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Artigo

A dehydrorotenoid produced by *callus* tissue culture and wild plant roots of *Boerhaavia coccinea*

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RESUMO: "Um desidro-rotenóide produzido por cultura de calos e por raízes de plantas silvestres de *Boerhaavia coccinea*". Cultura de calos foram estabelecidos de folhas e galhos finos de plântula de *B. coccinea* produzida *in vitro* e analisada para isoflavonóide. A quantificação do 6,9,11-triidroxi-6a,12a-desidro-rotenóide isolado das raízes de *B. coccinea* P Miller, coletada em seu habitat natural, e do mesmo rotenóide produzido na cultura de células estão descritos neste artigo. A análise rotineira em CLAE mostrou que a cultura de calos produziu o mesmo isoflavonóide encontrado nas raízes da planta do campo. A quantidade do metabólito secundário produzido *in vitro* foi de 955.35 µg/g de massa seca de *callus*, atingindo uma concentração de 2,5 vezes maior do que a quantidade do metabólito produzido pela planta em seu meio ambiente natural.

Unitermos: *Boerhaavia coccinea*, Nyctaginaceae, metabólitos secundários, cultura de células vegetais, isoflavonóide, desidro-rotenóide, rotenóide.

ABSTRACT: *Calli* cultures were established from leaves and stem of *B. coccinea* plantlet produced *in vitro* and analysed for isoflavonoid content. The quantification of 6,9,11-trihydroxy-6a,12a-dehydrorotenoid isolated from the roots of *Boerhaavia coccinea* P. Miller collected from its natural environment, and the same metabolite produced in *callus* tissue culture of the same plant are described in this paper. The rotinary quantitative HPLC analysis indicated that *callus* culture produced the same isoflavonoid compound found in the roots of intact wild growing plant. The amount of the secondary metabolite produced *in vitro* was 955.35 µg/g of dry cell weight, 2.5 times more than the highest amount concentration produced by the wild growing plant in its natural environment.

Keywords: *Boerhaavia coccinea*, Nyctaginaceae, secondary metabolite, plant callus culture, isoflavonoid, dehydrorotenoid, rotenoid.

INTRODUCTION

The *Boerhaavia* genus has been chemically and little pharmacologically studied. The presence of isoflavonoids compounds is very common in the roots of this genus (Lami et al., 1991). Coccineone-B is an isoflavonoid systematically named 6,9,11-trihydroxy-6a,12a-dehydrorotenoid that was isolated from the roots of wild growing *Boerhaavia coccinea* plant (Messana et al., 1986) (Figure 1).

This is a medicinal folk plant from the northeastern of Brazil used in the treatment of urinary diseases (Braga, 1960). In this study, the isoflavonoid was produced and accumulated *in vitro* using *callus* tissue culture techniques and compared to the amount of coccineone B produced in the root of intact plant during different months in a year.

The seasonal study of isoflavonoid compounds in the roots and *calli* of *B coccinea* brings about relevant information in the variation production and accumulation of this metabolite during different periods of the year. This phenomenon is followed by flowering or environmental changes that turn the chemical aspects of the plant. In general, plants produce high amounts of different metabolites with diversity of biological activity during these kind of changes (Barbosa-Filho et

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al., 2005).

This work was developed to compare the concentration of coccineone-B produced by *calli* culture of *B. coccinea* with that present in the roots of the same plant in its natural environment (Fedoreyev et al., 2000; Luczkiewicz; Glod, 2003). The results herein presented were obtained to evaluate the potentiality of the *calli* in producing high amount of isoflavonoid compound during seasonal changes.

MATERIAL AND METHODS

Plant material

Roots and seeds of wild growing *B. coccinea* were collected in Maceio-AL, Northeast region of Brazil. The specimen was certified by "Instituto do Meio Ambiente de Alagoa-IMA", and its voucher specimen was deposited at the herbarium of the IMA under the registry number MAC/8512.

Establishment of callus culture

C*allus* culture was initiated with explants prepared from aerial parts (leaves and stem) of plantlets obtained after *in vitro* seed germination. The explants were transferred to MS basal medium, supplemented with 0.5 mg of 2,4-D (2,4-dichlorophenoxyacetic acid) L^{-1}, 0.5 mg of BAP (benzylaminopurine) L^{-1}, 30 g sucrose L^{-1} and 10 g of agar L^{-1}; pH was adjusted to 5.7 (Murashige; Skoog, 1962). The cultivation was performed in the dark for 30 days at 27 ± 2 °C. Calluses emerging on the explants were excised and cultivated on the same medium. Subculturing was carried out every 30 days.

Obtaining of callus samples

Callus samples (cylindrical flask containing ca 5 g of fresh *callus*), for isoflavonoids quantification by HPLC analysis, were withdrawn in triplicate at the 20th day (half of exponential phase) and submitted to lyophilization for 48h using a freeze-dryer at -55 °C. Analyses were performed by sacrifice sampling.

Obtaining of chromatographic standard coccineone-B

Coccineone-B was isolated by phytochemical method from roots of *B. coccinea* as reported by Messana et al. (1986).

Samples preparation for HPLC

100 mg of freeze-dried *callus*, and room temperature dried roots (200 mg) of *B. coccinea* were submitted to extractions with chloroform (1 mL x 3) after been powdered by a mortar and pestle, and filtered under vaccum in a whatman paper quantitative filter nº 1. The chloroform extract was concentrated at room temperature. Prior to analysis, the dried extract was dissolved with 490 mL of acetonitrile and 10 mL (0.866 mg ≈ 9.413 μMol) of toluene, used as internal standard, and submitted to HPLC analysis.

HPLC analysis conditions

A volume of 5 mL of sample solution was injected into the analytical column RP-C-18 (3.9 mm x 30 cm) with 10 mM particle size mBondapak. The HPLC was a Waters equipped with an integrator and a UV detector operated at 300 nm with an injector U6K. A pump allowed a flow rate of 1.5 mL min^{-1} using acetonitrile-water (45:55 v/v) as mobile phase in an isocratic mode. Standard Curve was constructed with a solution containing COC-B in acetonitrile in the concentration range from 0.3 mg mL^{-1} to 500 mg mL^{-1}. Six different known concentrations from standard solution were injected separately with three replications, based on literature procedure (Yariwake et al., 2005; Batista et al., 2005; Lodhi et al., 1994). The limit detection was obtained using different decreasing concentratios.

RESULTS AND DISCUSSION

Three samples from *callus* tissue culture were submitted to isoflavonoids extraction procedure and analysed by HPLC, Table 1. The results were compared with the isoflavonoids seazonal producing by wild growing plant, Table 2.

The first sample came from a culture growth with 2,4-D. This growth regulator presents a function of accelerate the subdivision of the cell leading rapid growth of the *callus*. The second sample came from a culture growth with BAP, which presented a potential to induce coccineone-B to be produced and accumulated in cell tissue culture. The third sample came from an experiment developed to induce the growth of the callus in the presence of 0.5 μg/L of 2,4-D and 0.5 μg/L of BAP. All *calli* were analysed after 20 days of maintenance in a solid culture medium.

The results showed that BAP induced high level of the dehydrorotenoid concentrations better than 2,4-D and in the mixture of 2,4-D and BAP. The relevance of this quantitative analysis permitted to demonstrate that BAP induced isoflavonoids production in higher amount than wild plant.

The *calli* were analyzed to investigate the behaving of the isoflavonoid amounts to be compared with the same compound present in roots of *B. coccinea* during the different months. Dehydrorotenoid was produced by roots in high amount from August to December. From January to July this compound was produced in low concentrations or were not produced

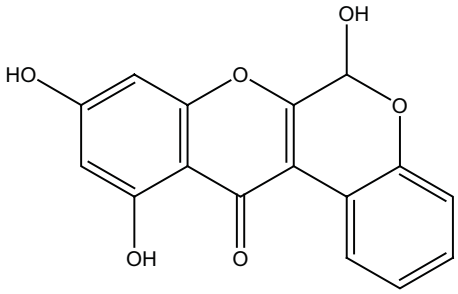

Figure 1. Chemical structure of coccineone-B (6,9,11-trihydroxy-6a,12a-dehydrorotenoid).

Table 1. Dehydrorotenoid analyses in µg of COC-B for g of dried *calli*.

Calli cultivated with	Amount of GR (mg L^{-1})	Fw of *calli* (g)	Dw of *calli* (g)	Amount of COC-B($\mu g.g^{-1}$)
2,4-D/BAP	0.5/0.5	8.97 ± 0.42	0.28 ± 0.02	35.49 ± 0.94
2,4-D	0.5	7.67 ± 0.68	0.36 ± 0.01	59.85 ± 0.57
BAP	0.5	6.34 ± 0.85	0.33 ± 0.02	955.35 ± 1.23

Quantification of 100 mg of dried *callus*. Results in µg of isoflavonoid per gram of dried cells. Fw: fresh weight of *callus*, Dw: dry

Table 2. Quantitative analysis of coccineone-B present in root samples of wild growing plant.

Months	Amounts in µg of dehydrortenoid per g of dw of roots	Standard deviation
Jan	4.82 ± 0.01	0.02
Feb	Nd	0.00
Mar	4.17 ± 0.02	0.04
Apr	1.18 ± 0.01	0.02
May	3.68 ± 0.11	0.19
Jun	4.95 ± 0.17	0.30
Jul	1.72 ± 0.06	0.10
Aug	379.33 ± 1.80	3.05
Sep	57.17 ± 0.92	1.60
Oct	28.63 ± 0.44	0.76
Nov	101.64 ± 0.75	1.30
Dec	33.91 ± 0.90	1.55

Nd = not detected.

Table 3. Statistical analysis of Internal Standard IS.

Mean	Sd (yEr±)	VC (%)
33,356 ± 767.22	2.544,58	± 7.62

VC = variance coeficient is the percentage value that deviates from mean, and is more representative than standard deviation.

as demonstrated in February, Table 2. The absence of this compound or its low presence during a few months may be related with the rainy season, when there is a considerable amount of rain incidence in this Region of Brazil. As described by Monteiro et al. (2006), tannins and phenolic compounds decrease in concentration in the rainy season when compared to dry season. As a rule, it is possible to see a relation between high isoflavonoid concentrations in November with the beginning of summer.

Calli cultures were withdrawn in triplicate for each experiment. As can be seen, by the relation dry weight/fresh weight, the friable *calli* presents too much water and the cultivation underwent 20 days, Table 1.

Calli cultures were established from the stem of *B. coccinea* plantlets obtained from seeds germination *in vitro* that is thought to be an important goal for plant biotechnology. Similar strategy was developed by Fedoreyev et al. (2000), exploiting the analytical HPLC for the reproducibility of the sample analysis. As described by Lima et al. (2006), natural resources have been extensively exploited for medicinal purposes and a great number of plants have been employed in folk medicine, although the active principles are found in very low isolated substances concentrations. In this case, plant cell culture was the most efficiente technique used to obtain higher amounts of active compound, that allowed inducing and accumulating the metabolite in a short time then mother plant did.

For this system of analysis the limit of detection was 0.04 mg mL^{-1} over the wide concentration range from 0.3 mg mL^{-1} to 500 mg mL^{-1}, with an average accuracy of 99.43 ± 0.48%. Statistical calculations with the areas of IS, were made and a relevant result was obtained to confirm the accuracy of the analysis, Table 3.

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