Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **28** (4): 613-6 (2009)

Short Communication

Received: January 17, 2009
Accepted: April 5, 2009

Production of Rutin and Kaempferol-3-*O*-glucuronide by Tissue Cultures of *Alpinia purpurata* (Vieill) K. Schum

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SUMMARY. **The accumulation of flavonoids was evaluated in organogenic cultures of *Alpinia purpurata* through HPLC analysis. Cultures were maintained in liquid MS medium in the following treatments: MS0 (control), TDZ, BAP and IAA + TDZ. Kaempferol-3-*O*-glucuronide content was higher than rutin for all *in vitro* treatments, except by control medium in which plants did not produce kaempferol-3-*O*-glucuronide. A remarkable increase in kaempferol-3-O-glucuronide content was verified using BAP 2 mg.l^{-1} (0.027 mg/100 mg dried extract) and IAA 2 mg.l^{-1} + TDZ 2 mg.l^{-1} (0.030 mg/100 mg dried extract) treatments. With the addition of 2 mg.l^{-1} BAP, rutin concentration also increased in the proportion 1:4 compared to control.**

INTRODUCTION

Advantages of secondary metabolites production through tissue cultures are that different physical factors as light, temperature and nutritive solutions can be easily controlled, additionally, *in vitro* cultures allow the use of growth regulators and elicitors to optimize the phytochemical production [1,2]. The ability to manipulate *in vitro* cultures consist in a high value alternative to produce therapeutic metabolites whose chemical synthesis is very expensive and long, making the process unworkable [1,3]. Cytokinins and auxins are responsible for regulation of growth and development plant process and their addition in culture media is an important step to large numbers of plants in short period of time; interfere with morphogenesis, physiology and induce changes on quantity and quality of secondary metabolites [4,5]. Several studies have been reported the influence of growth regulators in secondary metabolites production in field plants and plantlets grown *in vitro* [6,7]. Tissue cultures procedures are promising to large-scale production using clonal plants and cell cultures and it is a resource to study metabolic pathways [4].

Alpinia purpurata is widely used as ornamental. This species presents few references in folk medicine. According to Bermudez and Velazquez [8], *A. purpurata* leaves infusion are used to treat disturbs in respiratory tract. *Alpinia* genus comprises aromatic plants whose essential oils have been widely studied. [9] Approaches about phytochemistry of hydroalcoholic extracts of *A. purpurata* is very recent and incipient and results show the presence of flavonoids rutin and kaempferol-3-*O*-glucuronide [10,11]. Flavonoids are present in several species of *Alpinia* and they are referred as promising therapeutic agents in the treatment of cardiovascular diseases [12,13]. Flavonoids have been detected in plant tissue cultures [14,15], however this is the first evidence of the rutin and kaempferol-3-O-glucuronide flavonoids production by *in vitro* cultures. The aim of this study was to evaluate the production of flavonoids in tissue cultures of *A. purpurata* under effects of auxin and cytokinins.

KEY WORDS: Flavonoids, Micropropagation, Plant growth regulator, Zingiberaceae.

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MATERIALS AND METHODS

Plant material

Samples of the matrix plant of *Alpinia purpurata* (Vieill) K. Schum. were collected in the Universidade Federal do Rio de Janeiro (Rio de Janeiro, Brazil). A voucher specimen is deposited at the Herbarium of Rio de Janeiro Botanical Garden, under accession number RB 433485.

Chemicals

Kaempferol-3-*O*-β-glucuronide and kaempferol-3-O-β-rutinoside were isolated from *Alpinia zerumbet* species and identified by RMN [12,16]. Rutin was obtained commercially from Merck. Chemical for analysis by HPLC as methanol, phosphoric acid (85%) were of analytical grades. Distilled water was utilized as the extraction solvent and MilliQ water to HPLC mobile phase.

In vitro cultures

Tissue cultures were established according to Victório [17] from matrix plant. All culture media consisted of basic Murashige and Skoog [18] salts, 3% of sucrose, vitamins and myo-inositol. Media were supplemented with thidiazuron (TDZ 2 $mg.l^{-1}$), 6-benzylaminopurine (BAP 2 $mg.l^{-1}$) or indole-3-acetic (IAA 2 $mg.l^{-1}$) plus TDZ 2 $mg.l^{-1}$. Plantlets were subcultured every three months *in vitro,* at 25 ± 2 °C, photoperiod of 16 h, under white light (Duramax Universal) obtained from General Electric® fluorescent tubs (F-20 W, T-12), and light intensity of 30 µmoles $m\text{-}2\ s^{-1}$. Distance between shelves was standardized (30 cm). Plantlets at fourth subculture were used to initiate growth regulator treatments. Each treatment consisted of four sets with at least 15 plantlets. The effects of each treatment were evaluated according to following parameters: number of shoot, number of leaves, shoot height, leaf length and rooting.

Extraction and detection of flavonoids by high- liquid performance chromatography (HPLC)

Extracts were obtained from *in vitro* dried leaves. Macerated dry leaves were immersed in a Becker with 70% ethanol (1:20) and ultra-sonicated for 45 min in ultrasonic bath, at 60 °C. Then, crude extracts were filtered and dried using rotavapor and lyophilizator. Crude extracts were filtered using a Whattman filter paper (110 mm Ø) and ultra-sonicated before analysis by HPLC. Flavonoids standards and samples were dissolved in 70% methanol at 1 mg/ml and 50 mg/ml, respectively. Qualitative and quantitative analysis of flavonoids in crude extract was performed by HPLC technique using Shimadzu apparatus coupled to LC-10AD bomb and SPD-M10A-UV detector on column reverse-phase C_{18} (Lichrosorb, 25 cm x 5 mm, 5 µm de 100 Å silica). The chromatographic separation was developed using a mobile phase of solvents: A – H_2O (MilliQ) + H_3PO_4 0.1% (v/v) and B - MeOH: 1-10 min (30% B); 20 min (40 % B); 60 min (100% B) (60 min) at flow rate of 1 ml/min. The injection volume was set at 20 µL and detection was in UV absorbance at 254 and 360 nm. Flavonoids were detected through retention times, UV spectrum in compare with flavonoids standard spectra and by coinjection with authentic samples analyzed in the same conditions. For coinjection, it was prepared a mixture of extracts and flavonoids standard (1:1, v/v).

Statistical analysis

The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatments. Averages were compared using Dunnett`s test at 5% significance level.

RESULTS

Data from *in vitro* development of *A. purpurata* cultures treated with growth regulators are shown in Figure 1. Low concentration of TDZ (2 $mg.l^{-1}$) produced the highest number of shoots compared to other tested media, however without significant difference with control medium (MS0). Media containing TDZ (2 $mg.l^{-1}$) or BAP (2 $mg.l^{-1}$) produced lower amount of plant material due to a reduction in the number of leaves and leaf size (Fig. 1B and D). IAA in combination with TDZ contributed to increase the number of leaves (Fig. 1B).

Rutin content (RT = 31.129 min) was observed by HPLC in all plantlets cultured in vitro (Figure 2), showing greater concentration in BA 2 and IAA 2 + TDZ 2 treatments within four months. BA 2 treatment improved rutin production compared to control (Fig. 2). Treatments BAP 2 and IAA 2 + TDZ 2 increased the production of kaempferol-3-*O*-glucuronide when compared to TDZ 2 medium. Plantlets grown in MS0 did not produce kaempferol-3-*O*-glucuronide (Fig. 2).

DISCUSSION

Flavonoids are metabolites produced as part

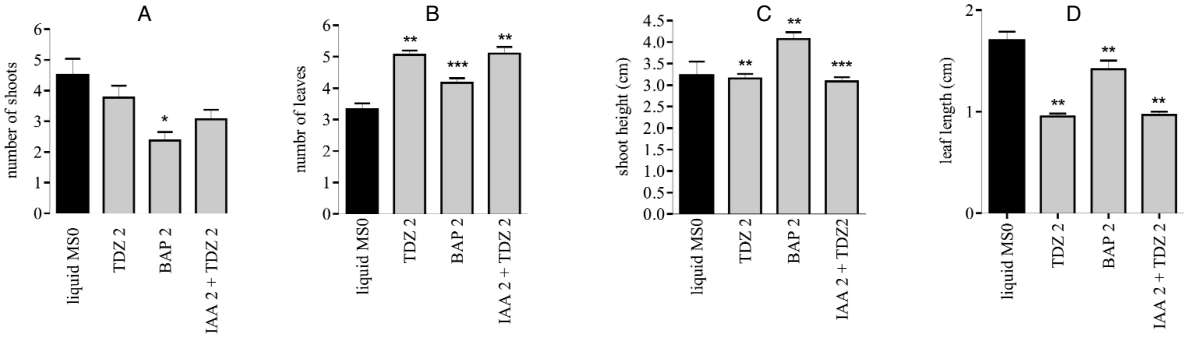

Figure 1. Effects of type and concentration of growth regulators on tissue cultures of *Alpinia purpurata* 4-month-old *in vitro*-grown. Statistical differences compared to control medium (*p<0.001, **p<0.01, ***p<0.05, Dunnett, n≥30).

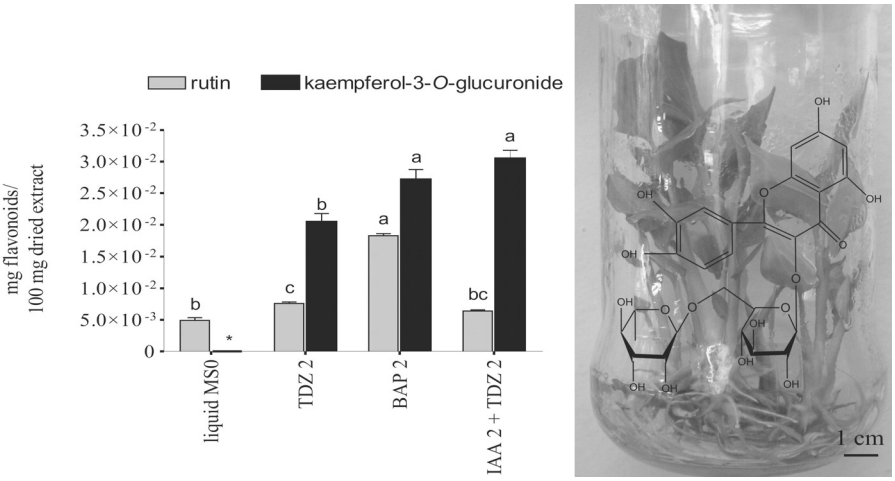

Figure 2. Flavonoids content obtained by HPLC from leaves of 4-month-old *Alpinia purpurata* plantlets. *Kaempferol-3-*O*-glucuronide not detected. Different letters indicate statistical differences ($p < 0.05$, Dunnett, n = 3) for each flavonoids separately. Four-month-old plantlets under TDZ 2 $mg.l^{-1}$ effects, showing rutin structure.

of plant defense, especially against the effects of ultraviolet radiation. In view of this, their contents are in greater concentration in the leaves the main part of plants exposed to solar incidence [19,20]. The use of leaves for tea and pharmaceutical preparations is very common and agreement with the high levels of secondary metabolites in this organ. Hydroalcoholic extracts of leaves of field *A. purpurata* showed chromatographic profiles similar to those of plantlets from tissue cultures, detecting important flavonoids referred to therapeutic uses [11]. The presence of flavonoids in plant tissue cultures have been described in several studies with the following families Vitaceae, Oxalidaceae, Lamiaceae, Saxifragaceae and other Droseraceae [14,21-23]. It is the first citation to Zingiberaceae family.

Although the morphological responses have not changed significantly, media containing BAP 2 or IAA 2 + TDZ 2 increased the production of kaempferol-3-*O*-glucuronide. These results presented in common the addition of TDZ or BAP in culture medium in combination or isolated. Reports have been suggested that auxins and cytokinins may stimulate enzymatic steps of the biosynthetic pathway of flavonoid glycosides. Some auxins have been referred by promoting the action of the enzyme that catalyzes the glucosyltransferase glycolization of flavonoids [14].

Rutin and kaempferol-3-*O*-glucuronide production in matrix plants was higher than results achieved in the current study to plantlets [11]. Low concentrations of flavonoids produced by cultures of *A. purpurata* may be related to reduce activity of photosynthetic apparatus due to low light intensity under *in vitro* conditions [24]. In studies with tissue cultures of *Nicotiana tabacum* [25] was found low production of carotenoids anteraxantina (1.4%) and zeaxanthin (5.1%) under light intensity of 60 µmoles.m-2.s^{-2}, against 9.6% and 12% respectively, for the

intensity of 200 µmoles.m-2.s^{-2}. Furthermore, low concentration of flavonoids in plantlets may be related to the reduction of leaf area of *A. purpurata* in tissue cultures that limits the exposure to light.

CONCLUSION

Tissue cultures of *A. purpurata* showed to be a place of inducing variation in flavonoids production. The flavonoids rutin and kaempferol-3-*O*-glucuronide were verified by HPLC in leaves of plantlets of *A. purpurata,* with pronounced increase of kaempferol-3-*O*-glucuronide in plantlets treated with BAP 2 or IAA 2 + TDZ 2. Rutin content increased in plantlets cultured in medium containing BAP 2.

Acknowledgements. C.P. Victório acknowledges the Ph.D. fellowship and financial support from CAPES/PROEX, Brazil.

REFERENCES

1. Collin, H.A. (2001) *Plant Growth Regul.* **34**: 119-34.
2. Canter, P,H,, H. Thomas & E. Ernst (2005) *Trends Biotechnol.* **23**: 181-5.
3. Konczak-Islam, I., S. Okuno, M. Yoshimoto & O. Yamakawa. (2003) *Biochem. Eng. J.* **14**: 155-61.
4. Zhao, J., W.H. Zhu, Q. Hu & X.W. He (2001) *Plant Growth Regul.* **33**: 33-41.
5. Hobbie, L., C. Timpte & M. Estelle (1994) *Plant Mol. Biol.* **26**: 1499-519.
6. Berhow, M.A. (2000) *Plant Growth Regul.* **30**: 225-32.
7. Luczkiewicz, M. & D. Glód (2003 *Plant Sci.* **165**: 1101-10.
8. Bermúdez, A. & D. Velázquez (2002) *Rev. Fac. Farm. (Univ. Los Andes, Mérida)* **44**: 2-6.
9. Victório C.P., S.G. Leitão & C.L.S. Lage. *J. Essent. Oil Res.* (in press)
10. Victório C.P., R.M. Kuster & C.L.S. Lage (2007) *J. Bras. Fitomed.* **5**: 137.
11. Victório, C.P., R.M. Kuster & C.L.S. Lage (2009) Detection of flavonoids in *Alpinia purpurata* (Vieill) K. Schum. leaves by high-performance liquid chromatography. *Braz. J. Med. Plants* **11**(2): 147-53
12. Mpalantinos, M.A., R.S. Moura, J.P. Parente & R.M. Kuster (1998) *Phytotherapy* **12**: 442-4.
13. Yang, Y.H. (2003) *Plant Growth Regul.* **39**: 253-61.
14. Kokubo, T., Y. Ambe-Ono, M. Nakamura, H. Ishida, T. Yamakawa, T. Kodama (2001) *J. Biosci. Bioeng.* **91**: 564-9.
15. Thiem, B. (2003 *Plant Sci.* **165**: 1123-8.
16. Mpalantinos, M.A. (2001) *Identificação de substâncias anti-hipertensivas de Alpinia zerumbet Pers.* Ph.D. thesis, Universidade Federal do Rio de Janeiro, Rio de Janeiro.
17. Victório, C.P., I.P. da Cruz, A. Sato, R.M Kuster & C.L.S. Lage (2008) Effects of auxins and cytokinins on *in vitro* development of *Alpinia purpurata* (Vieill) K. Schum and phenolic compounds production. *Plant Cell Cult. Micropropag.* **4**(2): 92-8.
18. Murashige, T. & F. Skoog (1962) *Physiol. Plant.* **15**: 473-97.
19. Havsteen, B.H. (2002) *Pharmacol. Therapeut.* **96**: 67-202.
20. Ferrer, J.L., M.B Austin, C. Stewart & J.P. Noel (2008) *Plant Physiol. Biochem.* **46**: 356-70.
21. Brisson, L., R.K. Ibrahim & M. Rideau (1988) *Plant Cell Rep.* **7**: 130-3.
22. Santos-Gomes, P.C., R.M. Seabra, P.B. Andrade & M.F. Ferreira (2002) *Plant Sci.* **162**: 981-7.
23. Krolicka, A., A. Szpitter, E. Gilgenast, G. Romanik, M. Kaminski & E. Lojkowska (2008) *Enzyme Microb. Tech.* **42**: 216-21.
24. Hazarika, B.N. (2006) *Sci. Hortic.* **108**: 105-20.
25. Ticha, I., F. Cap, D. Pacovska, P. Hofman, D. Heisel, V. Capkova & C. Schafer (1998) *Physiol. Plant.* **102**: 155–62.