli were previously reported for the species, but only in association with plant regeneration (Park *et al.* 2012; Jeong *et al.* 2009). Also, non-organogenic compact callus cultures were established (Ribeiro *et al.* 2010, 2015). However, to the best of our knowledge, among the methods of *in vitro* cultivation of *H. dulcis* described in the literature, no previous study has reported on the establishment of non-organogenic friable callus lines and cell suspension cultures.

Therefore, in this study, we report the effect of $AgNO_3$ on friable calli formation and the establishment of cell suspension cultures obtained from these calli. This is the first description of the effects of $AgNO_3$ on members of the Rhamnaceae family under *in vitro* conditions. This study also presents the effects of the absence of light, as well as auxins and cytokinins on *H. dulcis in vitro* cultures. Hence, we report the first method to establish plant cell suspension cultures of

the medicinal tree *H. dulcis*, a system that can add considerable value to the exploitation of its bioactive compounds.

Material and Methods

Culture conditions

The pH of the media was adjusted to 5.8 before adding agar (8 $g.L^{-1}$ Merck), dispensed into 8.3 × 6.5 cm flasks (30 mL per flask), and autoclaved (121 ºC, 104 KPa) for 15 min. Three explants were inoculated into each flask in a total of 12 explants per treatment. All experiments were conducted in triplicate. Cultures were incubated in a growth chamber under 16 h photoperiod provided by cool-white fluorescent tubes at 26 ± 2 ºC. Subcultures in the fresh media were performed after four weeks, and data were analyzed after eight weeks.

For liquid cultures, the flasks were maintained on a rotary shaker (New Brunswick Scientific) at 110 rpm at 26 ± 2 ºC and kept in the dark. Subcultures in fresh media were performed after four weeks, and data were analyzed after eight weeks.

Morphological characteristics of callus cultures (texture and color) as well as biomass growth based on dry weight (DW), were scored after 60 days. Dry weight was obtained after drying to constant weight at 45 ºC for 24 h.

The effect of auxins and cytokinins on the induction of friable callus

Two-month-old stem segments (5 mm) obtained from *in vitro* raised plantlets (Ribeiro *et al*. 2015b) were cultured on MS medium (Murashige & Skoog 1962) containing 30 $g.L^{-1}$ sucrose, supplemented with 1.25 $mg.L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 1.25 $mg.L^{-1}$ 4-amino-3,5,6-trichloropicolinic acid (PIC) or 2.5 $mg.L^{-1}$ α-naphthaleneacetic acid (NAA) in association with the cytokinins 6-furfuryladenine (KIN) or 1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ) at 0.65 or 1.25 $mg.L^{-1}$.

The effect of absence of light on friable callus formation

Two-month-old stem segments (5 mm) obtained from *in vitro* raised plantlets (Ribeiro *et al.* 2015b) were cultured on MS medium (Murashige & Skoog 1962) containing 30 $g.L^{-1}$ sucrose, supplemented with 1.25 $mg.L^{-1}$ PIC and 0.65 $mg.L^{-1}$ KIN and kept in the dark.

The effect of silver nitrate on callus friability

Two-month-old stem segments (5 mm) obtained from *in vitro* raised plantlets (Ribeiro *et al.* 2015b) were cultured on MS medium (Murashige & Skoog 1962) containing 30 $g.L^{-1}$ sucrose and supplemented with 1.25 $mg.L^{-1}$ PIC and 0.65 $mg.L^{-1}$ KIN and different concentrations of $AgNO_3$ (0, 2.0, 4.0, and 6.0 $mg.L^{-1}$).

Friable callus maintenance

In order to verify the need of $AgNO_3$ for friable callus maintenance, callus samples were subcultured to MS medium (Murashige & Skoog 1962) containing 30 $g.L^{-1}$ sucrose, supplemented with 1.25 $mg.L^{-1}$ PIC and 0.65 $mg.L^{-1}$ KIN in the absence of $AgNO_3$ or in the presence of 2.0 $mg.L^{-1}$ $AgNO_3$.

Establishment of cell suspension cultures

Friable callus samples (1.5 g) were obtained after eight weeks in MS medium (Murashige & Skoog 1962) containing 30 $g.L^{-1}$ sucrose, supplemented with 1.25 $mg.L^{-1}$ PIC, 0.65 $mg.L^{-1}$ KIN and 2.0 $mg.L^{-1}$ $AgNO_3$, under dark conditions. These were then transferred to Erlenmeyer flasks (50 mL) containing 10 mL of liquid medium. In a first experiment, the callus samples were transferred to media containing different concentrations of $AgNO_3$ (0, 2, 4 or 6 $mg.L^{-1}$). In the second experiment, callus samples were placed in media containing different concentrations of PIC (0.65, 1.25 or 2.5 $mg.L^{-1}$).

Phytochemical screening

Samples obtained from calli grown on MS medium (Murashige & Skoog 1962) containing 30 $g.L^{-1}$ sucrose and supplemented with 1.25 $mg.L^{-1}$ PIC, 0.65 $mg.L^{-1}$ KIN and 2.0 $mg.L^{-1}$ $AgNO_3$ were dried for 24 h at 45 ºC, powdered and extracted in ethanol (Merck) for seven days at 26 ± 2 ºC. The obtained solution was filtered (Whatman paper) and concentrated on a rotary evaporator under reduced pressure at 40 ºC. The presence of secondary metabolites was verified by the methods proposed by Barbosa *et al*. (2004). After, 5 mg of extract were diluted in 20 mL of distilled water and transferred to three test tubes (3 mL per tube) and the pH of the was adjusted to 3, 8.5 and 11. The second assay used to determine the presence of flavonoids were performed as follows. A sample

of 3 mL was transferred to two test tubes. The pH of the test tubes was adjusted to 1.5 and 11 and heated for three minutes. The color obtained after the reactions was used to identify the presence of flavonoids. To evaluate the presence of alkaloids, 5 mg of extract were diluted in 5 mL of a HCl solution (5%) and transferred to three test tubes (1 mL per tube) to each tube were added 10 drops of Bouchardt, Draggendorf or Mayer reagents. The presence of alkaloids was verified by the formation of precipitates. To assess the presence of saponins, 5 mg of extract were diluted in 15 mL of distilled water and vigorously agitated for two minutes. The formation of a persistent foam layer indicates the presence of saponins.

Statistical Analysis

The experiments followed a sequential and completely randomized experimental design (Compton 1994; Compton & Mize 1999). Results are presented as mean values ± S.D. Data were submitted to analysis of variance (ANOVA), followed by Tukey's test. The tests were performed using GraphPad Prism 7.0 software.

Results and Discussion

The effect of auxins and cytokinins on the induction of friable callus

As previously reported in *H. dulcis* cultures, auxin supplementation can control the type of callus that will be developed (Ribeiro *et al.* 2015b). In the present-work, we tested the hypothesis that different cytokinins could have the same effect and, in addition, allow the development of friable lines. Based on the evidence that BAP is not able to induce friable calli (Ribeiro *et al.* 2015b), we tested KIN and TDZ supplementation. The addition of KIN did not produce friable callus lines, and the cultures supplemented with this growth regulator presented both compact and friable areas (mixed callus). The supplementation of TDZ produced compact callus (Tab. 1). Combinations of NAA with KIN caused strong oxidation of the explants, and no callus formation was observed (Tab. 1). Although no friable calli were produced, the observation that TDZ only induced compact callus formation indicates the validity of the hypothesis that different cytokinins might influence the type of callus formed in *H. dulcis*. The combination of 2,4-D and PIC with KIN resulted in mixed callus formation, while the combination of all auxins with TDZ resulted in compact callus. The same combinations using BAP, instead of KIN, as source of cytokinin, also produced mixed callus (Ribeiro *et al.* 2015b). Despite the extensive use of callus culture as a plant biotechnology system, knowledge of the molecular mechanisms underlying callus formation is limited (Ikeuchi *et al.* 2013). The absence of purine ring, typical in cytokinins, on the structure of TDZ (Lu 1993) might indicate a different molecular mechanism and therefore be responsible for the differences observed, but the mechanism by which TDZ works is not fully described (Guo *et al.* 2011). In terms of morphological aspects, no difference was observed when comparing calli from the different media. Compact areas presented light green color, while the friable parts were creamish yellow (Fig. 1).

The effect of absence of light on friable callus formation

The maintenance of cultures in the absence of light induced a slight enhancement on callus friability when PIC was added to the culture medium. This effect, though, was not enough to sustain the formation of homogeneous friable calli, and the observed results were considered as mixed callus cultures. When NAA was added to the culture medium, the cultures grown in the absence of light maintained their compact morphology. The compact calli formed in this condition lost the green color and began to show a creamish color (Tab. 2). This change in color under dark conditions is not unusual since light is important in chloroplast formation. In callus cultures, the presence of light also has an essential role in chloroplast formation. The same responses to light conditions were observed in *Nicotiana tabacum* callus (Siddique & Islam 2015). The regulation of chloroplast gene expression was demonstrated in *Jatropha curcas* (Yong *et al.* 2011).

The effect of silver nitrate on callus friability

The exact mechanism of $AgNO_3$ activity on plants is still unclear. It is believed that silver ions compete for binding sites on membrane-localized ethylene receptors. Therefore, silver nitrate can block the activity of ethylene by reducing receptor capacity to bind to this hormone. $AgNO_3$ antiethylene properties are adequate for plant tissue culture because of its persistence, specificity, and lack of phytotoxicity at effective concentrations

Table 1 – Effect of auxins and cytokinins on the establishment of *Hovenia dulcis* friable callus cultures kept on MS medium after eight weeks.

Culture medium composition	Callus type	Percentage of friable callus induction	Dry weight (mg)
1,25 mg.L^{-1}2,4-D + 0.65 mg.L^{-1}KIN	Mixed	0	20.23 ± 21.20 c
1.25 mg.L^{-1}2,4-D + 1.25 mg.L^{-1}KIN	Mixed	0	37.05 ± 35.04 bc
1.25 mg.L^{-1}PIC + 0.65 mg.L^{-1}KIN	Mixed	0	33.41 ± 19.73 bc
1.25 mg.L^{-1}PIC + 1.25 mg.L^{-1}KIN	Mixed	0	36.51 ± 31.20
2.5 mg.L^{-1}NAA + 0.65 mg.L^{-1}KIN	-	0	-
2.5 mg.L^{-1}NAA + 1.25 mg.L^{-1}KIN	-	0	-
1.25 mg.L^{-1}PIC + 1.25 mg.L^{-1}TDZ	Compact	0	52.88 ± 67.98 abc
1.25 mg.L^{-1}2,4-D + 1.25 mg.L^{-1}TDZ	Compact	0	65.9 ± 64.74 ab
2.5 mg.L^{-1}NAA + 1.25 mg.L^{-1}TDZ	Compact	0	69.52 ± 37.16 a

Values represent mean ± SD of three replicates. Different small letters within each column indicate significant (P > 0.05) differences between treatments by ANOVA followed by Tukey's test.

(Beyer 1976; Kumar *et al.* 2009). Silver ions may be added to the culture medium as $AgNO_3$ or $Ag_2S_2O_3$ (Batista *et al.* 2013) The addition of $AgNO_3$ to the culture media promoted the formation of friable calli in all tested concentrations (Fig. 2). Dry weight analysis showed no significant differences in biomass growth among all tested concentrations; therefore, we considered the lowest concentration tested (2 mg.L^{-1}) to be the most suitable (Tab. 3). Naturally produced by plants, ethylene takes part in numerous cellular mechanisms, both *in vitro* and *in vivo*. One of the effects observed under *in vitro* conditions is related to callus formation (Kumar *et al.* 2009).

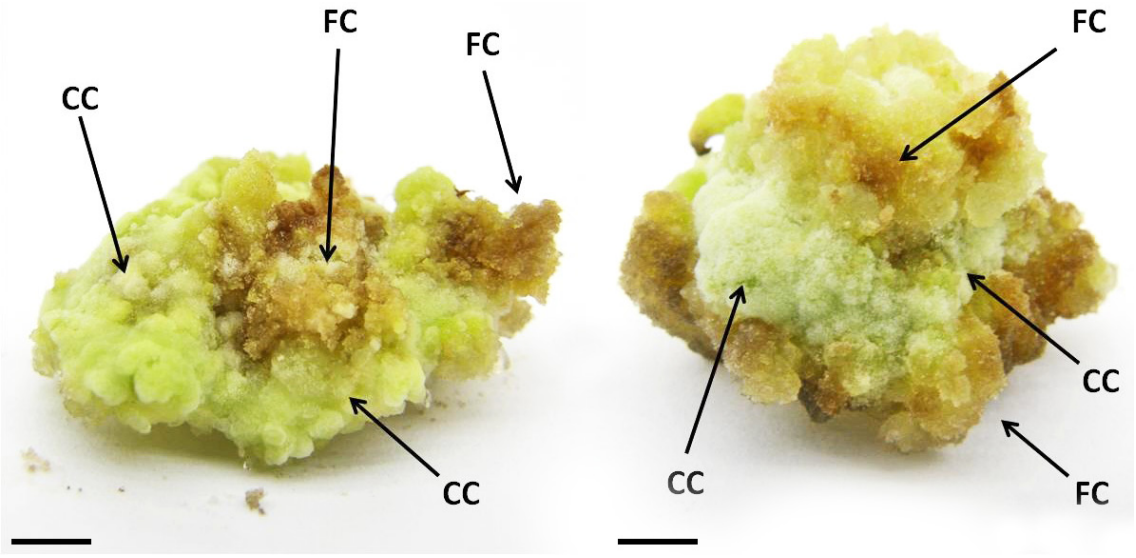

Figure 1 – Mixed callus of *Hovenia dulcis* showing morphologically diverse regions. Callus formation on MS medium supplemented with 1.25 mg.L^{-1} PIC + 0.65 mg.L^{-1} KIN after eight weeks of growth. CC = Compact Callus; FC = Friable Callus. Bar = 0.5 cm.

Table 2 – Effect of light absence on the friability of *Hovenia dulcis* callus cultures kept on MS medium after eight weeks.

Culture medium composition	Callus type	Percentage of callus induction	Dry weight (mg)
1.25 mg.L^{-1}PIC + 0.65 mg.L^{-1}KIN	Mixed	60	65.15 + 55.31
1.25 mg.L^{-1}NAA + 0.65 mg.L^{-1}KIN	Compact	95	105.00 + 88.40

Values represent mean ± SD of three replicates.

The correlation between the presence of silver ions and callus formation seems to be species-specific. The reduction of callusing was reported in some species, such as *Capsicum annuum* (Batista *et al.* 2013). In contrast, the promotion of callus friability here, as observed in the presence of $AgNO_3$, was previously reported. The addition of $AgNO_3$ to the culture media made long-term callus culture possible in *Brassica oleracea* (Williams *et al.* 1990) and *Zea mays* (Vain *et al.* 1989). All treatments of $AgNO_3$ increased the volume of calli by swelling in *Solanum lycopersicum* (Shah *et al.* 2014). The combination of an auxin with silver nitrate in the culture media promoted callus friability, reduced tissue browning and improved callus growth in the tree *Rollinia mucosa* (Figueiredo *et al.* 2000), corroborating our findings and suggesting that it may be a good strategy to enhance friability of calli from woody plants.

Friable callus maintenance

The maintenance of $AgNO_3$ on cultures was essential to keep the calli friable. When $AgNO_3$ was removed from cultures, callus continued growing, but the formation of compact regions

Figure 2 – *Hovenia dulcis* friable callus developed on MS media supplemented with 1.25 mg.L^{-1} PIC, 0.65 mg.L^{-1} KIN and 2.0 mg.L^{-1} $AgNO_3$ after eight weeks of growth. Bar = 0.5 cm.

Table 3 – Effect of silver nitrate on the friability of *Hovenia dulcis* callus cultures maintained on MS medium after eight weeks.

$AgNO_3$ (mg.L^{-1})	Viable cultures (%)	Callus type	Friable callus dry weight (mg)
0	100	Mixed	-
2	87.5	Friable	49.92 + 32.49 a
4	88.9	Friable	38.79 + 28.42 a
6	79.2	Friable	41.93 + 25.50 a

Values represent mean ± SD of three replicates. Different small letters within each column indicate significant (P > 0.05) differences between treatments by ANOVA followed by Tukey's test. - = No friable callus formation.

and the reversal for a mixed type was observed in 100% of cultures. Callus friability and biomass accumulation are important characteristics to establish cell suspension cultures. In order to assess the growth of cultures and biomass production, the literature presents several methods, both direct and indirect, that can be employed. Dry weight analysis at the end of the culture period has been widely used as a direct, and highly viable, method to estimate culture growth (Chawla 2009; Mohamad Puad & Abdullah 2018; Haida *et al.* 2019)

Establishment of cell suspension cultures

As presented in Table 4, the presence of $AgNO_3$ is not a requirement for the establishment of cell suspension cultures of *H. dulcis* from previously induced friable callus. A 100% formation in all tested concentrations was observed. From a morphological point of view, no observable differences were noted among the different treatments (Fig. 3). Dry weight analysis showed higher biomass production in the absence of $AgNO_3$, and we therefore opted to perform the second experiment in the absence of this compound. PIC supplementation promoted 100% of cell suspension culture formation regardless of the tested concentrations (Tab. 5). Dry weight analyses showed that the lower concentration (0.625 mg.L^{-1}) improved biomass production. Concerning morphological aspects, no differences were observed as a response to the different PIC concentrations.

It is widely known that friable calli are the most suitable material for the establishment of cell suspension cultures. The present study demonstrates the success of this strategy in producing *H. dulcis* cell suspension cultures. Growth regulator requirements proved to be similar for the establishment and maintenance of friable callus and suspension cultures, with only small differences in concentrations. These observations are also in accordance with several reports with different species, such as *Cyperus aromaticus* (Chan *et al.* 2010), *Nasturtium montanum* and *Cleome chelidonii* (Songsak & Lockwood 2004).

Table 4 – Effect of $AgNO_3$ supplementation on the formation of cell suspension cultures of *Hovenia dulcis* kept on MS medium after eight weeks.

$AgNO_3$ (mg.L^{-1})	CCS formation (%)	Dry weight (mg)
0	100	566.0 ± 80.02 a
2	100	304.6 ± 67.86 b
4	100	306.7 ± 119.2 b
6	100	298.4 ± 133.5 b

Values represent mean ± SD of three replicates. Different small letters within each column indicate significant (P > 0.05) differences between treatments by ANOVA followed by Tukey's test.

Figure 3 – *Hovenia dulcis* cell suspension culture developed on MS media supplemented with 0.625 $mg.L^{-1}$ PIC, 0.65 $mg.L^{-1}$ KIN and 2.0 $mg.L^{-1}$ $AgNO_3$ after four weeks of growth.

Phytochemical screening

Phytochemical screening showed the presence of saponins, flavonols, and catechins (Tab. 6), compounds that were previously isolated from *H. dulcis*. Cathechins and flavonols belong to the flavonoid class. The catechins isolated from *H. dulcis* include (-)-catechin, (+)-afzelechin (Li *et al.* 2005), (-)-epiafzelechin (An *et al.* 2007), and (+)-gallocatechin (Yoshikawa *et al.* 1996), with hovenitin I, II and III among the favonols (Yoshikawa *et al.* 1997). The cathechins were isolated mostly from stem samples while flavonols were obtained from fruits and seeds.

Saponins are the most representative class of secondary metabolites isolated from *H. dulcis*. The list includes the hodulosides I, II, III, IV, and V (Yoshikawa *et al.* 1992), the hodulosides VI, VII, VIII, IX, and X (Yoshikawa *et al.* 1993), hovenidulcioside A1 and A2 (Yoshikawa *et al.* 1995), and hovenidulcioside B1 and B2 (Yoshikawa *et al.* 1996). Hodulosides were isolated from leaves while hovenidulciosides were obtained from fruits and seeds. Previous findings suggest that auxins may have some degree of influence on the production of auxins *in vitro* (Furuya *et al.* 1983).

Although it is widely accepted that plant growth regulators may regulate the secondary metabolism *in vitro*, it is still not possible to generalize about the type and quantity of these compounds adequate for developing a productive culture and a case-by-case analysis has to be performed (Alvarez 2014).

The presence of secondary metabolites in the extracts obtained from *H. dulcis* calli shows that the cell cultures established in the present work are potential sources for the production of such compounds.

The proposed protocols revealed the feasibility of establishing an *in vitro* system for the production metabolites of medicinal interest from *H. dulcis* using callus and cell suspension cultures. To our knowledge this is an unprecedented work within the studies of plant biotechnology for the species.

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Table 5 – Effect of PIC supplementation on the formation of cell suspension cultures of *Hovenia dulcis* kept on MS medium after eight weeks.

PIC ($mg.L^{-1}$)	CCS formation (%)	Dry weight (mg)
0.625	100	657.6 + 14.24 a
1.25	100	573.0 + 75.02 ab
2.5	100	529.7 + 88.73 b

Values represent mean ± SD of three replicates. Different small letters within each column indicate significant (P > 0.05) differences between treatments by ANOVA followed by Tukey's test.

Table 6 – Chemical groups observed on extracts obtained from callus of *Hovenia dulcis*.

Chemical Class	Presence/Absence
Anthocyanins/Anthocyanidins	-
Flavones/Flavonols/Xhantones	+
Chalcones/Aurones	-
Flavanonols	-
Leucoanthocyanidins	-
Catechins	+
Flavanones	-
Alkaloids	-
Saponins	+

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