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Effect of Salicylic Acid, 2,4-D and 2i-P on the Production of Secondary Metabolites in *Garcinia brasiliensis* Mart. Callus

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HIGHLIGHTS

- Bacupari callus presents significative levels os phenolics and flavonoids.
- Specifically, it was idenficated fukugetin e 7-epiclusionone
- Growth regulators and salicylic acid affected the occurrence these substances.

Abstract: The aim of this work was to evaluate the effect of Salicylic acid, 2,4-D and 2-iP on the production of total phenolic and flavonoids as well in the levels of fukugetin and 7-epiclusionone in callus of *Garcinia brasiliensis* zygotic embryos. For this, Bacupari callus

were exposed to different concentrations (0.0; 0.1; 1; 10; 100 μM) of Salicylic acid (SA) in the presence or in the absence of 72 μM 2-iP and 28.73 μM 2,4-D. The highest concentration of total phenolic occurred in the treatment with 100 μM SA in callus subcultured in the absence of 2,4-D and 2-iP, and with 10 μM SA in callus subcultured in the presence of these regulators. Concerning flavonoids, 2,4-D and 2-iP supplementation without the presence of the SA was sufficient for the highest levels. Additionally, it was possible to identify the fukugetin and 7-epiclusianone. However, the variation in the levels was very high, especially for the fukugetin. Therefore, in most treatments, there was no statistically difference. Except for the treatment with 10 μM SA + 2,4-D and 2-iP, where there was a significant increase in 7-epiclusianone.

Keywords: Bacupari; phenols; flavonoids; 7-epiclusianone; fukugetin.

INTRODUCTION

The genus *Garcinia* has been shown by phytochemical studies to possess a great diversity of secondary metabolites, such as benzophenones, phenolic acids and flavonoids. Many of these compounds are recognized to possess pharmacological properties against various diseases [1,2,3].

In Bacupari, *Garcinia brasiliensis* Mart, biflavonoids and benzophenones can be highlighted. These compounds are very common in the *Garcinia* genus [4], having been found in thirty-two species [5,6]. In this context two substances has received great attention, the biflavonoid fukugetin and the tetraprenylated benzophenone 7-epiclusianone. Fukugetin is the most frequent biflavonoid in the Clusiaceae family including being used as chemosystematic marker [5]. *In vitro* tests have shown that fukugetin is a potent inhibitor of *Trypanosoma cruzi* [7] and *Leishmania amazonensis* [8,9]. It is also has activity against some gram-positive bacteria, such as *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus saprophyticus* [10]. In addition presents anti-inflammatory and anti-oxidant activity [11]. The 7-epiclusianone, isolated for the first time from *Rheedia gardneriana* Planch. & Triana fruits [6], has anti-anaphylactic [12], antimicrobial [13], anti-spasmodic [14], anti-proliferative [15] and leishmanicide [16], antinoceptive, antioxidant and cytotoxic [5] activities.

In *in vivo* plants, secondary metabolites are produced in very small amounts and are generally difficult to extract [17]. Therefore, *in vitro* plant cell culture has been used for the production of secondary metabolites of interest [18]. One strategy frequently used is the addition of elicitors, such as salicylic acid (SA), in the culture medium of *in vitro* cell culture [19]. SA acts as a phytohormone [20] and maintains auxin and cytokinin levels in plant tissues, increasing cell division and dry matter [21]. It also activates the enzyme L-phenylalanine ammonia lyase (PAL), which acts between primary and secondary metabolism, involved in the production route of compounds of secondary metabolism [22]. SA also acts on the antioxidant system, regulating various physiological and biochemical processes in plants [23], depending on its concentration, type of plant/explant, plant growth phase and environmental conditions [24].

Considering the medicinal properties of *Garcinia brasiliensis*, the objective of this study was to evaluate the effect of SA, 2,4-D and 2-iP on the total content of phenolics and flavonoids and on the production of fukugetin and 7-epiclusianone in callus obtained from zygotic embryos.

MATERIAL AND METHODS

Plant Material and Callus Induction

Garcinia brasiliensis fruits were collected in Viçosa, MG, in the second half of February 2015. The fruits were pulped and their seeds remained for 30 days at a temperature of 22°C for drying and subsequent seed coat removal.

For embryo disinfection, 70% alcohol was used for 2 minutes and, subsequently, sodium hypochlorite at a concentration of 2.5% active chlorine for 15 minutes. The embryos were then rinsed for three times in distilled and autoclaved water. This process was carried out in a laminar flow chamber previously sterilized with 70% alcohol and UV radiation for 20 minutes.

The embryos were transversely segmented and inoculated into 10-cm Petri dishes containing 30 mL of WPM culture medium [25], with 10 segments per plate. The culture medium was supplemented with 30 g L⁻¹ sucrose and 6 g L⁻¹ agar, with the combination of 72 µM 2-iP + 28.73 µM 2,4-D. The pH was adjusted to 5.8 ± 0.1 before autoclaving, which was carried out at 121°C, pressure of 1.0 atm for a period of 20 minutes.

Callus Subculture and Treatments

After 45 days of cultivation, the callus were separated from the zygotic embryo segment and inoculated into test tubes containing 20 mL of WPM culture medium with different concentrations of SA (0; 0.1; 1; 10; 100 µM) in the presence and in the absence of regulators 2-iP (72 µM) + 2,4-D (28.73 µM). Ascorbic acid (150 mg.L⁻¹) of were added to the medium to avoid oxidation, according to Werner et al [26]. The tubes were maintained at 25°C in the dark in a Biochemical oxygen demand chamber (B.O.D). For the determination of the increase in callus fresh mass, the difference between the mass at 90 days of incubation and the initial mass (mass before the subculture) was determined.

Phytochemical Analyses

For the extraction, 100 mg callus from the different treatments were collected. The samples were then macerated in 1 mL methanol and kept under constant stirring at 223 rpm for 2 hours on a Marconi MA-420 orbital shaker. Subsequently, the samples were centrifuged for 30 minutes at 14000 rpm. The supernatant was collected. The operation was repeated until the supernatant became colorless. The extracts were suitably mixed and dried at 45°C.

Total phenolic compounds was determined as described by Ainsworth and Gillespie as follows [27]. Aliquots of the extracts (250 µL) were mixed with 1.25 mL of 10% Folin-Ciocalteu reagent. After 8 minutes, 1.0 mL of 4% sodium carbonate was added. The samples were incubated for 2 hours in the dark. Absorbance was measured at 740 nm. The phenolic concentration was calculated from a standard curve of gallic acid.

To determine the total flavonoids content, aliquots of the extracts (250 μ L) were mixed with 2.44 mL of 80% ethanol, 50 μ L of potassium acetate (1M) and 50 μ L aluminum nitrate (10%). The samples were then incubated for 40 minutes at room temperature. Absorbance was measured at 415 nm. The concentration of flavonoids was calculated from a standard quercetin curve [28]. For both, phenolics and flavonoids, appropriated blanks were run in parallel.

The analysis of fukugetin and 7-epiclusionone was performed by high performance liquid chromatography in a Shimadzu LC-100 chromatograph, as described by Santa-Cecilia et al [29]. The identification of compounds was made based on retention time and absorption spectrum of *Garcinia brasiliensis* compounds previously isolated and characterized. All analyses were performed with callus at 90th days of cultivation after the subculture.

Experimental Design and Statistical Analyses

The experimental design was completely randomized, with 5 SA concentrations (0; 0.1; 1; 10 and 100 μ M) in the presence and in the absence of the growth regulators 2-iP (72 μ M) + 2,4-D (28.73 μ M), using 10 replicates for the obtention of fresh callus mass and 5 replicates for the phytochemical analyses. The data were submitted to analysis of variance at 5% significance by the Scott-Knott test, using the program Sisvar [30].

RESULTS AND DISCUSSION

Phytochemical Analyses

Several studies have shown that polyphenols present in medicinal plants, especially flavonoids, have aroused great interest in research due to their medicinal properties [31,11]. These compounds are among the most studied class of secondary metabolites, making them desirable targets for *in vitro* culture [32].

In this context, the table 1 presents the total content of phenolic and flavonoids compounds in callus of *Garcinia brasiliensis* zygotic embryos, subcultured at different salicylic acid (SA) concentrations, in the presence or in the absence of the growth regulators 2,4-D and 2-iP. It can be observed that the highest concentration of total phenolic compounds occurred in the treatment with 100 μ M SA in callus subcultured in the absence of 2,4-D and 2-iP, and with 10 μ M SA in callus subcultured in the presence of these regulators. Such result may be associated with the interactions of SA and phytohormones [33]. Anyway, SA showed a secondary metabolism elicitor effect as reported in the literature, especially in *in vitro* conditions [34,35,36]. It should be noted that a low concentration of phenolics was observed in the control, in the absence of 2,4-D and 2-iP. The lack of auxin and cytokinin may have had a negative influence on total phenolic production in callus, since studies by Aremu et al. [37] reported 12 types of bioactive phenolic compounds in *Tulbaghia simmleri* Beauverd, also with medicinal properties, and found that their occurrence, distribution and levels were strongly influenced by the treatment with cytokinins, emphasizing the importance of these molecules in the biosynthesis route of these compounds. It is important to highlight that control containing 2,4-D and 2-iP had a significantly higher amount of total phenolics when compared to the control in the absence of these regulators, showing the importance of auxin and cytokinin in the culture media. On the

other hand, the concentration 100 μM SA without the 2,4-D and 2-iP favored phenolic increase. This shows that media not supplemented with 2,4-D and 2-iP needed a higher concentration of the elicitor (Table 1).

Table 1. Total phenolics content in *Garcinia brasiliensis* callus subcultured with SA in the absence and presence of 72 μM of 2-iP and 28.73 μM of 2,4-D.

Treatments (SA)	Total Phenolics content ($\mu\text{g mg}^{-1}$ DW)	
	Without 2,4-D and 2-iP	With 2,4-D and 2-iP
Control	50.2c	153.6b
0.1 μM	121.3b	134.1b
1 μM	129.3b	129.0b
10 μM	127.0b	291.0a
100 μM	268.1a	145.8b

Same letters indicate that values do not differ by the Scott-Knott test at the 5% significance level.

Concerning flavonoids content, the levels was higher when compared to the phenolic compounds. This may be related to the large amount of biflavonoids produced by the Clusiaceae family, particularly in *Garcinia* [5] and, furthermore, because the solvent methanol does not extract all phenolic compounds present on the surface of callus cells [38]. In callus subcultured in the absence of auxin and cytokinin, there was significant difference in the concentration 100 μM AS (Table 2), indicating that highest concentration SA did influence the contents of these compounds. On the other hand, in the presence of 2,4-D and 2-iP, it was observed that auxin and cytokinin supplementation without the presence of the elicitor, was sufficient for the significant increase in flavonoids. Auxins and cytokinins may affect the production of these compounds by regulating initial steps of the chiquimate/phenylpropanoid pathway [39]. In the medicinal plant *Merwillia plumbea* Lindl., under *in vitro* conditions, 2-iP provided an increase in the production of caffeic acid Studies [40] and in callus of *Aronia melanocarpa*, both auxins and cytokinins significantly influence the production of free phenolic acids [41]. These results imply that the *in vitro* production of bioactive secondary metabolites, especially flavonoids, in callus of *Garcinia brasiliensis* was significantly influenced by the exogenous addition of different types and concentrations of cytokinins and auxins, and that the interaction between the elicitor SA and 2,4-D and 2-iP negatively affected flavonoid production, regardless of their concentration (Table 2).

Table 2. Total flavonoids content in *Garcinia brasiliensis* callus subcultured with SA in the absence and presence of 72 μM of 2-iP and 28.73 μM of 2,4-D.

Treatments (SA)	Total Flavonoids content ($\mu\text{g mg}^{-1}$ DW)	
	Without 2,4-D and 2-iP	With 2,4-D and 2-iP
Control	145.9b	438.2a
0.1 μM	188.6b	179.4b
1 μM	156.3b	169.2b
10 μM	197.9b	184.3b
100 μM	333.9a	160b

Same letters indicate that values do not differ by the Scott-Knott test at the 5% significance level.

Different hormones have some type of interaction with SA [42]. Crosstalk among different phytohormones results in synergistic and antagonistic interactions and plays an essential role in plant response to abiotic stress [43]. Auxin, for example, has an antagonistic interaction with salicylic acid [44]. This crosstalk between SA and auxin 2,4-D helps explain the significant reduction in flavonoid production, when compared to the control (Table 2). Additionally, the production of fukugetin and 7-epiclusianone was evaluated. As shown in Table 3, in most treatments, it was possible to identify these molecules. However, the variation in levels was very high, especially for the fukugetin. Therefore, in most treatments, there was no statistically difference. Except for the treatment with 10 μM SA with 2,4-D and 2-iP, where there was a significant increase in 7-epiclusianone, in which the addition of regulators 2,4-D and 2-iP favored the significant increase in this benzophenone, when compared to the medium without growth regulators.

This increase in the levels of 7-epiclusianone may be associated with the increase in phenolic compounds (Table 1). Such response shows that the metabolic pathways of the cell responsible for the synthesis of this benzophenone remained active in the callus [3]. The 7-epiclusianone is important in the prevention and treatment of cancer by induction of apoptosis and cell cycle blocking in G1 phase, preventing the transcription and translation of important cell cycle regulators involved in the progression of cancer cells [45]. It also has anti-HIV [46] and anti-anaphylactic [12] properties. Studies involving the antimicrobial activity of epiclusianone in *Garcinia brasiliensis* seeds showed that this compound has antifungal and antibacterial activities [13].

Table 3. Contents of fukugetin and 7-epiclusianone in *Garcinia brasiliensis* callus subcultured with SA in the absence and presence of 72 μM of 2-iP and 28.73 μM of 2,4-D.

Treatments (SA)	Fukugetin ($\mu\text{g mg}^{-1}$ DW)		7-epiclusianone ($\mu\text{g mg}^{-1}$ DW)	
	Without 2,4-D and 2-iP	With 2,4-D and 2-iP	Without 2,4-D and 2-iP	With 2,4-D and 2-iP
	Control	79.6b	n.i	117.1b
0.1 μM	216.3b	103.6b	135.1b	96.7b
1.0 μM	57.4b	39.9b	94.3b	132.9b
10 μM	17.5b	149.1b	166.0b	803.6 ^a
100 μM	n.i	n.i	130.7b	125.3b

Same letters indicate that values do not differ by the Scott-Knott test at the 5% significance level. N.i.: not identified.

Callus Fresh Mass

The addition of growth regulators, such as 2,4-D and 2-iP, is often required for callus growth, since they induce different responses in cells, including increased levels of proteins and mitotic activity [47].

In fact, the callus fresh mass was higher in the control with growth regulators when compared to that without these substances (Table 4). In the treatments without 2-iP and 2,4-D, SA did not cause changes in the callus fresh mass. However in the presence of the auxin and cytokinin there was a decrease in callus fresh mass in the treatments with 10 and 100 μM SA. Similar results were observed by Rezaei et al. [48] who verified that increased SA concentration resulted in a significant decrease in viability and cell growth, when compared to culture media without the presence of SA in *Taxus baccata* L. and Dong et al. [49], in which elicitation with SA resulted in a reduction in the mass of *Salvia miltiorrhiza* Bunge cells concomitant with the increased accumulation of phenolic compounds through the stimulation of PAL activity [50].

Table 4. Fresh mass of *Garcinia brasiliensis* calluses exposed to salicylic acid in the absence and presence of 2,4-D and 2-iP.

Treatments (SA)	Callus fresh mass (mg)	
	Without 2,4-D and 2-iP	With 2,4-D and 2-iP
Control	9.6c	30.8a
0.1 μM	6.0c	29.5a
1 μM	8.5c	25.5ab
10 μM	9.2c	12.8bc
100 μM	10.3c	15.2bc

Same letters indicate that values do not differ by the Scott-Knott test at the 5% significance level

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Conflicts of Interest: The authors declare no conflict of interest

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