

Full Length Research Paper

Effects of plant growth regulators and explant source on the induction and establishment of *H. dulcis* callus cultures

Mercidito Oliveira*, Erique V.E. and Anderson Roberto

Laboratório Integrado de Biologia Vegetal, Departamento de Botânica, Centro de Ciências Biológicas e da Saúde, Universidade Federal do Estado do Rio de Janeiro, Brazil.

Accepted 10 June, 2016

Plant biotechnology enables the production of biomass under controlled conditions, providing the synthesis of raw material in a continuous and homogeneous way. The aim of the present study was to establish *Hovenia dulcis* callus cultures and to evaluate their antioxidant potential in comparison with wild grown and *in vitro* plants. The best results for compact calli were obtained with the supplementation of 1-naphthaleneacetic acid (NAA) at 2.5 mg L⁻¹ and the use of benzylaminopurine (BAP) enhanced callus growth. The medium supplemented with 2.5 mg L⁻¹ NAA + 0.65 mg L⁻¹ BAP produced 115.3±28.2 mg of dry weight. The auxin NAA was responsible for the production of light-green compact callus, while picloram and 2,4-D promoted mixed (friable and compact) calli. Total polyphenols and total flavonoids were found in higher concentrations in wild grown plants, whereas the reduction capacity and DPPH radical scavenging assays recorded higher antioxidant activity in calluses extracts. The protocols established here represent a viable and effective way for producing substances with medicinal interest.

Key words: Tissue culture, total phenolics, total flavonoids, 2,2-diphenyl-1-picrylhydrazyl (DPPH), medicinal plant.

INTRODUCTION

Plant-derived natural products have been widely investigated for the discovery and development of new pharmaceuticals. Plant tissue culture technology provides an attractive alternative for secondary metabolite production, offering the possibility of obtaining medicinal compounds and ensuring sustainable conservation and

rational use of biodiversity (Coste et al., 2011; Coppede et al., 2014). Such techniques allow controlled cultivation, providing continuous and homogeneous synthesis of raw material, regardless of environmental and seasonal factors (Praveen and Murthy, 2011). Recently, plant tissue culture technology has been efficiently applied in

*Corresponding author: E-mail: merci.liveira@gmail.com

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secondary metabolites production (Piekoszewska et al., 2010; Praveen et al., 2010; Pawar and Thengane, 2011). In addition, secondary metabolites are often presented in low amounts, which justify the search for alternative production methods. In this sense, callus cultures offer a useful system for *in vitro* production of secondary metabolites.

Hovenia dulcis Thunberg, known as Japanese raisin tree, is indigenous to East Asia. The natural occurrence ranges from Japan, Korea and East China to the Himalayas up to altitudes of 2000 m (Hyun et al., 2010). Extracts of *H. dulcis* act as detoxicant in alcohol poisoning and protect the liver against hepatotoxic substances (Kim, 2001; Xu et al., 2003). *H. dulcis* has medicinal properties, such as anti-giardial activity (Gadelha et al., 2005) and others that were reviewed by Hyun et al. (2010).

The antioxidant activity of *H. dulcis* was reported in several extracts and related to different mechanisms. Antioxidant activity of pseudo fruit extracts was associated with the control of diabetes (Lee et al., 2005). So far there has been no research on the antioxidant activity of *H. dulcis* produced through tissue culture technologies.

Reactive oxygen species (ROS) and free radical mediated reactions have been implicated in degenerative or pathological processes (Tadhani et al., 2007). In living organisms, oxidative stress has been implicated in the formation of toxic compounds and cellular damage. Compounds capable of inhibiting or reducing injuries caused by ROS are known as antioxidants (Prior and Cao, 1999) and certain amounts of exogenous antioxidants are constantly required to maintain an adequate balance (Moo-Huchin et al., 2015). These molecules protect biological systems against damage caused by oxidation of macromolecules or cellular structures.

Taking into account the fact that *H. dulcis* is a tree and that its life cycle may be a step down for its exploitation, plant tissue culture techniques can supply the biomass necessary to its medicinal use. So far, callus and cell cultures of *H. dulcis* have not been studied in this context and may provide a reliable source of compounds of medicinal interest.

The purpose of this study was to evaluate the effects of plant growth regulators and explant source on the induction and establishment of *H. dulcis* callus cultures. This work also aimed to compare the antioxidant activity of cultured calluses, *in vitro* propagated plants and wild grown plants, in order to validate biotechnological strategies to produce bioactive compounds.

MATERIALS AND METHODS

Plant

Two-month-old seedlings obtained from *in vitro* germination and two-month-old *in vitro* propagated plantlets were used as explant sources. Seedlings were grown on MS medium (Murashige and

Skoog, 1962) and the regenerated plantlets were obtained and maintained on MS medium containing 0.5 mg L⁻¹ benzylaminopurine (BAP) and 0.5 mg L⁻¹ 6-furfuryl-aminopurine (KIN) (Castro, 2001).

Leaves and seeds of *H. dulcis* from *in vivo* plants were collected after ripening in Teresópolis, Rio de Janeiro, Brazil. A voucher specimen (HRJ1426) is kept at the Herbarium of Rio de Janeiro State University.

Callus induction

Leaf (5 mm²) and stem (5 mm) segments from both explant sources were inoculated in MS medium with 30 g L⁻¹ sucrose and supplemented with the auxins 2,4-dichlorophenoxyacetic acid (2,4-D), picloram (PIC) or 1-naphthaleneacetic acid (NAA) at different concentrations (0, 1.25, 2.5 or 5.0 mg L⁻¹). The medium pH was adjusted to 5.8 prior to autoclaving (121°C for 15 min) and solidified with 8 g L⁻¹ agar (Merck). Five explants were inoculated in flasks (65 x 83 mm) containing 30 ml of culture medium in a total of five replicates, and the experiments were repeated three times. The flasks were kept in a growth chamber at 26±2°C under 16 h photoperiod provided by cool-white fluorescent tubes (45 mol m⁻²s⁻¹). Subcultures to media with the same composition were performed after 30 days. Callus morphological characteristics (consistency and color) and biomass growth based on dry weight (DW), were scored after 60 days. Dry weight of the callus was obtained after drying to constant weight at 45°C for 24 h.

Establishment of callus lines

Based on the prior experiment, stem explants from *in vitro* propagated plants and epicotyls from seedlings obtained through *in vitro* germination were inoculated in MS medium supplemented with the cytokinin BAP (0.65 or 1.25 mg L⁻¹) associated with 2,4-D (1.25 mg L⁻¹), PIC (1.25 mg L⁻¹) or NAA (2.5 mg L⁻¹). Cultures were maintained and analyzed under the previously mentioned conditions.

The experiments followed a sequential and completely randomized experimental design (Compton, 1994; Compton and Mize, 1999). The results were submitted to D'Agostino & Pearson omnibus normality test, analysis of variance (ANOVA) and means were compared by Tukey's test. The tests were performed using GraphPad Prism 5.0 software.

Antioxidant activity

Compact calli obtained from two-month-old *in vitro* germinated seedlings, grown on MS medium supplemented with 2.5 mg L⁻¹ NAA + 0.65 mg L⁻¹ BAP, two-month-old *in vitro* regenerated plantlets cultivated on MS medium supplemented with 0.5 mg L⁻¹ BAP + 0.5 mg L⁻¹ of KIN and leaves of *in situ* tree were used as extraction sources. The material was dried at 45°C for 24 h. The samples (5 g) were immersed in 50 ml ethyl alcohol PA (Merck) for two weeks at 25±2°C and kept under agitation. Extracts were filtered on Whatman paper (No.1) and dried on rotary evaporator at 40°C, until constant weight and kept in the dark, at 10°C.

Evaluation of total phenolic compounds

Total phenolics were estimated according to the colorimetric method of Folin-Ciocalteu (Singleton and Rossi, 1965). The extracts were diluted in ethanol (90%), and a 100 µl sample was mixed with 100 µl of the Folin-Ciocalteu reagent (50%). After 5 min,

Table 1. Effect of explant source and auxins on the establishment of *H. dulcis* callus cultures grown on MS medium after 8 weeks.

Plant growth regulator (mg L ⁻¹)	<i>In vitro</i> plantlet stem segments			Seedlings epicotyls			Seedlings hypocotyls		
	Type	Induction (%)	Dry weight (mg)	Type	Induction (%)	Dry weight (mg)	Type	Induction (%)	Dry weight (g)
MSO	-	0	-	-	0	-	-	0	-
1.25 2,4-D	F/M	88	27.5±13.0 ^b	F/M	64	17.29±5.9 ^b	F/M	40	17.5±6.5 ^b
2.50 2,4-D	F/M	68.18	16.7±3.7 ^b	-	0	-	-	0	-
5.00 2,4-D	F/M	64	21.7±10.4 ^b	-	0	-	-	0	-
1.25 PIC	F/M	80	24.2±9.2 ^b	F/M	56	16.9±5.7 ^b	F	20	-
2.50 PIC	F/M	76	26.2±11.3 ^b	F/M	48	16.2±2.9 ^b	F/M	0	-
5.00 PIC	F/M	52	13.4±2.2 ^b	F/M	16	-	F/M	5	-
1.25 NAA	C/M	33.33	-	C	92	27.2±14.5 ^a	C	45.83	27.9±12.2 ^b
2.50 NAA	C	100	57.2±31.7 ^a	C	60	34.3±14.0 ^a	C	55	56.9±27.0 ^a
5.00 NAA	C	100	45.5±13.6 ^a	C	24	-	C	35	-

C: Compact callus; F: Friable callus; M: Mixed callus; -: No callus formation. Values represent mean ±SD of three replicates. Different small letters within each column indicate significant ($P < 0.05$) differences among treatments. MSO = Growth-regulator free MS medium.

2 ml of Na₂CO₃ (2%) were added and incubated at room temperature for 1 h. The absorbance was measured at 750 nm. Total phenolic compounds content was determined using a standard curve prepared with gallic acid and results were expressed as microgram of gallic acid equivalent (GAE) per milligram of dry weight. The samples were analyzed twice in duplicate.

Total flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay (Costa, 1982). The extracts were diluted in ethanol (90%), and 1 ml of sample was mixed with 1 ml of AlCl₃ (2%) and 2 ml of ethanol. Absorbance was measured at 425 nm after 5 min of incubation at room temperature. Total flavonoids were determined using a quercetin standard curve and results expressed as microgram of quercetin equivalent (QE) per milligram of dry weight. The samples were analyzed twice in duplicate.

Reducing power assay

The reducing power was determined based on the method of Yen and Chen (1995). A 400 µl sample (diluted in ethanol 20%) was added to 400 µl of phosphate buffer pH 6.6 (50 mM) and 400 µl of potassium ferrocyanide [K₂Fe(CN)₆] (1%). After 20 min of incubation 400 µl of trichloroacetic acid (TCA) (10%) and 4.4 ml of deionized water were added. After that 3 ml of the solution were withdrawn and mixed with 400 ml of 0.1% ferric chloride (FeCl₃). Absorbance was measured at 700 nm. The results were obtained using a gallic acid standard curve and samples were analyzed twice in duplicate.

DPPH radical scavenging assay

Free radical scavenging activity of extracts was quantified spectrophotometrically using DPPH assay (Brand-Williams et al., 1995). A 1 ml extract sample was dissolved in 50% dimethyl sulfoxide (DMSO)/50% ethanol, and mixed with 1 ml of DPPH. After 30 min of incubation, absorbance was measured at 517 nm, twice, in duplicate. The results were expressed as percentage of capture of DPPH radical calculated by the equation:

$$\frac{[1-(A_1-A_2)]}{A_0} \times 100$$

where A₀ = Absorbance of blank; A₁ = Absorbance of sample with DPPH; A₂ = Absorbance of sample without DPPH

RESULTS AND DISCUSSION

Auxins and callogenesis

Callus formation was not observed in leaf explants. The addition of 2,4 -D to cultures of regenerated plants resulted in the induction of friable calli at all tested concentrations, while only the lowest concentration (1.25 mg L⁻¹) promoted calli formation from *in vitro* seedlings. Despite being predominantly friable, these calli presented compact areas and therefore were classified as mixed callus. The addition of PIC induced the formation of the same types of calli on explants obtained from *in vitro* regenerated plants and on cultures initiated from *in vitro* germinated seedlings (Figure 1). The addition of NAA promoted the establishment of compact light green calli (Figure 1A and 1B) on all types of stem explants at all concentrations (Table 1).

Majority of callus induction processes described to date employ transcriptional or post-transcriptional regulators that lead to changes in gene expression or protein translation (Ikeuchi et al., 2013). Auxins are supposed to promote a physiological change in previously differentiated plant tissue, leading cells to differentiate and start division (Machakova et al., 2008). Lo Schiavo et al. (1989) suggested that an increase in DNA methylation in the presence of auxins could cause cellular reprogram-ming and such changes in DNA methylation were observed

Table 2. Effect of explant source and BAP on *H. dulcis* callus cultures grown on MS medium after 8 weeks.

Growth regulator (mg L ⁻¹)	<i>In vitro</i> plantlet stem segments			Seedlings epicotyls		
	Type	Induction (%)	Dry weight (mg)	Type	Induction (%)	Dry weight (mg)
1.25 2,4-D+0.65 BAP	M/F	100	73.3±54.3 ^a	F/M	100	32.2±15.5 ^b
1.25 2,4-D+1.25 BAP	M/F	93.33	100.6±48.19 ^a	F//M	40	43.5±31.3 ^b
1.25 PIC+0.65 BAP	-	0	-	-	0	-
1.25 PIC+1.25 BAP	M	100	80.6±35.9 ^a	F/M	76	55.63±36.5 ^b
2.50 NAA+0.65 BAP	C	91.66	110.8±40.92 ^a	C	100	115.3±28.2 ^a
2.50 NAA+1.25 BAP	C	80	75.0±25.3 ^a	C	100	95.6±19.04 ^a

C: Compact callus; F: Friable callus; M: Mixed callus; -: No callus formation. Values represent mean ±SD of three replicates. Different small letters within each column indicate significant ($P < 0.05$) differences among treatments.

in callus cultures (Lambe et al., 1997). Recently, genome-wide transcriptional analysis showed that genes involved in auxin signaling and meristem development were methylated within the callus (Li et al., 2011). The addition of exogenous auxin seems to be re-quired for *H. dulcis* callus induction since in the absence of such compounds callus formation was not observed.

In the present study, friable calli were observed when 2,4-D and PIC were added to the culture medium, while only NAA induced compact callus which is in contrast with Jeong et al. (2009) findings, where 2,4-D promoted the formation of compact callus in leaf explants. Similar results were reported for other species such as *Gossypium hirsutum*, where 2,4-D promoted only friable callus while NAA was responsible for compact callus formation (Baksha et al., 2006). This response may be related to 2,4-D strength, since other studies suggested this growth regulator as more suitable to promote callus friability (Cardoso and Oliveira, 1996; Salman, 2002). The degree of activity of individual auxins in different processes is very variable. It may differ not only from species to species, but also from organ to organ, tissue to tissue, cell to cell and, with the age and physiological state (Davies, 2004). Often, after uptake into plant tissues, 2,4-D and NAA are converted to conjugates, and this process may regulate levels of free active substances (Machakova et al., 2008).

NAA has been mentioned as weaker auxin when compared with 2,4-D and PIC. Thus, it may be inferred that endogenous cytokinin action would be responsible for cell aggregation, which could explain compact callus formation when NAA was added to the culture medium. This consideration could also explain the green color of the compact callus, since cytokinins, in culture, tend to induce chlorophyll formation in callus, while auxins may be inhibitory (Machakova et al., 2008). Abdellatef and Khalafallah (2008), working with *G. hirsutum*, found that 2,4-D induced callus ranging from yellow to brown while the NAA produced callus ranging from yellow to green, and IAA, which is considered weaker than the NAA, promoted green callus. The increase of IAA concentration led to the reduction in chloroplast development in callus

(Wolzny et al., 1973). These data corroborate our findings and could explain the formation of compact, green callus on media supplemented with NAA.

Dry weight analysis (Table 1) showed that tested concentrations of PIC and 2,4-D had no significant difference regarding biomass accumulation. In the present paper, the best results for compact callus were obtained with the supplementation of NAA at 2.5 or 5.0 mg L⁻¹ (Table 1). Explant source also had no significant effect on callus growth. Considering the collected data, the culture media supplemented with 1.25 mg L⁻¹ 2,4-D, 1.25 mg L⁻¹ PIC and 2.5 mg L⁻¹ NAA were selected to continue the study, and as explant source, stem segments from *in vitro* regenerated plants and epicotyls of *in vitro* seedlings.

Cytokinin and callogenesis

The addition of BAP had no effect on callus morphology (Table 2). None of the media containing 2,4-D or PIC were able to establish homogeneous friable callus cultures. The cultures inoculated in MS medium supplemented with 1.25 mg L⁻¹ PIC + 1.25 mg L⁻¹ BAP showed a high degree of oxidation. Dry weight measurements showed that BAP concentration had no significant effect on biomass production (Table 2). Therefore, the medium supplemented with 2.5 mg L⁻¹ NAA + 0.65 mg L⁻¹ BAP was considered the most suitable to produced *H. dulcis* compact callus. This medium was considered preferable since the same results were obtained using less growth regulators, which may induce somaclonal variation. This genetic modification was observed in *Amorphophallus albus* plants obtained from callus induced by combinations of NAA and BAP (Hu et al., 2008).

The addition of cytokinin to the media in order to increase culture growth proved to be effective. In many species, the association of cytokinins and auxins has been reported to promote the best growth and maintenance of callus (Park et al., 2002; Salman, 2002; Santos et al., 2011).

Compact callus cultures obtained on the present study were subcultivated for at least 6 months and no morphological

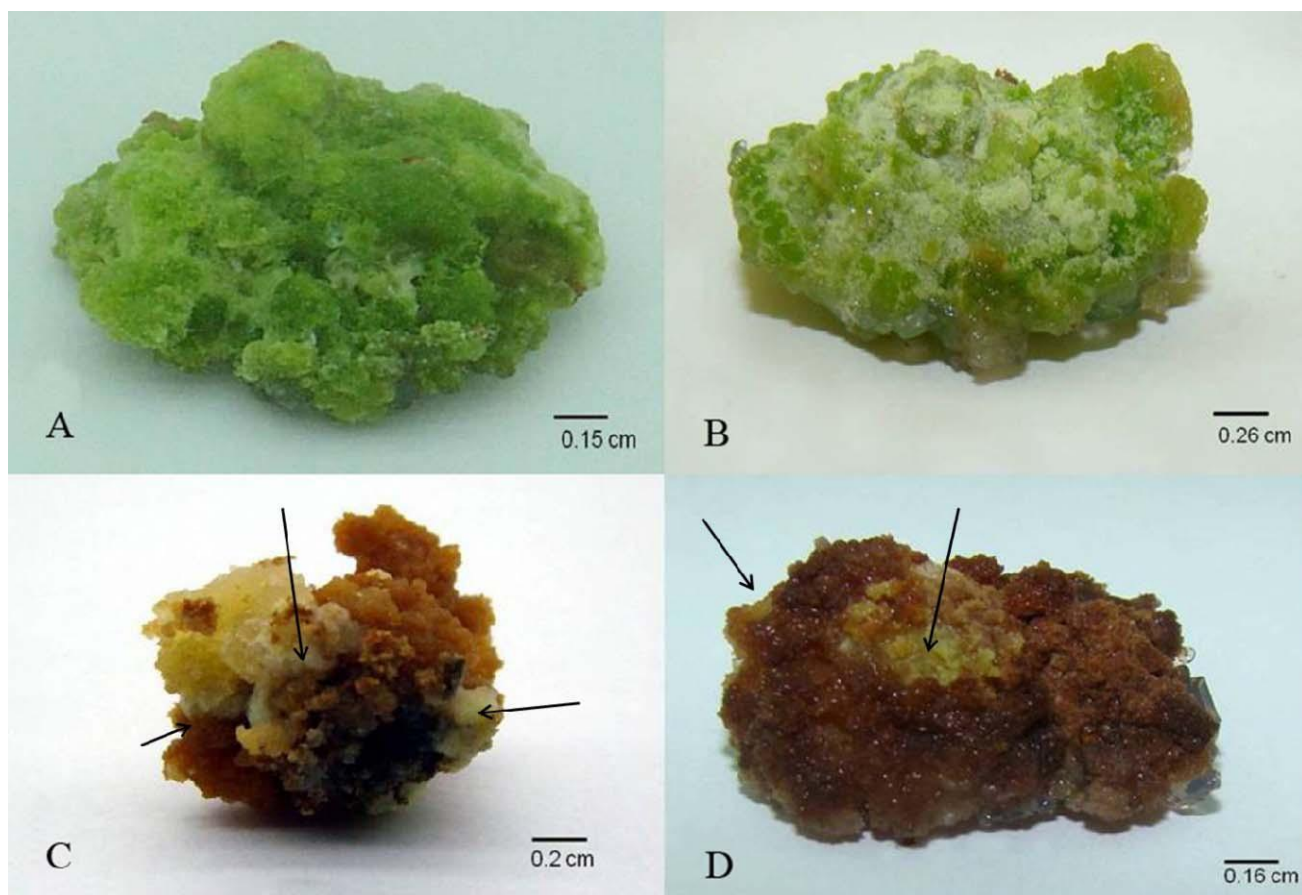


Figure 1. *H. dulcis* callus cultures established on MS medium supplemented with different auxins and BAP, after 8 weeks. Light-green compact callus grown on MS + 2.5 mg L⁻¹ NAA (A) and 2.5 mg L⁻¹ NAA + 1.25 mg L⁻¹ BAP (B). Mixed (friable and compact) callus grown on MS + 1.25 mg L⁻¹ PIC (C); and 1.25 mg L⁻¹ BAP 1.25 mg L⁻¹ PIC (D). Arrows indicate compact areas.

changes were observed. These results are highly desirable, since Jeong et al. (2009) observed oxidation in *H. dulcis* non-organogenic calli after 20 days.

Antioxidant activity

The Folin-Ciocalteu method, used to analyze polyphenol content, revealed that wild grown plants had the highest concentration of these compounds with the equivalent of 59.73±1.37 mg of gallic acid/mg extract (Figure 2A). Phenolic compounds are commonly found in plants and are important in defense against infections and injuries. They are recognized as antioxidants (Kahkonen et al., 1999) and their presence is considered an indication of antioxidant activity of plant extracts. The Folin-Ciocalteu has become a routine method and is considered sensitive and accurate (Huang et al., 2005). As observed for phenolic compounds, *in situ* plants also presented the highest flavonoid concentration with the equivalent to 12.20 ± 0.87 mg of querciting/mg extract (Figure 2B).

In some cases, the antioxidant activity can be

significant and not correlated with the presence of total polyphenols. Wanasundara et al. (1995), working with canola, concluded that polyphenol content was not the crucial factor in determining the antioxidant capacity. The authors found a fraction with low polyphenol content with higher antioxidant activity in comparison with fractions of higher polyphenol content. These data are consistent with what was observed in *H. dulcis* callus extracts. The reducing power assay showed that callus extracts had the highest capacity of reducing iron, yielding 0.420 + 0.012 mg of gallic acid equivalent/mg extract (Figure 2C). The DPPH radical scavenging assay showed that callus extracts had the highest percentage of radical scavenging. The compounds present in callus extract were able to scavenge 57.31% of available radicals (Figure 2D).

The data obtained from these assays suggest that the highest antioxidant activity observed in the callus extracts is due to compounds different from polyphenols or flavonoids that were found in lower amounts in callus. Saponins are one of the major groups of secondary metabolites present in *H. dulcis*. Among several

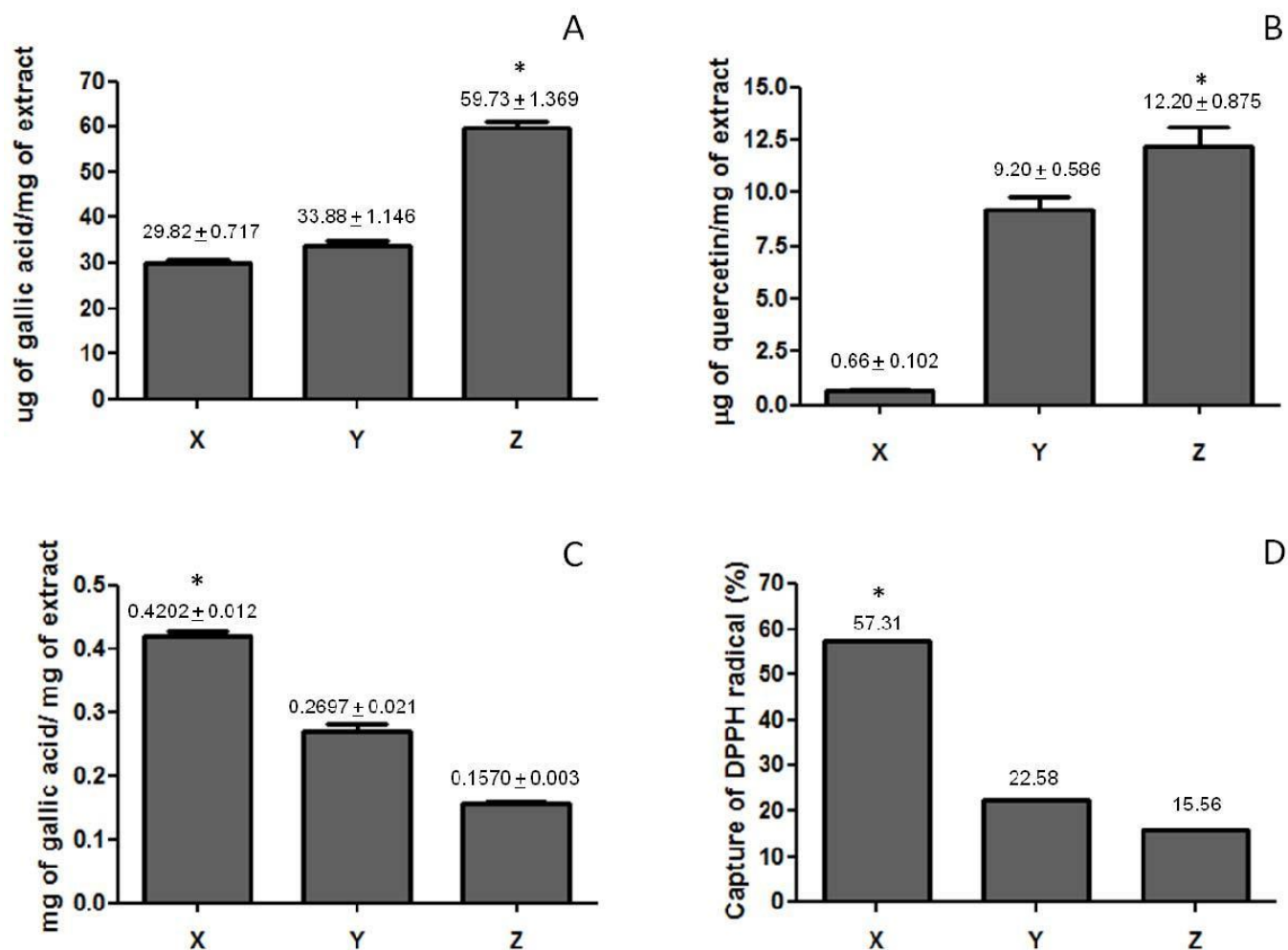


Figure 2. Antioxidant activity of *H. dulcis* extracts from compact callus (X), *in vitro* propagated plants (Y) and field grown plants (Z) evaluated by the total polyphenols assay (A), total flavonoids assay (B), reducing power assay (C) and DPPH radical scavenging assay (D). Values represent mean±SD of three replicates (A, B and C) and mean (D). * indicate significant (P < 0.001) differences between samples by the Tukey test.

antioxidant properties (Sparg et al., 2004; Ayaz et al., 2014) and therefore, might be responsible for the observed results. The highest antioxidant activity of callus in comparison to other plant materials is in accordance to what was observed in *Sylibum marianum* (Abbasi et al., 2010).

Conclusions

In conclusion, the auxins 2,4-D and PIC produced mixed friable beige and light green compact callus, while NAA induced compact green callus formation. The addition of BAP to the culture medium increased biomass growth. Callus extracts presented the highest antioxidant potential, which shows that *in vitro* production of compounds of medical interest by callus culture is a viable alternative in comparison to traditional methods, being able to exceed the productivity of *in situ* plant. Further

investigation on the phytochemistry of these calli is of interest in order to elucidate which molecules are responsible for the higher antioxidant activity.

ACKNOWLEDGEMENTS

This work was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors are grateful to Maria Francisca Santoro Assunção for the valuable technical assistance. The authors wish to thank Dr. Timothy P. Moulton for english corrections.

Conflict of interest

All authors declare that they have no conflict of interest.

Abbreviations:

ROS, Reactive oxygen species; **BAP**, benzylaminopurine; **KIN**, 6-furfuryl-aminopurine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **PIC**, 4-amino-3,5,6-trichloro picolinic acid; **NAA**, 1-naphthaleneacetic acid; **DW**, dry weight; **DPPH**, 2,2-diphenyl-1-picrylhydrazyl.

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