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Total Phenols, Flavonoids and Antioxidant Activity in *Annona muricata* and *Annona purpurea* Callus Culture

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Abstract: Callus cultures of *Annona muricata* and *Annona purpurea* were induced in Murashige and Skoog (MS) medium supplemented with different concentrations of 1-naphthylacetic acid (NAA), 6-benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) utilized hypocotyls with explant. The highest percentage of callus formation was the treatment supplemented with 3 mg L^{-1} NAA for *A. muricata* (100%) while for *A. purpurea* in lower percentage (75%). BA stimulated the formation of shoots in all the evaluated concentrations, being the concentration of 2 mg L^{-1} the one that induced the greater formation of shoots for *A. muricata* (23 shoots/explant) and *A. purpurea* (28 shoots/explant). The content of total phenols, flavonoids and antioxidant activity was measured in the callus obtained from both species. The results showed that a higher content of total phenols was quantified in callus of *A. purpurea* (27.8 mg g^{-1} dw) compared to *A. muricata* (23.2 mg g^{-1} dw). The highest content of total flavonoids was observed in the callus of *A. purpurea* (8.0 μg g^{-1} dw). Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydracil radical assay. The concentration required for 50% inhibition (IC50) of the 2,2-diphenyl-1-picrylhydracil radicals were 4.22 μg mL^{-1} in methanolic extracts of callus of *A. muricata*, while in extracts of callus of *A. purpurea* was 2.86 μg mL^{-1}, in both cases was greater than that found for leaves. Callus culture of the species studied in this work represents an alternative for the production of natural antioxidants.

Keywords: Annonaceae; antioxidant activity; 6-benzyladenine; secondary metabolites; tissue culture

1 Introduction

The Annonaceae family, characterized by growing mainly in tropical and sub-tropical climate, comprises 130 genera and approximately 2,300 species. Micropropagation of anonaceae has been reported, in seeds from *A. cherimolu cv.* 'Concha Lisa', multiple shoot formation was obtained from hypocotyl segments (34 mm long) [1]. Adventitious shoot formation occurred directly without undergoing the callus phase. The maximum percentage of shoots was obtained with liquid MS medium supplemented with 2 mg L^{-1} BA, 0.5 mg L^{-1} NAA and 1000 mg L^{-1} PVP or liquid N medium containing 0.1 mg L^{-1} BA and 1 mg L^{-1} NAA, [1,2]. Bejoy and Hariharan [3] have produced adventitious shoot buds

directly from the hypocotyl of *A. muricata* without a callus phase. When BA was used alone no buds developed, but when NAA and BA were both present, growth of shoots with leaves was observed.

These plants have acquired importance because they contained secondary metabolites such as phenols, essential oils, acetogenins and alkaloids [4]. Phenols are secondary metabolites; they include numerous compounds such as phenolic acids, flavonoids and tannins [5]. Recently, the physiological potential of phenolic compounds has attracted considerable attention due to its important role in the prevention of degenerative diseases, particularly cancer, cardiovascular and neurodegenerative diseases [6,7]. Phenolic compounds are potent antioxidants that complement the functions of vitamins and enzymes in the body, as a defense against oxidative stress caused by the excess of reactive oxygen species [7]. Interest in the development of processes for bioactive compounds production derived from natural sources has increased significantly in recent years [8]. However, the challenges to produce bioactive compounds using vegetables grown in the field or greenhouse are related to the difficulties to grow healthy plants, free of pests and diseases, well nourished, but at the same time with high production of metabolites.

An alternative is the *in vitro* culture; however, it is necessary to obtain a culture in which sufficient biomass for the extraction of the bioactive compounds. Callus culture could be used a viable alternative when a culture with higher production of the metabolites is obtained. Additionally, callus culture could be used to study plant responses when subjected to different types of biotic or abiotic stress. Callus culture can also be used to improve the responses of plants to different stress, evaluating the growth of biomass and the production of metabolites of interest. The objective of the present work was to evaluate the effect of different plant growth regulators on callus induction in *A. muricata* and *A. purpurea*. In addition, the content of total phenols, total flavonoids and antioxidant activity in the callus culture of the two Annonaceae species were quantified.

2 Materials and Methods

Germination. For evaluation of mechanical scarification and gibberellic acid effects on germination percent (Gp) and germination time (Gt) of *A. muricata* and *A. purpurea* seeds, eight hundred seeds were selected for each of the two species, through the buoyancy test in water. Seeds were placed in tap water for 12 hours; eliminating the seeds that floated. Four hundred seeds underwent a scarification process for which a small cut was made until observing the endosperm. Another four hundred seeds were used for evaluating the effect of Gibberellic Acid (GA_3). One hundred seeds were placed in 0, 10, 20, and 40 mg L^{-1} of GA_3. The germination test was done for 2 months for *A. muricata* and 3 months for *A. purpurea* for scarified and non-scarified seeds. The variables measured for each treatment were germination percent (Gp) and germination time (Gt). Germinated seed was considered when the radicle emerged and the measurements were taken every 3 days.

Effect of Naphthalene Acetic Acid (NAA), Benzyl Adenine (BA) and 2,4-Dichorophenoxiacetic acid (2,4-D) on callus induction and shoot number. Explants were obtained from *in vitro* germinated seedlings. For disinfection, seeds were washed with commercial detergent for 5 minutes, drained with purified water to remove the detergent. In a laminar flow hood, seeds were placed in a 2% solution of tween 80 for 15 min, washed with purified water and further in a 95% ethanol solution for 5 min, then three washes with sterile water and in a 30% chlorine solution for 20 min and finally three washes with sterile water. Disinfected seeds were placed in inorganic salts MS [9] medium supplemented with 30 g L^{-1} sucrose, 2.5 g L^{-1} phytagel and 20 mg L^{-1} of GA_3 for *A. muricata* and 40 mg L^{-1} for *A. purpurea*; The pH was adjusted to 5.7 before autoclaving. Twenty ml of medium was emptied in glass bottles and autoclaved for 1.2 kg cm^{-2} pressure for 15 min. One seed per bottle and 40 replicates for each species was used. To carry out the germination and plantlet growth bottles were placed at room temperature (25°C ± 2) and darkness. Plantlets were used as explant source for callus induction and for evaluation of the morphogenic response in both *A. muricata* and *A. purpurea*. Aseptic hypocotyls of approximately 12 cm were taken and cut into segments of 1 cm, callus induction was carried out in MS medium supplemented with different concentrations of NAA, BA and 2,4-D (Tab. 2). Percent of callus induction was evaluated one month after and shoot number two months after.

Total Phenols (TP), Total Flavonoid (TF) and Antioxidant Activity (AA) were evaluated in leaves from the middle part in trees of 2 years of age and callus in both *Annonaceae* species. Ten g of fresh leaves and 10 g of callus were dehydrated to -40ºC and 0.250 mbar during 48 h in a freeze dryer (FreeZone 4.5, LABCONCO). The samples were sealed in vacuum bags for further analysis.

Preparation of extracts. Crude extracts were obtained by macerating 3 g dry plant material in 30 mL methanol for phenolic compounds and 96% ethanol for flavonoids for 48 h at room temperature according to the methodology reported by [10] with some modifications. The supernatants were filtered and dried in an oven at 30ºC for 8 h.

Total Phenols and Flavonoids quantification. The Folin-Ciocalteu method was used to determine the content of total phenols [11]. Each dried extract was resuspended with 3 mL of solvent; The sample was diluted (1:10), 25 µL was taken, 475 µL H_2O, 1250 µL of 10% Na_2CO_3 solution were added, and finally 250 µL of the Folin reagent, the mixture was homogenized, and it was left for 2 h in dark. Finally, the absorbance was read at 765 nm in a spectrophotometer (HACH DR 5000). The concentration was obtained from a standard curve with Gallic Acid. The total phenolic content was expressed as Gallic Acid equivalents (GAE mg g^{-1} dry weight). The flavonoid content was determined by the Aluminium Trichloride colorimetric method [12]. A 200 µL of diluted sample was taken, 600 µL 95% Methanol, 40 µL of 10% $AlCl_3$ solution, 40 µL of 1M Potassium Acetate solution were added and finally 1120 µL of H_2O. The mixture was homogenized and allowed to stand for 30 min in the dark. Finally, the absorbance was measured at 415 nm on a spectrophotometer (HACH DR 5000). The concentration was obtained from a standard curve with Quercetin. The total Flavonoids content was expressed as Quercetin mg g^{-1} dry weight (dw) (QE mg g^{-1}). Quercetin and Folin-Ciocalteu's reagent were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

DPPH Antioxidant Capacity. The total antioxidant capacity of the extract was carried out using 2,2-diphenyl-1-picrylhydracil radical (DPPH) [13]. A solution of 0.1 mM DPPH in methanol was prepared and 1.0 mL of this solution was mixed with 1.0 mL of extract at different concentrations (0.5-20 µg mL^{-1}) dissolved in methanol, DPPH in methanol was used as a negative control. The sample was left in the dark at room temperature (25°C) for 30 min and measured at 517 nm on a spectrophotometer (HACH DR 5000) calibrated with Methanol. All tests were performed in triplicate. DPPH in Methanol was used as a negative control. Percent inhibition of the DPPH radical was calculated using the following equation:

$$\text{AAr \%} = \frac{Ao - A1}{Ao} \times 100$$

AAr % = antiradical activity; A_o = control absorbance; A_1= extract absorbance

Statistical analysis. The effect of the treatments was determined by analysis of variance (ANOVA) with a significance level of 95%, and the mean comparisons were conducted by Media Significant Difference test, using the Statgraphic centurion XV1 software.

3 Results

Mechanical scarification induced different effects on the germination percentages (Gp) in seeds of annonaceae species. In *A. muricata* induced a positive effect whereas in *A. purpurea* was negative. With respect to germination times (Gt), the scarification induced a minor time of germination in both annonaceae species. GA_3 concentration was an important factor to induce an increase in Gp and a reduction in Gt in two species. The increment in Gp was proportional to GA_3 concentration, however, in *A. muricata*, the Gp was 7.4 times with 40 mg L^{-1} GA_3 in comparison with control without GA_3. In *A. purpurea*, the Gp was 4.9 times (Tab. 1). Also, Gt was influenced by GA_3, but the effect was different in both species. In *A. muricata*, Gt decreased 12 days, whereas in *A. purpurea* Gt decreased 35 days.

Table 1: Effect of mechanical scarification and gibberellic acid on seed germination percentage (Gp) and germination time (Gt) of *A. muricata* and *A. purpurea*

	A. muricata		*A. purpurea*	
	Gp	Gt	Gp	Gt
	—%—	—days—	—%—	—days—
Scarification				
With	44.0 ± 6.2 a[2]	27 a	26.5 ± 0.97 b	70 a
Without	26.3 ± 6.2 b	40 b	34.8 ± 0.97 a	85 b
LSD [1] (0.05)	3.96	9.3	5.64	11.3
GA_3 (mg L^{-1})				
0	9.0 ± 6.2 c	27 b	10.0 ± 1.38 d	70 b
10	24.5 ± 6.2 b	18 a	24.5 ± 1.38 c	40 a
20	65.5± 6.2 a	15 a	39.0 ± 1.38 b	35 a
40	66.5± 6.2 a	15 a	49.0 ± 1.38 a	35 a
LSD (0.05)	5.6	6.7	8.25	10.4

[1] LSD: Least significant difference ($p < 0.05$).

NAA and 2,4-D induced callus and induction percent was proportional to both plant growth regulators. To induced a 100% callus formation was needed 3 mg L^{-1} NAA or 3 mg L^{-1} 2,4-D for *A. muricata*. Whereas for *A. purpurea*, the higher callus induction percentage was observed with 3 mg L^{-1} 2,4-D (Tab. 2). Higher shoot numbers (23 and 28) were found with 2 mg L^{-1} BA in *A. muricata* and *A. purpurea*, respectively. The chronological morphogenic responses of *A. muricata* induced by 2,4-D is presented in Fig. 1. Similar responses were found in *A. purpurea*.

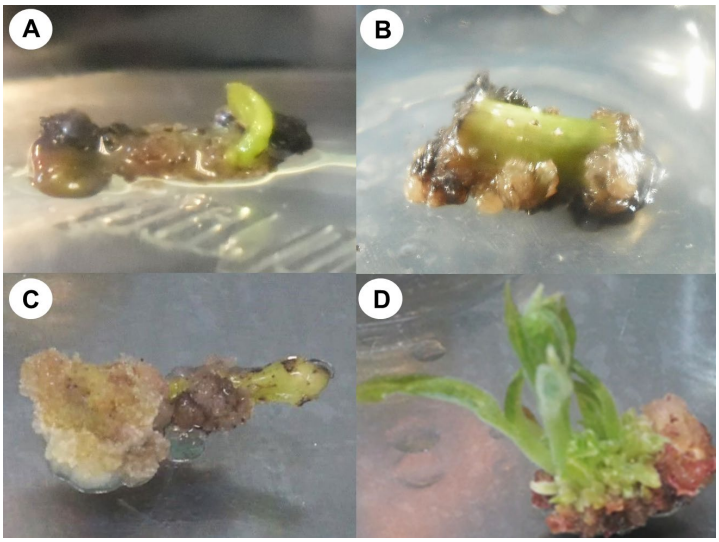

Figure 1: Morphogenic response of *A. muricata* induced by the addition of 3 mg L^{-1} of NAA to the MS culture medium. A) 15 days, B) 30 days, C) 45 days and D) 60 days after the implementation of the *in vitro* culture

Table 2: Effect of 1-Naphthylacetic Acid (NAA), 6-benzyladenine (BA) and 2,4-Dichlorophenoxyacetic Acid (2,4-D) on callus induction and shoot number in *A.muricata* and *A. purpurea*

Treatment	*A. muricata*		*A. purpurea*	
	Shoot —number—	Callus —%—	Shoot —number—	Callus —%—
NAA (mg L^{-1})				
0	0.0 d [b]	0.0	0.0 d	0.0
1	4.3 ± 1.0 c	75.0	3.0 ± 1.0 c	50.0
2	6.0 ± 1.0 b	75.0	5.0 ± 1.0 b	75.0
3	12.0 ± 1.0 a	100.0	7.0 ± 1.0 a	75.0
LSD [a] (0.05)	5.58	5.58	3.75	3.75
BA (mg L^{-1})				
0	0.0 d	nd	0.0 d	nd
0.5	8.0 ± 0.97 c	nd	12.0 ± 2.0 c	nd
1	18.6 ± 0.97 b	nd	17.0 ± 2.0 b	nd
2	23.0 ± 0.97 a	nd	28.0 ± 2.0 a	nd
LSD (0.05)	9.41	-	12.25	-
2,4-D (mg L^{-1})				
0	0.0 d	0.0	nd	0.0
1	0.56 ± 0.15 c	50.0	nd	50.0
2	0.83 ± 0.15 b	75.0	nd	75.0
3	1.40 ± 0.15 a	100.0	nd	100.0
LSD (0.05)	0.7	0.7	-	0.74

[a] LSD: Least significant difference ($p < 0.05$).

[b] Values with the same letter are not significantly different between the treatments.

nd: no detected.

Total phenols and total flavonoids contents were higher in leaves in comparison with callus culture in both *Annonacea*. However an interesting result was found in *A. purpurea* antioxidant activity, since although the content of total phenols and flavonoids in callus is much lower than in the leaves, both showed IC_{50} values very similar, suggesting that metabolites produced by callus culture are different from those produced in the leaves and also have higher antioxidant activity (Tab. 3).

Table 3: Total phenols (TP), total flavonoid (TF) and antioxidant activity (AA) in leaves and callus of *Annona muricata* and *A. purpurea*

Explant	TP GAE mg g^{-1} DW	TF QE µg g^{-1} DW	AA IC_{50} (µg mL^{-1})
	A. muricata		
Callus	23.20 ± 0.70 b	6.60 ± 0.20 b	4.22
Leaves	70.80 ± 0.60 a	9.80 ± 0.20 a	3.08
LSD [a] (0.05)			
	A. purpurea		
Callus	27.80 ± 1.02 b	8.0 ± 0.30 b	2.86
Leaves	88.10 ± 1.60 a	11.1 ± 0.20 a	2.08
LSD (0.05)			

[a] LSD: Least significant difference ($p < 0.05$).

TP: Total phenolic compounds expressed as gallic acid equivalents (GAE mg/g plant).

TF: Total flavonoids compounds expressed as quercetin equivalents (QE µg/g plant).

Values with the same letter are not significantly different between the treatments.

4 Discussion

Micropropagation techniques have been useful for clonal propagation of many fruit trees, including Annona species. Shoots were obtained with liquid MS medium supplemented with 2 mg L^{-1} BA, 0.5 mg L^{-1} NAA and 1000 mg L^{-1} PVP or liquid N medium containing 0.1 mg L^{-1} BA and 1 mg L^{-1} NAA, when *Annona cherimola* L. hypocotyls were used [1,2]. Bejoy and Hariharan [3] have produced adventitious shoot buds directly from the hypocotyl of *A. muricata* without a callus phase. Anjum and Pandey [14] reported that MS medium was supplemented with 5 mg L^{-1} concentration of cytokinins (BAP) callus formation was induced in *Annona reticulata* (L). Differentiation of shoot bud from shoot tips was observed in 45% cultures on MS medium supplemented with 1.5 mg L^{-1} on concentration of BAP. Lemos and Jennet [15] reported adventitious bud and shoot proliferation were achieved from hypocotyls of seedlings of *A. muricata* these authors report the formation of 18 shoots when hypocotyls were induced in MS medium supplemented with 0.5 mg L^{-1} BA. Our results differ with that reported in other annonaceae species; we find that BA induces shoot formation in *A. muricata* and *A. purpurea* (23 and 28 shoots per explant respectively) when hypocotyl is used as an explant, being the highest number reported for any species of annonaceae. Our results suggest that shoots formation is affected by the type of explant and growth regulator required, as well as the concentration and auxin-cytokinin ratio, the induction of callus depends on the genotype and endogenous hormonal content of the tissue used.

The content of total phenols and flavonoids in leaves of both *Annonaceae* species was higher than that obtained in callus. Hassimotto [16] evaluated the antioxidant activity of different frozen fruits and vegetables in Brazil, reporting that commercial pulp of *A. muricata* contains 120 ± 8 mg of gallic acid /100 g FW. Formagio [17] reported that methanol extract of *Annona dioica* leaves had high levels of total phenols and flavonoids with values of 187.77 mg/g and 733.20 mg/g of dry plant, respectively. On the other hand, Luján-Hidalgo [18] reported the content of total phenols and flavonoids in a range of concentrations from 94.9-193.1 GAE mg/g dw and 29.39 to 44.17 QE mg/g dw, in plants of *A. purpurea* cultivated in the soil and fertilized with vermicompost and rock phosphoric. The difference obtained in the concentration of the metabolites produced by *in vitro* culture is due to the fact that in plant cell cultures, substances are not always produced qualitatively and quantitatively equal to those elaborated by

the mother plants. The production and the profile of chemical compounds can be unstable, because within the whole plant the plant cells have a different biochemical and physiological environment than the cells that grow in culture media. In addition, as many of the metabolites are synthesized when integrated to differentiation events, sometimes some degree of organization in the cultures is needed for the metabolite to be synthesized, which is why the concentration in calluses is lower [19]. A large variety of chemical compounds with biological activity including many alkaloids such as morphine (pain killer), codeine (antitussive), papaverine (phosphordiesterase inhibitor), ephedrine (stimulant), ajmaline (antirrhythmic), quinine (antimalarial), reserpine (antihypertensive), galanthamine (acetylecholine esterase inhi bitor), scopolamine (travel sickness), berberine (psoriasis), caffeine (stimulant), capsaicin (rheumatic pains), colchicines (gout), yohimbine (aphrodisiac), pilocarpine (glaucoma), and various types of cardiac glycosides (heart insufficiency) [20], can be produced in the *in vitro* culture, the expression of genes from different metabolic pathways has been observed during the *in vitro* culture of some species.

On the other hand, the plant material, type of solvent used and extraction method are factors that may affect the yield of extraction of phenolic content [21]. The extraction time can also affect the extraction performance due to the prolonged exposure of light and the stability of the phenolic compounds, however, in this research; the samples were protected from light during the entire extraction process and quantification. For extraction, the solvent is chosen according to the type of phenolic compounds present. The less polar flavonoids (isoflavones, flavanones, flavones and flavonols, methylated) are extracted with chloroform, dichloromethane, diethyl ether or ethyl acetate, while the flavonoids glycosides and aglycones, more polar, are extracted with alcohols or alcohol-water mixtures. The glycosides increase their solubility in water and aqueous solutions alcoholic [22]. According to Hassimotto [16] the main phenolic compounds present in the pulp of *A. muricate* are the catechins, highly soluble in polar solvents such as methanol. Hernández-Rodríguez [23] evaluated the effect of different solvents and extraction time on the extraction yield of total phenolic compounds in fruits of *Prunus serotina* Erhr, reporting however that there is not significant statistical difference using acetone-water (7:3 v/v) and methanol-water (4:1 v/v) while the optimal extraction time was 30 min. Several authors have reported the use of selective extractions for flavonoids, Henry-García [10] optimized the extraction process of flavonoids present in leaves of *A. muricana*, reporting an extraction efficiency of 87% using 96% ethanol as solvent.

The antioxidant or free radical scavenging activity of flavonoids has been related to the number and position of free hydroxyl groups, which could be a result of their hydrogen donating ability [24,25]. The antioxidant activity and radical-scavenging activity of flavonols depend highly on their structure, especially the presence of a free C-3-OH, a free C-4'-OH, a double bond between C-2 and C-3, and an O-dihydroxy group in the B-ring [26].

According to Hassimotto [16] the main phenolic compounds present in the pulp of *A. muricata* are the catechins, a type of flavonoids with great antioxidant activity. Vega [27] report the isolation and characterization of the four foliar flavonoids, kaempferol, 3-O-[3",6"-di-O-p-hydroxycinnamoyl]-β-galactopyranosyl-kaempferol, 6"-O-p-hydroxycinnamoyl-β-galactopyranosyl-kaempferol and 3-O-β-galactopyranosyl-kaempferol from a methanolic extract of the leaves of *A. dioica* collected in Paraguay. The results (Tab. 3) show that the extract of both Annonaceas possesses significant free radical scavenging activity, with an IC50 of 2.0 and 4.2 μg mL^{-1} DPPH compared to the IC50 value (17.84 μg mL^{-1}) reported by [17] on leaves of *A. dioica*. Roesler [28] evaluated some compounds identified in ethanolic extracts of *A. crassiflora* using ESI-MS and among them the most representative was caffeic acid and gallic acid with IC50 of 1.9 μg mL^{-1} and 1.4 μg mL^{-1} of DPPH. Luján-Hidalgo [18] obtained a maximum inhibition percentage of 75.17 for the DPPH radical in leaves of *A. purpurea* L.

5 Conclusion

The standardization of a highly reproducible regeneration system in *A. muricata* and *purpurea* using hypocotyl segments during the present investigation may be useful in genetic improvement programs of

these fruits plant. The results obtained in callus culture are very interesting, since the extract presents a very high antioxidant activity, despite the fact that the concentration of total phenols is low, which means that the secondary metabolites biosynthesized in the callus culture are of high quality. The present work revealed the presence of secondary metabolites with antiradical activity from different extracts of *Annona muricata* and *purpurea*, cultivated under *in vivo* and *in vitro* conditions. The present investigation has great commercial application, the material produced could be used for phytochemical characterization and this could help to select the callus culture of these two annonaceae species as a source of natural antioxidants and nutraceuticals to enhance health benefits.

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