Polygala paniculata: a source of methyl salicylate produced through plant tissue culture[1]

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ABSTRACT

The establishment of *Polygala paniculata* L. tissue culture allowed a rapid propagation of raw plant material under aseptic and standardized conditions. Plantlets were subjected to simultaneous distillation-extraction (SDE) and volatiles composition analyzed by GC/FID and GC/MS. The effect of BAP (6-benzylaminopurine) on *in vitro* development and volatiles production was evaluated. The main feature of volatile compounds was the significant amount of methyl salicylate. Plantlets cultured on control MS (Murashige & Skoog) medium showed more than 80% of salicylate, while the addition of BAP at 2 and 4 mg L^{-1} concentrations reduced methyl salicylate production to 71% and 21%, respectively. Tissue culture of *P. paniculata* proved to be a potential source of methyl salicylate.

Key words: BAP, micropropagation, volatile compounds, elicitor, Polygalaceae.

RESUMO

Polygala paniculata: um recurso de salicilato de metila produzido por cultura de tecidos vegetais

O estabelecimento de cultura *in vitro* de *Polygala paniculata* L. permitiu a propagação rápida de matéria-prima vegetal sob condições assépticas e padronizadas. As plantas *in vitro* foram submetidas à extração e destilação simultâneas (EDS) e a composição química do óleo essencial foi analisada por CG/DIC e CG/EM. Foram avaliados os efeitos de BAP (6-benzilaminopurina) sobre o desenvolvimento *in vitro* e produção de voláteis em *P. paniculata*. A principal característica do óleo essencial consistiu na presença marcante de salicilato de metila. Plantas cultivadas em meio controle MS (Murashige & Skoog) produziram mais do que 80% de salicilato, enquanto o uso de BAP, reduziu a produção de salicilato de metila a 71% e 21% para as concentrações de 2 e 4 mg L^{-1}, respectivamente. A cultura de tecidos de *P. paniculata* pode ser indicada como fonte de salicilato de metila.

Palavras-chave: BAP, propagação *in vitro*, voláteis, elicitores, Polygalaceae.

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INTRODUCTION

Methyl salicylate also known as salicylic acid methyl ester is used in flavoring for foods, candies, beverages and pharmaceuticals. It is also an odorant, perfume and ultraviolet absorber in cosmetics. This compound is used in traditional medicine, mainly as anti-inflammatory, analgesic, expectorant and anti-rheumatics (Effmert *et al*., 2005). Volatile compounds obtained from natural products are important in industry, including high value products useful to pharmaceutical, cosmetic and food companies.

Polygala (Polygalaceae), native to tropical America, comprises different species widely used worldwide for medicinal purposes (Wang *et al*., 2008). *Polygala paniculata* L., known as milkwort, root beer plant, and called "barba-de-são-pedro" and "vassourinha-branca" in some parts of Brazil, is a small herb growing in *roadsides, rainforests and wastelands* (Marques, 1988). In Brazil, it is used as topical preparations to treat physical traumas (Lorenzi & Matos, 2008). The main volatile compound detected in *P. paniculata* root was methyl salicylate (Nogueira *et al*., 2005). This volatile is the main component of Gelol®, a common medicine in Brazil. Previous studies have shown scientific evidence for its analgesic, antiedematogenic and gastroprotective activities (Nogueira *et al*., 2005; Lapa *et al*., 2007; Lorenzi & Matos, 2008).

Tissue culture techniques have some advantages as physical and biological manipulation of medicinal plants under standardized conditions, use of elicitors and plant growth regulators to optimize bioactive compounds and subculturing of elite genotypes (Affonso *et al*., 2007; Victório *et al*., 2008; Victório *et al*., 2009; Victório *et al*., 2011). The purpose of this study was to evaluate *in vitro* organogenesis of *P. paniculata* under standardized conditions and volatiles production using the growth regulator BAP (6-benzylaminopurine).

MATERIAL AND METHODS

Plant material

Seeds collected from *Polygala paniculata* L. wild plants were used as source of plant material. A voucher specimen is deposited at the Herbarium of Rio de Janeiro Botanical Garden (RB), under accession number 347639.

Culture medium and in vitro conditions

In vitro germination procedures were according to Nogueira *et al*., 2005. Nodal segments excised from *in vitro* seedlings were inoculated in 141 x 72 mm jars: 5 explants per jar containing 50 mL of MS (Murashige & Skoog, 1962) supplemented with 2% (w/v) sucrose, 1.3 µM of thiamine-HCl, 3 µM of pyridoxine, 4.1 µM of nicotinic acid, 0.6 mM of myo-inositol, pH at 5.8 ± 0.1 and autoclaved at 120ºC and 1.1 kgf cm^{-2}. Media were solidified with 7.8 g L^{-1} of agar. Cultures were maintained at 25 ± 2ºC, under white light (Sylvania F20 W T12), intensity of 30 µmoles m^{-2} s^{-1}, 16 h photoperiod, for 60 days. BAP (6-benzylaminopurine) was used in different concentration: MS0 (control), BAP 1 (1 mg mL^{-1}), BAP 2 (2 mg mL^{-1}), BAP 4 (4 mg mL^{-1}) and BAP 8 (8 mg mL^{-1}). Morphogenesis was evaluated within 60 days in culture, considering the shoot number per explant, shoot length, shoot fresh weight (120 days) and presence of roots and callus. At least, thirty random explants were used per treatment. Data were subjected to analysis of variance (ANOVA) and means were compared by the Dunnett's test at 5% probability level.

Simultaneous distillation-extraction (SDE)

Fresh leaves (2.5 g) from 120-day-old plantlets were homogenized with 70 mL of distilled water and subjected to SDE (Godefroot *et al*., 1981) for 1h 30 min, using 2 mL of dichloromethane as an organic collecting solvent, according to Boix *et al*. (2010).

Volatiles analysis

Analytical GC was carried out on a Varian Star 3400 gas chromatograph fitted with a DB-5/MS column (30 m × 0.25 mm, 0.25 µm film thickness) and equipped with flame ionization detection (FID). Temperature was programmed from 60 – 290ºC-10 min. Sample injection of 1 µL was performed at 270ºC. Hydrogen was used as carrier gas, at linear flow 1 mL min^{-1}. GC/MS analyses were performed using a Shimadzu Model GC MS-QP 5000 apparatus under the following conditions: column, DB-5/MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 µm); carrier gas, helium at 1 mL min^{-1}; injection of 1 µL; injector temperature, 260°C; interface 200ºC; column temperature, 60 – 290ºC-10 min; mass spectra, 70 eV.

Identification of volatiles components was based on retention indices (RI) relative to *n*-alkanes (C_8-C_{19}) (Van den Dool & Kratz, 1963) and computer matching with the National Institute of Standards and Technology (NIST 98) library as well as by comparison of mass spectra fragmentation patterns with those reported in the literature (Adams, 1995). Quantification was performed from CG/FID profiles using relative area (%).

RESULTS AND DISCUSSION

The organogenic response of *P. paniculata* to different BAP concentrations was evaluated considering shoot number, shoot length, fresh weight, rooting and callogenesis (Figure 1 and 2). The lowest BAP concentration was enough to produce a high level of shoots, without significant differences in comparison

with the control. The greatest shoot lengths were obtained with the addition of BAP 1, 2 or 4 mg L^{-1}. Positive correlation was found between increasing BAP concentration and shoot fresh weight. Plantlets from the control medium showed 12.5% of rooting within 60 days. An enhancement of rooting was observed after 60 days of plantlets in culture. Proliferation rate increased dramatically after 60 days and counting of shoot number became impractical. Plantlets cultured in medium BAP 4 for 120 days showed the highest fresh weight per shoot of 2.5 mg (Figure 1C).

P. paniculata is a potential source of methyl salicylate. In nature, *P. paniculata* accumulates volatiles consisting of more than 50% of methyl salicylate (Nogueira, 2004). Under *in vitro* conditions, plantlets also produced a high concentration of this volatile, consisting of unique constituents detected by GC (Figure 3). This study indicated a high methyl salicylate production in leaves and stems. Chromatographic profiles showed a few hydrocarbons that appeared in some samples after 30 min. Methyl salicylate has been identified in several *Polygala* species (Weinhold *et al*., 2008). Increasing in fresh weight with growing BAP concentrations was inversed to the amount of methyl salicylate (Figure 2 and 3). Plantlets cultured in higher BAP concentration showed great water accumulation, but a reduced volatile production.

Methyl salicylate was rapidly detected after 7 min by GC/MS, in an intense peak. The use of BAP may induce hydrocarbons biosynthesis, when compared with plants grown in the control medium. These data agree with those reported for leaf volatiles from *A. zerumbet* cultured in medium containing cytokinin, where data evidenced a large number of hydrocarbons in leaf volatiles when KIN and BAP were applied (Victório *et al.*, 2011). Effects of cytokinins on the level of volatile compounds have been reported in tissue cultures of medicinal plants (Affonso *et al*., 2007). However, in this study, the addition of cytokinin did not improve methyl salicylate production *in vitro* cultures of *P. paniculata*, conversely a reduction in its content was observed in growing concentrations of BAP (Figure 3).

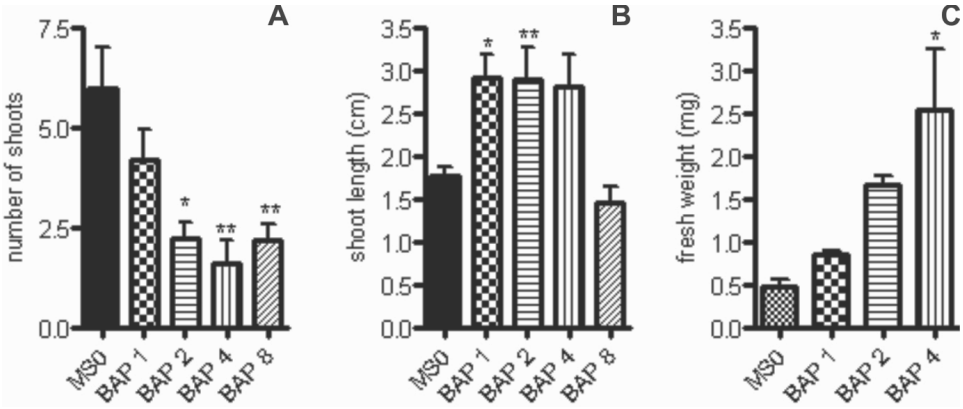

Figure 1. Effects of BAP (mg L^{-1}) on *in vitro* development of *Polygala paniculata*: A. shoot number (60 days), B. shoot length (60 days), C. shoot fresh weight (120 days). Data indicate mean ± SD, *p< 0.01; **p< 0.05 compared with MS0, Dunnett's test.

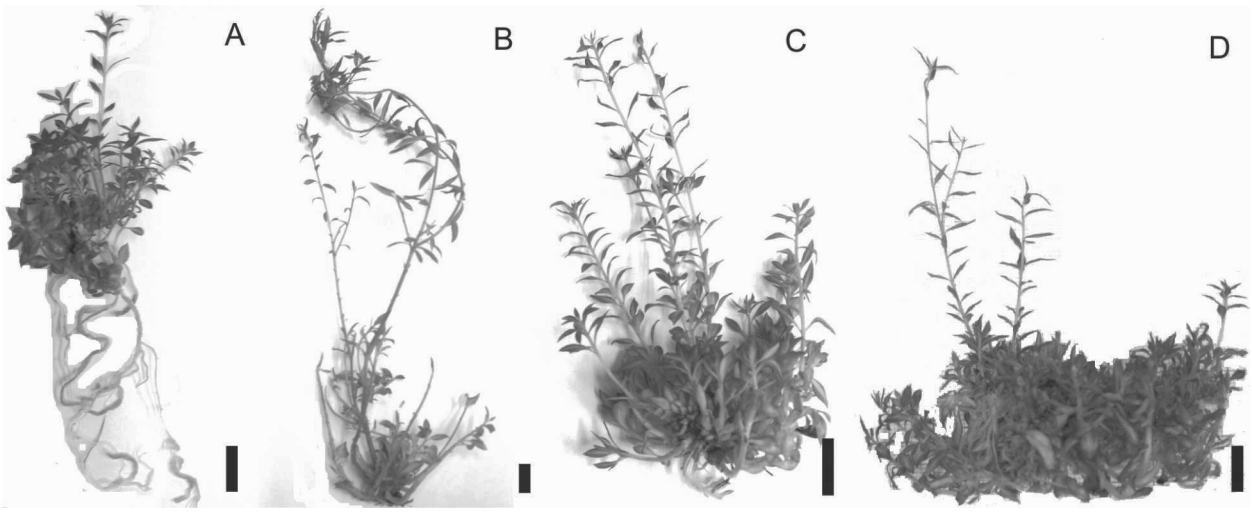

Figure 2. *In vitro* organogenesis in *Polygala paniculata*, 120-day-old plantlets. A. MS0, B. BAP 1, C. BAP 2, D. BAP 4 (mg L^{-1}).

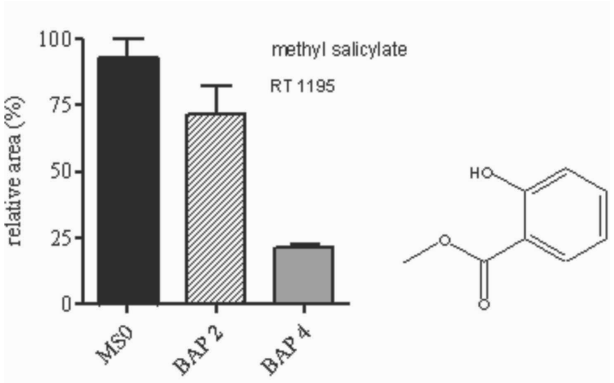

Figure 3. Leaf volatiles composition (%) of 120-day-old *Polygala paniculata* plantlets. Each value is the average of two extractions and analyses.

Several reports have indicated methyl salicylate as an important elicitor of production of secondary metabolites in plants towing to an induction of a wide range of defense responses against insect herbivores and microbial pathogens (Boer & Dicke, 2004; Wang *et al*., 2007). Still, the results about methyl salicylate production in *P. paniculata* cultures may be used as a natural source of allelopathic agent, via co-cultivation with other plants.

CONCLUSION

The production of methyl salicylate by tissue culture is an interesting alternative to manipulate its constant production under standardized conditions. A SDE/GC methodology for leaf volatiles analysis was developed for *P. paniculata* for the first time. The results obtained in the current study showed that plantlets of *P. paniculata* produced a high level of methyl salicylate and BAP negatively influenced, reducing its total contents.

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REFERENCES

Adams RP (1995) Identification of essential oil components by gas chromatography/mass spectroscopy. Allured Publ. Corp., Carol Skream, IL. 469p.

Affonso VR, Bizzo HR, Lima SS, Esquibel MA & Sato A (2007) Solid phase microextraction (SPME) analysis of volatile compounds produced by in vitro shoots of *Lantana camara* L. under the influence of auxins and cytokinins. Journal of Brazilian Chemical Society, 18:1504-1508.

Boer JG & Dicke M (2004) The role of methyl salicylate in prey searching behavior of the predatory mite *Phytoseiulus persimilis*. Journal of Chemical Ecology, 30: 255-271.

Boix YF, Victório CP, Kuster RM & Lage CLS (2010) Volatile compounds from *Rosmarinus officinalis* L. and *Baccharis dracunculifolia* DC. growing in Southeast coast of Brazil. Química Nova, 33: 255-257.

Effmert U, Saschenbrecker S, Ross J, Negre F, Fraser CM, Noel JP, Dudareva N & Piechulla B (2005) Floral benzenoid carboxyl methyltransferases: From *in vitro* to in plant function. Phytochemistry, 66:1211-1230.

Godefroot M, Sandra P & Verzele M (1981) New method for quantitative essential oil analysis. Journal of Chromatography 203: 322–335.

Lapa FR, Freitas CS, Baggio CH, Missau FC, Pizzolatti MG, Santos ARS & Marques MCA (2007) Gastroprotective activity of the hydroalcoholic extract obtained from *Polygala paniculata* L. in rats. Journal of Pharmacy and Pharmacology, 59:1413-1419.

Lorenzi H & Matos FJA (2008) Plantas medicinais no Brasil: nativas e exóticas, 2 nd ed., São Paulo, Nova Odessa, Instituto Plantarum. 544p.

Marques MCM (1988) Polígalas do Brasil V, seção *Polygala* (Polygalaceae). Arquivos do Jardim Botânico do Rio de Janeiro, 29:1-114.

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plantarum, 15:473-497.

Nogueira FLP, Fernandes SBO, Reis GM, Matheus ME, Fernandes PD, Lage CLS & Menezes FS (2005) Atividade analgésica e antiedematogênica de *Polygala paniculata* L. (Polygalaceae) selvagem e obtida por micropropagação. Revista Brasileira de Farmacologia, 15:310-315.

Nogueira FLP (2004) Estudo biotecnológico e farmacológico de *Polygala paniculata* L. Dissertação de mestrado. Universidade Federal do Rio de Janeiro, Rio de Janeiro, 86p.

Van den Dool H & Kratz PD (1963) A generalization of the retention index system including linear temperature programmed gas liquid partition chromatography. Journal of Chromatography, 11: 463-471.

Victório CP, Cruz IP, Kuster RM & Lage CLS (2008) Effects of auxins and cytokinins on *in vitro* development of *Alpinia purpurata* (Vieill) K. Schum and phenolic compounds production. Plant Cell Culture & Micropropagation, 4:92-98.

Victório CP, Kuster RM & Lage CLS (2009) Production of rutin and kaempferol-3-*O*-glucuronide by tissue cultures of *Alpinia purpurata* (Vieill) K. Schum. Latin American Journal of Pharmacy, 28:613-616.

Victório CP, Kuster RM & Lage CLS (2011) Leaf and root volatiles produced by tissue cultures of *Alpinia zerumbet* (Pers.) Burtt & Smith under the influence of different plant growth regulators. Química Nova, 34:430-433.

Victório CP, Arruda RCO, Riehl CAS & Lage CLS (2011) Leaf volatiles and secretory cells of *Alpinia zerumbet* (Pers.) Burtt et Smith (Zingiberaceae). Natural Product Research, 25:939-948.

Wang YL, Wang XD, Zhao B & Wang YC (2007) Enhancing antioxidative capacity of *Lepidium meyenii* calli by addition of methyl salicylate to culture medium. Acta Physiologiae Plantarum, 29:417-423.

Wang H, Gao J, Kou J, Zhu D & Yu B (2008) Anti-inflammatory activities of triterpenoid saponins from *Polygala japonica*. Phytomedicine, 15:321-326.

Weinhold TS, Bresciani LFV, Tridapalli CW, Yunes RA, Hense H & Ferreira SRS (2008) *Polygala cyparissias* oleoresin: comparing CO_2 and classical organic solvent extractions. Chemical Engineering and Processing: Process Intensification, 47:109-117.