Original Article

Analysis of Phenolics in *Calligonum polygonoides In Vitro* Cultured Roots

Abstract

Background: *Calligonum polygonoides* L. subsp. *Comosum* (L'Hér.) Sosk. is an endangered plant species belonging to family Polygonaceae. Although the plant is rich in phytoconstituents and has multipurpose medicinal applications, but *in vitro* root culture studies and phytochemical investigations of these cultures are rare. **Objectives:** To establish *in vitro* root, callus and cell suspension cultures from *in vitro* germinated fruits of *C. polygonoides* to investigate the production of phenolics through root, callus and cell suspension cultures and attempt to enhance cell capacity to accumulate phenolics. **Materials and Methods:** Modified Murashige and Skoog medium supplemented with 1 mg l-1 indole-3-butyric acid was used to establish the root culture. Elicitation of cell suspension culture was performed using salicylic acid and yeast extract. The phenolic compounds in root, callus and cell suspension cultures were evaluated using reversed phase high performance liquid chromatography technique. **Results:** The unorganized cell suspension culture contained fewer amounts of phenolic compounds than the differentiated roots tissue. Elicitation produced quantitative reprogramming of phenolic content. **Conclusion:** The present study provides a chance to improve secondary metabolite yield from this valuable natural plant.

Keywords: *Analysis, Calligonum polygonoides, high-performance liquid chromatography, phenolics*

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Introduction

Calligonum polygonoides L. subsp. *comosum* (L'Hér.) Sosk. (Polygonaceae) is a small shrub, which has reputation in folklore medicine as a stimulant and astringent, under the local names "ghardaq," "rusah," "arta," or "Wargat Al-shamas."[1,2] Leaves and stems are chewed to treat gummosis, whereas young shoot infusion is used as tonic.[3] It was reported that *C. polygonoides* possesses hypoglycemic,[4] cytotoxic, antioxidant,[1] antimicrobial,[5] anti-ulcer, anti-inflammatory,[6] antifungal,[7] mosquitocidal,[8] and estrogenic activities.[9] Chemical analysis from previous studies revealed the presence of (+)-catechin, dehydrodicatechin A, kaempferol-3-*O*-rhamnopyranoside, quercitrin, β-sitosterol-3-*O*-glucoside, isoquercitrin, kaempferol-3-*O*-glucuronide, and miquelianin in the aerial parts of the plant.[1] Campesterol, stigmasterol, (3β,5α,24S)-stigmastan-3-ol, and stigmast-4-en-3-one were isolated from the roots,[10] whereas β-sitosterol, kaempferol, quercetin, taxifolin, gallic acid, and astragalin were isolated from leaves.[11]

C. polygonoides has been quoted in the *Red Data Book* of the International Union for Conservation of Nature and Natural Resources as an endangered plant species.[12] Plant tissue culture is found to be an attractive alternative approach to traditional methods of propagation as it offers a controlled supply of biochemical, independent of plant availability. Apart from open field cultivation hindrances, cell suspension culture systems could be used for large-scale culturing of plant cells from which secondary metabolites could be extracted, so it can ultimately provide a continuous, reliable source of natural products.[13] Furthermore, elicitation is one of the most important approaches to enhance the yield of secondary metabolites produced by *in vitro* cultures.[14] Previous attempts for studying phenolic production from tissue culture of *C. polygonoides* shoot were fruitful;[15] however, no report was available concerning the accumulation of phenolic constituents by root culture. Therefore, achieving a protocol for *in vitro* establishment of root culture and subsequent cell cultures from the fruit explants will definitely assist investigations of phytoconstituents of such valuable plant species. Thus, the objectives of this study were to establish *in vitro* root culture, callus to be used in cell suspension cultures from *in vitro* germinated plantlets using the fruit as an explant, investigate the production of phenolics through *in vitro* root and cell

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suspension cultures, and attempt to enhance cell capacity to accumulate phenolics using salicylic acid and yeast extract.

Materials and Methods

Plant materials

C. polygonoides fruits were collected from western desert, Egypt, on April 2012 during flowering season and kindly authenticated by Dr. Abdelhalim Mohamed (Plant Taxonomy Department, Agricultural Research Center, Cairo, Egypt) for whom we are thankful. Voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University under the registration number BUPD-40. The fruits were surface sterilized by dipping in a solution of commercial Clorox (The Clorox Co., Oakland, California, USA) (5%) for 5 min followed by H_2O_2 for 15 min.[15] The sterilized fruits were germinated aseptically on quarter-strength Murashige and Skoog (MS) basal liquid medium,[16] supplemented with 30 g/L sucrose and 7 g/L agar. The pH of the medium was adjusted to 5.7. The fruits were kept at 27°C ± 2°C in the dark.

Establishment of root, callus, and cell suspension cultures

C. polygonoides roots obtained from 14-day-old *in vitro* germinated plantlets were cultured in liquid MS media supplemented with 1 mg/L indole-3-butyric acid (IBA) in the dark at 25°C on 100 rpm rotary shaker then subcultured regularly every 3 weeks under the same conditions. Segments (1 cm long) obtained from 14-day-old root were cultured on the same liquid MS media to obtain callus. Cell suspension culture was established from 30-day-old callus. About 1 g callus was added to 100-mL Erlenmeyer flasks containing 30 mL full strength of the same liquid MS medium and incubated at 27°C ± 2°C in the dark on a rotary shaker at 100 rpm. Cells were subcultured on the same media under the aforementioned conditions every 3 weeks.

Growth time course for cell suspension culture

Growth ratio of the cultured cells was assessed by harvesting cells at specified times (7, 14, 21, and 28 days) after inoculation. Final fresh weight of the harvested cells was determined, and the growth ratio was calculated according to the following equation:

Growth ratio = final fresh weight/initial fresh weight

Treatment of cell suspension cultures with elicitors

Two grams fresh weight cells were inoculated into 100-mL Erlenmeyer flasks containing 30-mL liquid MS medium. The 7-day-old cultured cells were separately treated for 48 h with filter sterilized aqueous solution of crude yeast extract (Sigma-Aldrich, St. Louis, Missouri, United States) (50 and 100 µg/mL) or salicylic acid (100 and 200 µg/mL). Control received equivalent volumes of solvent.

Phenolic extraction and high-performance liquid chromatography analysis

In vitro root and cell suspension cultures were separately harvested. The cells of suspension cultures were separated from the medium by filtration. All cultures were separately extracted with 75% aqueous methanol (3 × 10 mL) at room temperature and filtered. Each filtrate was separately pooled and evaporated to dryness. One gram of each dried extract was separately dissolved in 1 mL methanol (high-performance liquid chromatography [HPLC] grade, Sigma-Aldrich), clarified using Millipore filters (0.22 µm) and subjected to HPLC analysis to quantify their phenolic content. The used HPLC (Agilent 1260 Infinity) instrument was equipped with an Agilent 1260 Infinity Preparative Pump (G1361A), Agilent 1260 Diode Array Detector VL (G1315D), Agilent 1260 Infinity Thermostatted Column Compartment (G1361A), and Agilent 1260 Infinity Autosampler (G2260A). Separation and quantitation were performed on a ZORBAX Eclipse Plus C_8 Analytical Column (Agilent, Santa Clara, USA) (250 × 4.6 mm ID, 5 µm particle size). An aliquot of 50 µL was injected. Phenolics (Sigma-Aldrich) were quantified at 275 nm using peak area by comparing to a calibration curve derived from commercially available standards (taxifolin, isoquercitrin, astragalin, quercetin, and kaempferol). Ambient temperature was used. Elution was carried out at a flow rate of 1 mL/min using water:formic acid (99.97:0.03, v/v) as solvent A and methanol:formic acid (99.97:0.03, v/v) as solvent B in a gradient mode as following: 0–5 min with 80% A, 5–20 min with 80%–50% A, 20–25 min with 50%–20% A, and 25–30 min with 20%–0% A.

Results

Establishment of root, callus, and cell suspension cultures

Fruits of *C. polygonoides* showed an excellent ratio of germination (100%) on quarter strength MS media after treatment with Clorox (5%) for 5 min followed by H_2O_2 for 15 min [Figure 1A]. The roots of the *in vitro* germinated plantlets [Figure 1B] showed fast growth when cultured in MS liquid media supplemented with 1 mg/L IBA [Figure 1C]. Callus produced from root segments was very friable, white to off-white in color [Figure 1D]. Growth parameters of cell cultures in liquid MS medium are shown in Figure 2. It showed high growth ratios with an exponential phase between 7th and 14th day of culture. The growth ratios were almost constant after 21 days of inoculation.

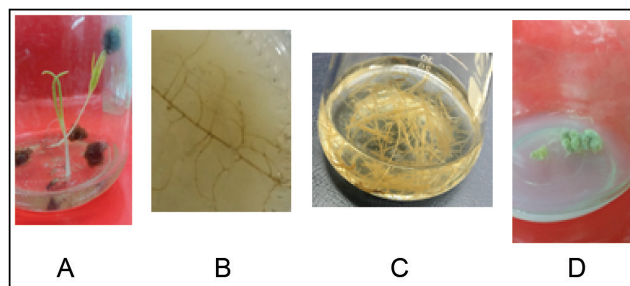

Figure 1: (A) *In vitro* germinated *Calligonum polygonoides* L. fruits. (B) Root from the *in vitro* germinated seedling. (C) Root culture. (D) Callus induced from the *in vitro* root segments

Production of phenolics and high-performance liquid chromatography analysis

The capacity of root and cell suspension cultures of *C. polygonoides* to produce secondary metabolites was investigated by determining their phenolic content using HPLC. Levels and profiles of phenolics varied significantly with respect to differentiation state of the cultures [Table 1]. The unorganized callus and cell suspension cultures contained less amounts of all phenolic compounds than differentiated tissues of *in vitro* roots. Astragalin and kaempferol were the major compounds detected in roots, whereas astragalin, kaempferol, and taxifolin were the majors in case of callus. Quercetin was not detected in both callus and cell suspension cultures, whereas the latter did not accumulate isoquercetin.

Effect of elicitors on the production of phenolics

Cell suspension cultures of *C. polygonoides* undergo quantitative reprogramming in response to elicitation with salicylic acid (100 and 200 µg/mL) and yeast extracts (50 and 100 µg/mL), added to 7-day-old cultures for 48 h [Table 1]. Salicylic acid and yeast extracts induced the accumulation of isoquercitrin and quercetin, which was not detected primarily in the untreated control. Incubation of the cells with salicylic acid, at a concentration of 100 µg/mL, produced an increase in taxifolin, astragalin, and kaempferol levels by approximately 2.7-, 1.1-, and 1.5-fold, respectively, whereas an increase of approximately 4.2-, 1.3-, and 2.4-fold, respectively, was observed when 200 µg/mL salicylic acid was used in comparison to that of control cells. On the contrary, incubation of the cells with crude yeast extract, at a concentration of 50 µg/mL, increased taxifolin, astragalin, and kaempferol levels by approximately 1.0-, 1.0-, and 1.4-fold, respectively, whereas an increase of approximately 3.0-, 1.6-, and 2.2-fold, respectively, was observed when 100 µg/mL crude yeast extract was used in comparison to that of control cells. Extensive browning and considerable loss of viability were detected in all yeast extract cultures after 48 h. This browning of cells as well as the culture medium is a common feature observed after treating cell suspension cultures with fungal elicitors.[15]

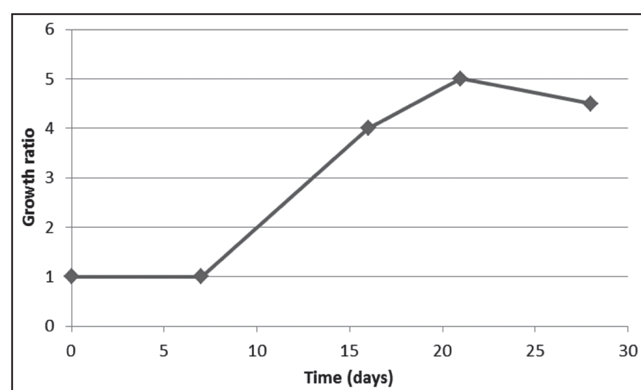

Figure 2: Time growth course of *Calligonum polygonoides* L. cultured cells cultivated in Murashige and Skoog (MS) liquid medium supplemented with 1 mg/L indole-3-butyric acid. Mean values ± standard error

Discussion and Conclusion

This study provides a strategy to get benefit from one of the valuable medicinal plant species, *C. polygonoides*, through the application of *in vitro* culture techniques using the fruit as an initial explant. Root was successfully established from *in vitro* germinated plantlets. Callus when cultivated in MS liquid medium produced cell suspension culture, which showed stable growth and accumulated phenolic constituents. This phenolic content was higher when the cells were organized as roots. Our results were in agreement with previous studies where the production of secondary metabolites was higher in differentiated structures than in non-differentiated cells.[15,17,18] The expression of secondary metabolic pathways in redifferentiated cultures is actually not surprising because it mimics exactly what the plant does. However, the inconvenience of manipulating plants or parts of them and their relatively slow growth remain the main disadvantages of this plant tissue culture system.[19] To overcome these problems, a great effort has been directed toward increasing secondary metabolite production from cell culture using various techniques such as elicitation.

Elicitation is an effective strategy to enhance the production of secondary metabolites such as alkaloids, terpenoids,

Table 1: High-performance liquid chromatography determination of phenolic content in different *in vitro* cultures of *Calligonum polygonoides* L.

Tissue type		Compounds (in µg/g) dry extract				
		Taxifolin	Isoquercitrin	Astragalin	Quercetin	Kaempferol
Root		181.36 ± 4.6	436.81 ± 1.5	704.98 ± 0.9	391.05 ± 5.7	708.25 ± 4.6
Callus		80.2 ± 1.1	39.4 ± 0.9	115.5 ± 0.7	n.d.	91.2 ± 3.2
Cell suspension		29.56 ± 3.2	n.d.	79.1 ± 5.4	n.d.	54.2 ± 5.7
Cell suspension treated with (in µg/mL)						
Salicylic acid	100	79.82 ± 3.1	37.14 ± 6.1	85.74 ± 2.2	38.54 ± 2.8	83.44 ± 5.4
	200	125.44 ± 3.1	96.44 ± 2.6	106.35 ± 4.1	82.02 ± 2.5	128.36 ± 3.5
Yeast	50	30.04 ± 0.5	43.09 ± 0.8	80.15 ± 3.5	n.d.	76.17 ± 2.5
	100	88.54 ± 8.0	102.13 ± 6.3	124.66 ± 4.0	56.05 ± 0.2	120.61 ± 1.3

n.d. = not detected

Values were expressed as mean ± standard deviation in triplicates at 275 nm

The content of all flavonoids is expressed as µg% w/w quercetin equivalent, except taxifolin calculated in µg% w/w of taxifolin equivalent

flavonoids, and phenolic compounds.[20] Cell suspension cultures are preferred due to its rapid growth cycles. They have been used for generating large amounts of cells for quantitative or qualitative analysis of growth responses and metabolism of novel chemicals.[21] It can ultimately provide a continuous, reliable source of natural products.[13] This was in agreement with our results where the capacity of cell suspension culture to accumulate phenolics was enhanced after application of salicylic acid and yeast extract, thus providing a chance to improve yield.

In conclusion, this study with *C. polygonoides* is another example for the power of tissue culture technique to offer an alternative and renewable source for secondary metabolites from a valuable medicinal plant and provide a good chance to improve such secondary metabolite yield, which is mandatory for using biotechnological methods instead of field crops as a basic source of this important pharmaceutical raw material. Our results are reported for the first time.

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Conflicts of interest

There are no conflicts of interest.

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