Influence of plant growth regulators and light on callus induction and bioactive phenolic compounds production in *Pyrostegia venusta* (Bignoniaceae)

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Received 14 January 2015; revised 22 February 2016

Callus culture allows production of bioactive compounds in a short time when there is limited availability of natural sources. In this work, we analyzed the influence of plant growth regulators and light on callus induction and the phenolic compounds contents in the calli of *Pyrostegia venusta*. Leaf explants were placed on MS medium containing 2,4-dichlorophenoxiacetic acid and 6-benzylaminopurine in the presence and absence of light. Callus induction was observed in media with plant growth regulators. The calli’s colour and consistency ranged from green and compact to yellow and friable, respectively in the presence and absence of light. The interaction between 4.52 µM 2,4-D and 8.88 µM BAP in the absence of light provided the best friable calli, with total phenol and flavonoid contents at 0.25 ± 0.01 µg ATE mg⁻¹ DW and 0.15 ± 0.02 µg RE mg⁻¹ DW, respectively. Calli presented higher flavonoid contents than those in the initial explants. HPLC-DAD analyses showed bioactive phenolic compounds as gallic, caffeic and benzoic acid derivatives in hydromethanolic extracts of calli.

**Keywords:** Auxin, Benzoic acid, Caffeic acid, Callogenesis, Cytokinin, Gallic acid, Flamevine, Flavonoids, Orange trumpetvine, Phenols

Though acquiring secondary metabolites witnessed increased global interest, production and extraction of such compounds continue to be compromised by seasonal variations and environmental factors to which natural populations are exposed. Moreover, the chemical synthesis of several plant-derived compounds is often impractical due to their high structural complexity. The technology of plant tissue culture provides strategies to minimize these problems, favours continuous production of commercially important secondary metabolites by optimizing cultures, and controls the chemical and physical factors to which plant cells are subjected. These techniques are explored as models for controlled bioactive substances production from plants, since they may be potentially manipulated to alter the quality and quantity of metabolites produced.

Plant growth regulators (PGRs), especially auxin and cytokinin are important for accumulation of biomass and production of secondary metabolites in *in vitro* culture of plant cells and tissues. Changes in the types and concentrations of auxin and cytokinin, coupled to their interaction play important roles in the plant growth and formation of plant metabolites. Auxins may have synergistic, additive and antagonistic interactions with cytokinines in the regulation of the physiological response, which often depends on the species and the type of plant tissues. Generally, auxins are associated with cell growth and root formation, whereas cytokinins account for cell division and shoot growth. Auxin 2,4-D used alone or in combination with cytokinins has been widely used to stimulate callus induction and the maintenance thereof. High auxin and cytokinin concentrations generally promote cell proliferation with callus formation. However, the concentration and PGRs combinations must be determined for each species.

**Pyrostegia venusta** (Ker Gawl.) Miers, commonly called flamevine or orange trumpetvine, is a seasonal species and its use in traditional medicine and acquisition of seedlings are quite limited. The literature has consistently documented its potential as a medicinal plant and many pharmacological activities are related to phenolic compounds, mainly flavonoids, such as hesperidin, homoeriodictiol, rutin, quercetin, catechin and acacetin-7-O-glycoside found in different organs of the plant. Recent studies showed absence of genotoxic activity in the ethanolic extracts of flamevine flowers and high ability to scavenge free radicals of methanolic extracts of leaves, flowers and roots. Other biological activities have been reported such as improvement of flu symptoms, stimulation of melanogenesis, antimicrobial activity and anti-inflammatory and antinociceptive activities.
In spite of a recent study by Loredo-Carrillo et al.\textsuperscript{14} on callus induction in \textit{Pyrostegia venusta}, effects of auxin-cytokinin and light interaction have not been investigated so far. The current investigation evaluates the influence of different supplementations of PGRs 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) on callus induction and the phenolic compounds production in \textit{P. venusta} calli in the presence and absence of light.

\textbf{Materials and Methods}

\textbf{Reagents and materials}

Chlorogenic, \textit{trans}-cinnamic, ferulic, caffeic and tannic acids, apigenin, rutin, (+)-catechin hydrate, luteolin, chrysins, quercetin, 2,4-dichlorophenoxyacetic acid, 6-benzilaminopurine, methanol and formic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The Folin-Dennis reagent was prepared according to AOAC\textsuperscript{15}.

\textbf{Plant material}

The explants were obtained from leaves of \textit{Pyrostegia venusta} (Ker Gawl.) Miers (Bignoniaceae) collected in the Brazilian Cerrado region in Divinópolis, Midwest Minas Gerais State, Brazil (20°10'45.9"S and 44°55'07.2"W). The voucher specimen was identified by Andréia Fonseca Silva of Herbarium PAMG (PAMG 56307).

\textbf{Establishment of callus}

The leaves were treated with 70% ethanol (1 min), 1% NaClO (5 min) and washed five times in sterilized water for asepsis. The leaf segments (6 mm) were placed on basal medium MS\textsuperscript{16} with 30 g L\textsuperscript{-1} sucrose plus 2,4-D and/or BAP (Table 1) to induce callus formation. The pH was adjusted to 5.8 ± 0.1 with NaOH 0.1 N after adding PGRs and medium was solidified with 7 g L\textsuperscript{-1} agar.

The explants were transferred to a growth chamber and kept in the presence or absence of light, at 27 ± 2°C under 16:8 h light/dark regime, with a light intensity of 40 µmol m\textsuperscript{-2} s\textsuperscript{-1}. After 60 days of inoculation, the color, consistency, callus induction (%), fresh and dry weights, total phenols and flavonoids contents and the chromatographic profile of phenolic compounds in the calli were determined. The completely randomized experimental design comprised different concentrations of 2,4-D and BAP in the presence and absence of light, with 20 replications.

\textbf{Preparation of hydromethanolic extracts}

Approximately, 200 mg of dried calli were extracted with 10 mL methanol:water (1:1) by cold maceration with constant stirring for 4 hours in a shaker apparatus\textsuperscript{17}. The extract was filtered and the final volume was completed to 10 mL with methanol:water (1:1).

\textbf{Determination of total phenols content}

Phenols were quantified with 100 µL of hydromethanolic extract, following AOAC\textsuperscript{15} procedure. Total phenols content was calculated by a calibration curve with 100 mg L\textsuperscript{-1} tannic acid solution as standard. Determinations were performed in triplicate and the result was given in microgram equivalents of tannic acid per milligram of dry matter (µg ATE mg\textsuperscript{-1} DW).

\textbf{Determination of total flavonoids content}

Total flavonoids assay was performed according to Woisky and Salatino\textsuperscript{18} and flavonoid content was calculated by a calibration curve with 100 µg mL\textsuperscript{-1} rutin in a methanol solution of 2 % aluminum chloride as standard. Determinations were performed in triplicate and the result was given in microgram equivalents of rutin per milligram of dry matter (µg RE mg\textsuperscript{-1} DW).

\textbf{HPLC-DAD analysis}

Chromatographic profiles were obtained in a modular system liquid chromatography Shimadzu Prominence HPLC (Shimadzu Corp., Kyoto, Japan). The separation of compounds was performed with a reversed-phase column Gemini C18 (4.6 X 250 mm, 5 µm, Phenomenex\textsuperscript{8}, Torrance, CA, USA) conditioned at 35°C. The mobile phases comprised A: water: formic acid (99.9:0.1) and B: methanol: formic acid (99.9:0.1) at the proportion of 0% B (0-5 min); 0-100% B (5-30 min); 100% B (30-35 min). A 20 µL injection volume and a flow rate of 1.0 mL min\textsuperscript{-1} were employed. Separations were monitored at three wavelengths to detect phenolic substances; 254 nm and 328 nm for phenolic acids and flavan-3-ols and 350 nm for flavones, flavonols and chalcones\textsuperscript{19}. The determination of phenolic compounds in the samples was performed by comparing retention times and UV spectrum of standards previously injected and by data from the literature\textsuperscript{20-22}.

\textbf{Data analysis}

Data were analyzed by the System Analysis of Variance for Balanced Data (Software SISVAR 5.1)\textsuperscript{23} at \(P \leq 0.05\) significance level. Mean rates were further separated by Scott-Knott Test when differences were significant.
Table 1 — Callus induction, biomass production (fresh weight - FW; dry weight - DW), total phenolics and flavonoids, consistency and color of callus induced with different concentrations and combinations of 2, 4-D and BAP in the presence and absence of light after 60 days of culture.

| Treatments | Combination of PGRs | Callus induction (%) | Fresh weight (g) | Dry weight (g) | Phenolics (μg TAE mg⁻¹ DW) | Flavonoids (μg RE mg⁻¹ DW) | Consistency | Color | Callus induction (%) | Fresh weight (g) | Dry weight (g) | Phenolics (μg TAE mg⁻¹ DW) | Flavonoids (μg RE mg⁻¹ DW) | Consistency | Color | Consis
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<td>C1</td>
<td>4.52 μM 2,4-D</td>
<td>55 d</td>
<td>1.97±0.22 a</td>
<td>0.17±0.01 a</td>
<td>0.18±0.01 i</td>
<td>0.08±0.01 d</td>
<td>Compact</td>
<td>Green</td>
<td>5 j</td>
<td>0.43±0.04 b</td>
<td>0.02±0.01 i</td>
<td>0.13±0.01 c</td>
<td>0.08±0.01 c</td>
<td>Frangible</td>
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<td>C2</td>
<td>9.05 μM 2,4-D</td>
<td>65 c</td>
<td>1.57±0.27 a</td>
<td>0.11±0.01 b</td>
<td>0.16±0.01 i</td>
<td>0.12±0.01 c</td>
<td>Compact</td>
<td>Green</td>
<td>15 i</td>
<td>0.64±0.29 b</td>
<td>0.04±0.02 c</td>
<td>0.10±0.01 f</td>
<td>0.06±0.01 d</td>
<td>Frangible</td>
<td>LY</td>
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<tr>
<td>C3</td>
<td>18.10 μM 2,4-D</td>
<td>25 i</td>
<td>0.93±0.30 b</td>
<td>0.07±0.03 c</td>
<td>0.20±0.01 d</td>
<td>0.19±0.01 a</td>
<td>Compact</td>
<td>Green</td>
<td>35 h</td>
<td>0.03±0.02 c</td>
<td>0.01±0.02 c</td>
<td>0.01±0.02 f</td>
<td>0.07±0.01 d</td>
<td>Frangible</td>
<td>LY</td>
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<tr>
<td>C4</td>
<td>4.44 μM BAP</td>
<td>50 e</td>
<td>0.14±0.04 c</td>
<td>0.01±0.02 d</td>
<td>0.20±0.01 h</td>
<td>0.13±0.01 b</td>
<td>Compact</td>
<td>Green</td>
<td>40 g</td>
<td>0.53±0.14 b</td>
<td>0.06±0.02 b</td>
<td>0.19±0.01 d</td>
<td>0.10±0.01 h</td>
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<td>C5</td>
<td>8.88 μM BAP</td>
<td>45 f</td>
<td>0.39±0.13 c</td>
<td>0.06±0.02 c</td>
<td>0.25±0.01 f</td>
<td>0.18±0.01 a</td>
<td>Compact</td>
<td>Green</td>
<td>55 e</td>
<td>0.53±0.14 b</td>
<td>0.06±0.02 b</td>
<td>0.12±0.01 f</td>
<td>0.09±0.01 b</td>
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<td>C6</td>
<td>17.79 μM BAP</td>
<td>30 h</td>
<td>0.13±0.09 c</td>
<td>0.02±0.01 d</td>
<td>0.25±0.02 f</td>
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<td>Compact</td>
<td>Green</td>
<td>35 h</td>
<td>0.28±0.11 c</td>
<td>0.04±0.01 c</td>
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<td>55 d</td>
<td>0.40±0.20 c</td>
<td>0.04±0.02 c</td>
<td>0.22±0.01 g</td>
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<td>Compact</td>
<td>Green</td>
<td>70 c</td>
<td>0.45±0.20 b</td>
<td>0.04±0.02 c</td>
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<td>Frangible</td>
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<td>C8</td>
<td>4.52 μM 2,4-D +8.88 μM BAP</td>
<td>40 g</td>
<td>0.35±0.11 c</td>
<td>0.05±0.03 c</td>
<td>0.27±0.02 e</td>
<td>0.14±0.01 b</td>
<td>Compact</td>
<td>Green</td>
<td>75 b</td>
<td>0.64±0.10 b</td>
<td>0.06±0.01 b</td>
<td>0.25±0.01 b</td>
<td>0.15±0.02 a</td>
<td>Frangible</td>
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<tr>
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<td>4.52 μM 2,4-D +17.79 μM BAP</td>
<td>70 b</td>
<td>0.06±0.02 c</td>
<td>0.02±0.01 d</td>
<td>0.28±0.01 d</td>
<td>0.13±0.01 b</td>
<td>Compact</td>
<td>Green</td>
<td>85 a</td>
<td>0.44±0.11 b</td>
<td>0.04±0.01 c</td>
<td>0.21±0.01 c</td>
<td>0.15±0.01 a</td>
<td>Frangible</td>
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<td>C10</td>
<td>4.52 μM 2,4-D +8.88 μM BAP</td>
<td>45 f</td>
<td>0.18±0.11 c</td>
<td>0.02±0.01 d</td>
<td>0.41±0.01 b</td>
<td>0.11±0.01 c</td>
<td>Compact</td>
<td>Green</td>
<td>75 b</td>
<td>0.15±0.07 c</td>
<td>0.02±0.01 c</td>
<td>0.15±0.01 e</td>
<td>0.09±0.01 c</td>
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<td>C11</td>
<td>4.52 μM 2,4-D +8.88 μM BAP</td>
<td>30 h</td>
<td>0.10±0.06 c</td>
<td>0.01±0.02 d</td>
<td>0.28±0.01 b</td>
<td>0.14±0.01 b</td>
<td>Compact</td>
<td>Green</td>
<td>40 g</td>
<td>0.43±0.14 b</td>
<td>0.03±0.01 c</td>
<td>0.28±0.01 a</td>
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<td>Frangible</td>
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<td>C12</td>
<td>4.52 μM 2,4-D +17.79 μM BAP</td>
<td>55 d</td>
<td>0.14±0.05 c</td>
<td>0.05±0.01 c</td>
<td>0.23±0.01 g</td>
<td>0.11±0.01 c</td>
<td>Compact</td>
<td>Green</td>
<td>50 f</td>
<td>0.16±0.05 c</td>
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<td>45 f</td>
<td>0.03±0.02 c</td>
<td>0.01±0.00 d</td>
<td>0.28±0.01 d</td>
<td>0.11±0.01 c</td>
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<td>Green</td>
<td>55 e</td>
<td>0.18±0.07 c</td>
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<td>Frangible</td>
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<td>C14</td>
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<td>55 d</td>
<td>0.03±0.02 c</td>
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<td>0.59±0.02 a</td>
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<td>Compact</td>
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<td>75 a</td>
<td>0.27±0.10 c</td>
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<td>60 d</td>
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<td>Frangible</td>
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*Means in each column followed by the same letter are not significantly different at P <0.05 by the Scott-Knott test. **Standard deviation values for these data were omitted, since they are less than 0.009. LY, Light Yellow and DY, Dark Yellow.
Results and Discussion

Callus induction

Callus induction was observed in media containing 2,4-D and/or BAP, in the presence and absence of light (Table 1). The calli’ colour ranged from green to yellow. Whereas in the presence of light the calli were compact and green (Table 1), they became friable and light yellow or dark yellow in the absence of light (Table 1). Friable calli with a yellow colour may be considered organogenic and may develop into organs or embryos, as previously reported24,25.

The callus induction ranged between 25 and 75 % in presence of the light and between 5 and 85 % in the absence of light. The highest induction of friable callus (85 % and 75 %) were observed in 4.52 µM 2,4-D + 17.75 µM BAP (C9), 4.52 µM 2,4-D + 8.88 µM BAP (C8) and 9.05 µM 2,4-D + 4.44 µM BAP (C10) in the absence of light. On the other hand, the calli induced with 4.52 µM 2,4-D + 8.88 µM BAP (C8) presented the highest dry weight when compared with treatment C9 and C10. The lowest callus induction (5%) was registered on medium with 4.52 µM 2,4-D in absence of light (Table 1).

There was not generally direct relationship between callus induction (%) and callus weight. However, a relationship was observed between callus growth (expressed by callus weight) with light, and PGRs, since the combination between light and low concentrations of 2,4-D (4.52 µM and 9.05 µM) and absence of light and higher concentration of 2,4-D (18.10 µM) + BAP (17.75 µM) promoted higher callus weight (Table 1). Current results corroborate with Loredo-Carrillo et al.14 that produced compact and green calli from P. venusta with higher fresh weight in media containing 1 and 2 mg L-1 2,4-D (4.52 and 9.05 µM 2,4-D, respectively).

The association of PGRs and light conditions for callus induction should be carefully investigated since these conditions not only affect the cultures growth but also the callus’s color and consistency. In P. venusta, the green color and the compact consistency of the calli are associated with light, since these aspects are characteristic of all treatments in the presence of light, regardless of the type and concentration of the regulator used. Calli induced in the light were compact. This fact limits their use in future studies related to in vitro secondary metabolites production employing cell suspension cultures26.

Phenolic compounds

Phenols and flavonoids were detected in all calli produced (Table 1). In the presence of light, the calli induced in 9.05 µM 2,4-D + 4.44 µM BAP (C10), 18.10 µM 2,4-D + 8.88 µM BAP (C14) and 18.10 µM 2,4-D + 17.75 µM BAP (C15) showed higher total phenol contents, and the production was stimulated 2.1 times on media containing 18.10 µM 2,4-D + 8.88 µM BAP, when compared to the initial explant. In all treatments, calli showed higher flavonoid contents than the initial explant and the production was stimulated 4.5 times on media containing 18.10 µM 2,4-D (C3), 8.88 µM BAP (C5) and 18.10 µM 2,4-D + 8.88 µM BAP (C14). However, the treatments carried out in presence of light produced compact calli, which were not interesting for the production of these metabolites in cell suspensions. In the absence of light, the calli provided the same or lower phenols amounts than in the initial explant. The flavonoid amounts were markedly higher, especially in calli induced with 4.52 µM 2,4-D + 8.88 µM BAP (C8) and 4.52 µM 2,4-D + 17.75 µM BAP (C9). The production was stimulated 3.75 times when compared with initial explant and the calli induced in absence of light were friable. These results show the potential for in vitro production of high amounts of flavonoids from P. venusta’s calli and disagree with some reports which suggest that the formation of specialized tissues is a requisite for the production of secondary metabolites, especially phenolics27.

Phenols and flavonoids contents showed an inverse relationship with cell growth given by calli weight (Table 1), especially in presence of light, and higher total phenols and flavonoids contents were observed in calli with lower weight. The relationship between cell growth and secondary metabolites production has been reported in the literature; however it’s still not well understood14. Loredo-Carrillo et al.14 described that decrease in growth may be related to the use of energy from sucrose in the culture medium for the synthesis of secondary metabolites.

The current studies confirm the biotechnological potential of P. venusta and agree with Loredo-Carrillo et al.14 that indicated a significant increase in in vitro flavonoids production in P. venusta’s calli in the presence of light and the elicitation of cultures with 2.5 % polyethylene glycol. However, in this work also, we showed the possibility to produce calli with higher flavonoids contents in the absence of light and without elicitation.
Hydromethanolic extracts from the initial explant and calli from treatment C8 (4.52 µM 2,4-D + 8.88 µM BAP) in the absence of light were subjected to HPLC-DAD for the analysis of phenolic compounds (Fig. 1). Results from the initial explant (Fig. 1A) suggested the presence of nine phenolic substances including three benzoic acid derivatives (di- and tri-hydroxylated) (peaks 1–3), ferulic acid derivative (peak 4), rutin (peak 5) and four derived from p-coumaric acid (peak 6–9). Derivatives of p-coumaric acid in calli extracts induced by C8 treatment (Fig. 1B) were not detected. Just a few peaks were observed in hydromethanolic extracts from calli. They may be assigned to derivatives of gallic (peaks 1 and 2), benzoic (peaks 3 and 4) and caffeic (peak 5–9) acids. This is the first report on the presence of phenolic acids in calli of *P. venusta*. Although Loredo-Carrillo et al. detected and quantified the presence of phenolics and sterols in *P. venusta's* calli, the substances were not characterized.

Phenolic compounds are extensively studied and there is evidence of their role in disease prevention, including heart disease. Caffeic acid derivatives observed only in calli extracts from the treatment C8 such as coumaric acid derivative have antinociceptive activities and may act as potent chemoprotective agents against skin cancer. Studies about the bioactivity of gallic acid and its derivatives indicate antioxidant functions and antibacterial, antiviral, anti-inflammatory and antitumor activity in several cell lines. Benzoic acids are capable of inhibiting bacterial growth and are widely used to acidify food products.

Secondary metabolites, especially phenolic compounds are involved in plant responses to biotic and abiotic stresses and provide a significant contribution to the antioxidant activity in plant tissues.

**Conclusion**

Auxin (2,4-D) and cytokinin (BAP), as well as the presence or absence of light strongly influence the
induction, growth, consistency, colour and phenolic compounds production in Pyrostegia venusta calli. Friable callus containing higher amounts of bioactive phenolic compounds in P. venusta can be produced in media supplement with 4.52 µM of 2,4-D associated to 8.88 µM BAP, in the absence of light. The results encourage additional agronomic and biochemical studies to understand the mechanisms involved in the regulation of the bioactive compounds production in P. venusta calli, with the perspective of developing new protocols to obtain in vitro cell cultures with high concentrations of bioactive phenolic from a biotechnological approach.

Acknowledgement
This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

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