Saponins Production in Shoot and Callus Cultures of Gypsophila Paniculata

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Abstract: Shoot tips of G. paniculata were cultured on MS-medium contained 0.5 mg/l each of NAA and BAP for establishment of shoot cultures. The same explants were cultured on MS-medium supplemented with 1.5 mg/l each of 2,4-D and kinetin to initiated callus cultures. Both cultures were grown on different culture media with different concentrations of growth regulators. Callus growth was high when grown on medium contained 1.5 mg/l 2,4-D. Saponins content was determined by HPLC analysis. The results indicated that the highest content of saponins in shoot cultures was observed when shoots grown on medium contained 5 mg/l IAA. However, saponins of callus tissue grown on medium contained 1.5 mg/l each of 2,4-D and kinetin showed the highest content of saponins.

Key Word: Caryophyllaceae, Gypsophila paniculata, triterpenoidal saponins, gypsogene 3, O-glucuronide, 2,4-D, kinetin.

INTRODUCTION

Gypsophila paniculata L. (baby's breath) is a perennial plant belonging to the family Caryophyllaceae. It is an herb which can reach 1.5 m in height, the most important part being underground. The roots are ramified with long, thick branches. The upper shoot is erect, with leaves reduced to an enlarged and flattened petiole. It has white or pink flowers, which are composed of a short calyx split almost to the bottom. Flowering occurs in July and August[2].

Gypsophila paniculata saponins have been used for a long time under the generic name of saponin. They have the typical properties of saponins, such as detergent, emulsive, hemolytic, and membrane-toxic substances[3].

Gypsophila paniculata is well known to contain gypsogene 3, O-glucuronide (Fig. 1) with various application of commercial interest. Saponin pure white (Merck) has also been widely employed as a standard for hemolytic tests in most saponin determinations, and was previously reported to be extracted from roots and rhi zomes of G. paniculata[7]. They are used for the composition of photosensitive surfaces in photography, and of shampoos[8]. More recently, these saponins were described as being apparently responsible for spermicidal and anti-inflammatory effects[1,12].

Tissue culture was studied as a mean of rapid clonal multiplication of Gypsophila paniculata. In vitro propagation of G. paniculata from shoot tip explants of cv. Perfecta and cv Fairy Bristol and Red Sea was investigated[17].

However, callus tissues were induced from leaf segment of G. paniculata cv. Perfecta[17].

The aim of the study is to focus on tissue culture of Gypsophila paniculata producing triterpenoidal saponins as a good model to study the biosynthetic behavior of triterpenoidal saponins, to detect the differences in biosynthetic expression between callus and multiple shoot line.

MATERIALS AND METHODS

Plant Material, Sterilization and Culture Medium: Gypsophila; (Gypsophila paniculata L.) mature plants were kindly provided by Floriculture Department –
Faculty of Agriculture – Cairo University. Shoot tips were washed by running tap water for half hour. Under sterile conditions of laminar flow hood, explants were surface sterilized by 70% Ethanol for 1 min, followed by 20% commercial clorox (contained 5.25% sodium hypochlorite) for 20 min. After three successive rinses in sterile distilled water, shoot tips (about 0.25 cm in length) excised and placed in glass tubes containing 20 ml of MS-medium[10] supplemented with 3% sucrose, 100 mg/l inositol, 0.7% agar and contained 0.5 mg/l each of α-naphthalenacetic acid (NAA) and 6-benzylaminopurine (BAP) to establish shoot cultures. The pH of the media was adjusted to 5.8 using 1 N of either NaOH or HCL, prior autoclaving for 20 min at 121°C.

**Establishment of Gypsophila Shoots Cultures:** Shoot cultures were incubated in a growth chamber at 25 ± 2°C under a 16-h photoperiod (irradiance of about 40 µmol m⁻² s⁻¹ provided by cool white fluorescent lamps). After elongation of the shoot cultures (within 30 days), the axillary buds developed and formed new shoots which excised and sub cultured each on the same proliferation fresh medium at 4-week intervals up to three months (Fig 2). The proliferated shoots were transferred to different culture MS-media as shown in Table (1). After two months in that media shoots were collected and subjected to extraction and isolation of saponins.

**Initiation of Callus Cultures:** Shoot tip explants were cultured on callus initiation MS-medium supplemented with 1.5 mg/l each of 2,4-D (2,4-dichlorophenoxyacetic acid) and kinetin (6-furfurylamino purine). Sufficient callus growth was achieved after one month of cultivation. The proliferated calli were routinely sub cultured by refreshing the initiation medium after 30 days up to three months. Callus tissues were transferred to MS-medium containing different concentrations of 2,4-D and / or kinetin. After two months in that media callus tissues were collected and subjected to extraction and isolation of saponins.

**Extraction and Isolation of Gypsogenin 3-O-glucuronide from Callus and Shoots Explants of Gypsophila paniculata:** Saponins were extracted from the dried, ground proliferated shoots and callus tissues. The extraction was done separately three times, first with pure methanol and then twice with methanol/water (MeOH: H₂O) (20: 80 v/v) for 24 hrs each time at 25°C. After filtering, the combined methanolic extracts of each sample was concentrated under vacuum. The residue was suspended in water and extracted with ethyl acetate (EtOAc) (2×25 ml). The water solution is subjected to further purification with a single and rapid purification step on a Sep-Pack C₁₈ reversed phase cartridge. The aqueous extract, adjusted to pH 2-3 with acetic acid, then passed through the cartridge, which was then rinsed with methanol: water (60:40 v/v) containing 0.1% of acetic acid. The saponins were then eluted by 2 ml of methanol and were then ready for injection into the HPLC[4]. Ten micro liters was injected on to the column. Gypsogenin-3-O-glucuronide was purchased from Sarsyntex (Mergignac, France). High performance liquid chromatography (HPLC) was performed using a Perkin Elmer series 200 apparatus. Separations were performed on Phenomenex C18 Column (250 × 4.6 mm i.d, Luna). The flow rate of the mobile phase was 1ml min⁻¹. Saponins were detected using a UV variable wavelength detector (Waters®) at λ= 210 nm. The gradient elution of mobile phase consists of acetonitrile: water containing 0.1% trifluoroacetic acid (TFA) was as follows.

**Table 1:** Levels and ratios of growth regulator (mg / l) of media tested.

<table>
<thead>
<tr>
<th>Growth regulators</th>
<th>Media code</th>
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<tr>
<td></td>
<td>MS1</td>
</tr>
<tr>
<td>NAA</td>
<td>0.5</td>
</tr>
<tr>
<td>BA</td>
<td>0</td>
</tr>
<tr>
<td>IAA</td>
<td>0</td>
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<tr>
<td>IBA</td>
<td>0</td>
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</tbody>
</table>
Time | Acetonitrile water (0.1% TFA).
---|---
0.0 | 20% 80%
25 min | 80% 20%

Retention time of authentic sample of gypsogenin-3-O-glucuronide is 9.55 min. Calculation of total saponins was done by peak area calculations.

**RESULTS AND DISCUSSIONS**

**Saponins Accumulation in G. paniculata Shoot Cultures:** HPLC analysis of saponins content of shoot cultures grown in different culture media (Fig. 3) showed that the highest content of saponins (1.6 mg/100g dry weight) was observed when shoot cultures grown on medium contained 5 mg/l IAA (MS8) followed by medium contained 0.5 mg/l each of NAA and BA (MS3) where the saponins content was 1.452 mg/100g dry weight. On the other hand growing shoot cultures on medium contained 0.5 mg/l NAA and 1.0 mg/l BA (MS4) gave the lowest content of saponins 0.01 mg/100g dry weight.

From the above results it could be observed that, shoot cultures can produce saponins in all media used. The content of saponins was differed between cultures according to the growth regulators used and their concentrations. In this respect, Pauthe-Dayde et al.,\(^{[11]}\) studied saponins production in *in vitro* cultures of four species of Gypsophila and reported that cultures of *G. paniculata* accumulated up to 1.5 mg of gypsogenin 3,0-glucuronide per g/DW, whereas the three other species showed a smaller production or lost the ability to produce it after one year of subculture. They also, added that cells of *G. paniculata* are quite different from that of mature plant in which only the roots contain gypsogenin-saponins. Also, Henry et al.,\(^{[4]}\) reported that roots of *G. paniculata* are being the only biosynthetic site in the whole vegetative plant which produces gypsogenin-saponins. Recently, Rady and Nazief,\(^{[13]}\) studied rosmarinic acid content of the *in vitro* produced *Ocimum americanum* plants grown on different culture media. They found that MS medium with BA at a concentration of 1 mg/l and 0.25 mg/l IAA supports maximum rosmarinic acid production in plants produced from cultures grown on that medium.

**Effect of Growth Regulators on G. paniculata Callus Growth:** Effect of various concentrations of 2,4-D, kinetin, in the culture media were tested for their growth promoting activities (as fresh and dry weights) in shoot-tips derived callus cultures of *G. paniculata* grown in that media for two months (Fig.4). It was observed that shoot-tips derived callus gave the highest value of fresh weight (8.10 g.) when grown in medium contained 1.5 mg/l 2,4-D (MS12). Minimum growth of shoot-tips derived callus was observed when callus tissue grown in medium contained 1.5 mg/l Kin (MS13) or medium without growth regulators (MS 10).

The dry weights of *G. paniculata* tissues were closely paralleled the fresh weight responses; the callus tissue had higher dry weights (0.32 g) when grown in medium contained 1.5 mg/l 2,4-D + 1.5 mg/l Kin; (MS 11). The lowest dry weight (0.15 g) was recorded with medium contained 1.5 mg/l Kin; (MS 13). The effect of growth regulators on callus growth of different plant species was studied in several research reports. In this respect, Tamura et al.,\(^{[14]}\) optimized conditions needed for growth of *Thymus vulgaris* callus cultures. They found that 1 mg / l NAA or 2,4-D and 1 mg/l kinetin with 3 % sucrose and 0.9 % agar on MS-
medium at 25°C and 2000 lux were the best condition for callus growth. Youssef et al. found that raising levels of kinetin to that of 2,4-D in the culture medium reduced fresh weights of all types of Nigella sativa calli. Recently, Kapchina-Toleva, and Stoyanova studied the effect of cytokinins and cytokinin antagonists on in vitro cultured Gypsophila paniculata L. and found that the application of Kin and N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) stimulated bud opening and increased fresh and dry masses. Cytokinin antagonists reduced the number of sprouted buds and bud fresh and dry masses.

**Saponins Accumulation in G. paniculata Callus Cultures:** From HPLC analysis, it is clear however that, the saponins production in G. paniculata callus cultures is influenced by different growth regulators levels in the culture media Fig. (5). Saponins of callus tissue grown on medium contained 1.5 mg/l each of 2,4-D and Kinetin (MS11) showed the highest content (0.87 mg/100g dry weight) of saponins, whereas the lowest content (0.128 mg/100g dry weight) was recorded when callus grown on medium contained 1.5 mg/l Kinetin (MS13). However, no accumulation of saponins in callus tissue grown in medium contained only 2,4-D (MS12).

In this respect, different authors studied the effect of growth regulators on the accumulation of secondary metabolites in various species. Mohagheghzadeh et al. reported that in vitro cultures of Zataria multiflora biosynthesized rosmarinic acid (55-355 mg/100g dry wt.) and the highest accumulation were reached on MT (Modified Tobacco medium) containing 2 mg/l NAA. Generally, it could be concluded that the presence of auxin and cytokinin in the culture medium is very vital for accumulation of saponins in callus tissue of G. paniculata. Presence of auxin or cytokinin alone in the medium had negative effect in saponins content of callus tissues.

In conclusion, G. paniculata was shown to synthesize gypsogenin saponins, both in undifferentiated callus and multiple shoot cultures which initiated from shoot tip explants. The biosynthetic behavior of both cultures was different. Such differing biosynthetic behavior may be a model for comparative studies on the regulation of saponins biosynthesis, to enhance saponins production in vitro. The presence of gypsogenin saponins in both callus and shoot cultures is surprising as, in the vegetative state of the mature plant, only the roots contain gypsogenin saponins (7.5 mg/g dry weight in 1 year-old plants to 40 mg/g dry weight in 4-year-old plants, and other parts of the plant are not able to produce any gypsogenin saponins). In our study we found gypsogenin saponins in both callus and shoot cultures which prove that in vitro culture can contribute in producing saponins from G. paniculata. It could be suggested that selection of cell lines, addition of precursors to the medium and the change of growth regulators levels in the culture medium may be more effective for enhancing the saponins production in G. paniculata shoots or callus cultures.

**REFERENCES**


