



Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana*

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ABSTRACT

The objective was to enhance the production of the phenolic compounds in plant cell suspension cultures of *T. peruviana* at shake flask scale. The effects of salicylic acid (SA), methyl-jasmonate (MeJA) and the combination of both (SA/MeJA) were studied. Elicitor concentration, elicitation time and harvest time of cells were optimized. Phenolic compound content (PCC), flavonoid content (FC) and antioxidant activity (AA) were determined by the folin-ciocalteu method, flavonoid-aluminum complexation method and the ABTS assay, respectively. Differences between intracellular metabolite profiles due to the mentioned treatments were analyzed by Thin-layer chromatography and High-performance liquid chromatography. Highest PCC, FC and AA were obtained under the following treatments: 3 μ M MeJA > 3 μ M MeJA/300 μ M SA > 300 μ M SA > control, when elicited on the 4th day and harvested 96-h post-elicitation. It was demonstrated that exposure to 3 μ M MeJA increase 1.49-fold of PCC, 1.66-fold of AA and 2.55-fold of FC compared to the control culture.

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1. Introduction

Thevetia peruviana (Pers.) K. Schum is an ornamental shrub belonging to the Gentianales order, Apocynaceae family. It is widely distributed in the tropical and sub-tropical regions of Central and South America, Asia, and Africa [1]. This plant is pharmacologically recognized for containing cardiotonic glycosides such as peruvoside and thevetin [2,3], especially concentrated in its seeds. These metabolites have a positive inotropic effect, like in digoxin [1,4]. Therefore, the un-regulated consumption of the fruits has been reported as toxic [5]. *T. peruviana* also produces phenolic compounds with potential usage as antimicrobial [6,7] and antineoplastic drugs [8,9]. Additionally, flavonoids have been identified in the fruits and leaves of this plant, and they are capable of inhibit the integrase enzyme and reverse transcriptase associated to DNA polymerase of the human immunodeficiency virus HIV-1 [10].

Phenolic compounds (PC) are one of the most important secondary metabolites in plants. These compounds are related to the mechanisms of environmental adaptation and stress under *in*

vivo growth conditions [11]. However, the quality and quantity of the PC and other secondary metabolites produced in field crops is extremely variable and depends on the biotic and abiotic conditions [12]. *In vitro* cultivation of plant cells is a viable alternative to increase the growth rate of biomass and the stability during the continuous production of PC and other metabolites. Additionally, *in vitro* cultivation allows for the manipulation of growth variables, as well as the use of precursors and/or elicitors. These variables might change the biosynthetic pathways of the compounds, optimizing its production [13].

An elicitor can be defined as a compound (natural or synthetic) that initiates or improves the biosynthesis of specific metabolites when introduced in small concentrations to a living cells system [14,15]. Jasmonates (JA) and salicylic acid (SA) are signaling molecules that respond to the biotic and abiotic stress of the plants. These molecules can be used to induce catalytic reactions by specific enzymes involved in the biosynthesis of PC [16]. JA and SA have been used in the stimulation of flavonoids and polyphenols production in cell suspension, calluses and tissue cultures of diverse plant families

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[17–21]. A recent study published by Rincón et al. [22] reported an increase in the production of PC from callus culture of *T. peruviana* elicited with a combination of 100 μM of JA and 10 μM of abscisic acid. Similarly, MeJA, a derivative of jasmonic acid, has also been used to elicitate cell suspension culture of *T. peruviana*, resulting in an increased production of peruvoside, a cardiotonic glycoside [23].

In this study, the effect of SA and MeJA was evaluated on the production of PC in a plant cell suspension culture of *T. peruviana* at shake flask scale. The concentration and time of addition of the elicitors that improve the production of PC was established, as well as the time of cultivation of the cells post-elicitation.

2. Materials and methods

2.1. Reagents and materials

Folin-Ciocalteu reagent (2.0 N), salicylic acid, methyl jasmonate (95%) and quercetin were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Gallic acid, acid 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic, ABTS) and glass HPTLC (High Performance TLC) Silica Gel 60 F₂₅₄ plates were purchased from Merck (Darmstadt, Germany).

2.2. Callus culture

Calluses were obtained from fruit pulp of *T. peruviana* plants cultivated at the Universidad Nacional de Colombia, Medellín (6°15'44"N 75°34'37"O). Fruits were disinfected following the protocol outlined previously by Arias et al. [23]. Briefly, fruits were submerged in ethanol (70%) for 5 min. Then transfer to a solution of NaClO at 10% (v/v) for 5 min. In-between and after the disinfectants, the fruits were rinsed three times with sterile distilled water. Afterwards, the explants were transferred aseptically onto Schenk and Hildebrandt solid medium (SH) supplemented with 2 mg/L 2,4-D, 0.5 mg/L kinetin, 7 g/L agar, 30 g/L sucrose and 1 g/L myoinositol (pH 5.8), sterilized at 20 psi for 15 min. Cultures were maintained on photoperiod (12 h light/12 h darkness) at 25 °C. Sub-cultures were performed every 3 weeks until friable calluses were obtained.

2.3. Cell suspension culture

Ten grams of fresh friable calluses (g FW) were transferred to 100 mL of SH liquid sterile medium supplemented with 2 mg/L 2,4-D, 0.5 mg/L kinetin, 30 g/L sucrose and 1 g/L myoinositol (pH 5.8), in 250 mL flasks. Cell suspension cultures were maintained on an orbital shaker at 110 rpm (New Brunswick™ Innova® 2300), natural photoperiod and 25 °C. Sub-cultures were made every 2 weeks.

2.4. Growth kinetics

Growth kinetic was done in 250 mL flasks, using an inoculum (cell suspension culture) of 6 days from the last subculture, and an initial concentration of 2–3 g dry weight per liter (DW/L) in 100 mL of supplemented SH liquid sterile medium. The other conditions of the cultures were maintained as previously described. Cellular growth was determined by measuring the DW of biomass every 2 days for 18 days, in duplicate. The content of each flask was filtered using a vacuum system and quantitative filter paper. Biomass was rinsed three times with distilled water and was dried in a convection oven at 60 °C for 48 h [24]. Results were expressed as g DW/L.

2.5. Elicitor treatment method

SA and MeJA were prepared in an aqueous solution of ethanol 50% (v/v) and were filter-sterilized through a 0.45 μm Millipore

filter (Minisart®, Sartorius, Germany). Experiments were done with 6 days cell suspensions at a concentration of 2–3 g DW/L in 250 mL flasks with 100 mL of SH liquid sterile medium, under the same conditions described previously. A factorial experiment based on completely randomized design was used to study the effect of the elicitation conditions on the PC production. The evaluated factors were: elicitor concentration, elicitation time and harvest time of the cells post-elicitation (see supplementary file). All the experiments were carried out in triplicate using a destructive sampling method, which consisted of processing the complete sample at each harvest time. Ethanol solution 50% (v/v) was used as control in these experiments. Non-significant elicitor effect was found when ethanol 50% was used when compared to a culture without ethanol (data not shown).

2.5.1. Effect of elicitor concentration on PC production

Concentration of each elicitor was initially evaluated. MeJA was used in concentrations of 0, 1, 3 and 5 μM ; while SA was used in concentrations of 0, 100, 300 and 600 μM . Elicitor concentration ranges used in these experiments were chosen by reviewing published studies [17,18] and by a previous screening performed in our laboratory. Elicitors were added during the exponential growth phase (day 8). Cells were harvested 24 h and 96 h after the addition of the elicitor.

2.5.2. Effect of the elicitation time on PC production

Elicitors were added at three different times: at the beginning of the culture (day 0) and during the exponential phase (days 4 and 8), using the best concentration of each elicitor, as it was previously established. Cells were harvested 24 h and 96 h after the addition of the elicitor.

2.5.3. Effect of the harvest time on PC production

The best cultivation time (24–48 – 72–96 and 120 h) after addition of the elicitor was determined, using the concentration and elicitation time that were previously established. In addition, flavonoid content (FC) and antioxidant activity (AA) were measured for each cultivation time.

2.6. Analytical methods

2.6.1. Intracellular metabolites

The biomass was dried in a convection oven at 60 °C for 48 h and was pulverized using mortar and a pestle. The powder (0.3 g) was extracted with 15 mL of an aqueous solution of ethanol 50% (v/v), in an ultrasonic bath (40 kHz) at 30 °C for 30 min, to obtain the intracellular metabolites. The extracts were centrifuged at 3000 rpm for 10 min; the supernatant was collected and stored in polypropylene tubes at -4 °C, protected from light. The extracts were used for the quantitative determination of phenols and flavonoids. An extract of the dried and powder fruit pulp of *T. peruviana* was also prepared to determine the metabolites present in the initial explant, following the methodology prescribed for the intracellular extraction of the *T. peruviana* biomass.

2.6.2. Extracellular metabolites

Extracellular metabolites were determined directly from the cell culture medium without any previous extraction step.

2.6.3. Phenolic compounds content (PCC)

These compounds were determined with the folin-ciocalteu method, using gallic acid as standard [25]. A volume of 2 mL of samples (culture medium or intracellular extracts) were mixed with 2.5 mL of the folin-ciocalteu reagent at 10% (v/v), in different test tubes. After 2 min, 2 mL of 7.5% (p/v) Na₂CO₃ were added; followed by incubation during 10 min at 50 °C. The absorbance of

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