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Methyl jasmonate and salicylic acid synergism enhances phloroglucinol content in shoot cultures of *Astragalus fruticosus* Forssk

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Introduction: *Astragalus fruticosus* Forssk. is a plant species with potential for producing valuable secondary metabolites, such as phloroglucinol, which has pharmaceutical applications. *In vitro* techniques, including multiple shoot induction and elicitation, offer promising methods to enhance the production of such compounds. This study investigates the use of signal molecules methyl jasmonate (MJ) and salicylic acid (SA) to increase phloroglucinol yield in cultured shoots of *A. fruticosus*.

Methods: Stem and leaf explants of *A. fruticosus* were cultured on Murashige and Skoog (MS) medium supplemented with 1 mg/L 6-benzylaminopurine (BAP) for over 8 weeks to induce multiple shoots. Elicitation was performed by treating the cultured shoots with MJ and SA at concentrations of 50 and 100 µg/mL, individually and in combination (final concentration of 50 µg/mL), over 1–3 weeks. Phloroglucinol content and biomass production were measured to assess the efficacy of the elicitation treatments.

Results: Elicitation with MJ at 100 µg/mL resulted in a 5.06-fold increase in phloroglucinol content after 3 weeks compared to the unelicited control. Other treatments with MJ, SA, and their combination also enhanced phloroglucinol production, though to a lesser extent. The highest biomass production was observed under the same conditions that maximized phloroglucinol yield.

Discussion: This study represents the first report of elicitation in *A. fruticosus* multiple shoot cultures using MJ and SA to enhance phloroglucinol production. The significant increase in phloroglucinol content, particularly with 100 µg/mL MJ, highlights the effectiveness of elicitation as a yield enhancement strategy. These findings suggest potential for optimizing in vitro protocols for large-scale production of phloroglucinol, offering valuable insights for biotechnological applications in pharmaceutical development.

KEYWORDS

methyl jasmonate, phloroglucinol, salicylic acid, *Astragalus fruticosus*, biomass

Introduction

Astragalus fruticosus Forssk (family Leguminosae) is a rare perennial herb and is one of the 37 species wild growing in Egypt (Salehi et al., 2021). *Astragalus* is famed for its medicinal applications, with hepatoprotective, antioxidative, immunostimulant, antiperspirant, diuretic, tonic, and antiviral properties (Li et al., 2014; Pistelli, 2002), which have been attributed to its secondary metabolites including triterpenes, saponins, phenolics, flavonoids, and polysaccharides (Lysiuk and Darmohray, 2016).

Phloroglucinol is a major phenolic compound in *A. fruticosus* Forssk. It displays many pharmacological activities with anticancer, anti-inflammatory, anti-allergic, anti-microbial, neuro-regenerative, vasodilating, and antioxidant activities besides its use in cosmetics, pesticides, paints, cements, and dyeing. Moreover, phloroglucinol and its derivatives could serve as artificial sweeteners, plasma substitutes, and in vitamin preparations (Singh et al., 2009; Li et al., 2011). Worldwide, there is an increasing demand for plant-based medicines, especially in primary healthcare (Zaheer and Giri, 2015). The field cultivation of *A. fruticosus* Forssk faces challenges such as long growth time and the uncontrollable production of valuable secondary metabolites. Therefore, an alternative method for the more efficient production of phenolic compounds such as phloroglucinol from *A. fruticosus* Forssk is urgently required.

Plant cell, tissue, and organ culture has recently been successfully used for the sustainable production of bioactive compounds of commercial interest (Loyola-Vargas and Ochoa-Alejo, 2018). *In vitro* shoot cultures can provide an alternative source to natural plant populations for the large-scale production of secondary metabolites. However, to the best of our knowledge, there are no reports on the production of phloroglucinol from the shoot cultures of *A. fruticosus* Forssk. Little is known so far about the strategies for biomass production and secondary metabolites stimulation using elicitors.

Elicitation is a process of creating a scenario of artificial pathogen attack using different means to promote secondary metabolite production (Sidhu, 2011). Elicitors are external stimuli that cause changes in plant cells, leading to a series of reactions that result in the accumulation of secondary metabolites (Largia et al., 2015). Among the commonly used elicitors are methyl jasmonate (MJ) and salicylic acid (SA), which are effective biotechnological tools for the induction of secondary metabolites in plant cultures (Sivanandhan et al., 2013). Methyl jasmonate, a volatile methyl ester of jasmonic acid, is involved in the signal transduction pathway which triggers specific enzymes that catalyze different biochemical reactions in plants to synthesize low-molecular weight defense compounds such as terpenoids, alkaloids, polyphenols, and quinones (Bai et al., 2025). Salicylic acid is a signal molecule that induces plant resistance to stress factors and pathogens through the stimulation of gene expression related to the biosynthesis of secondary metabolites in plants (Gadzovska et al., 2012). Recently, multiple shoot culture systems have been used for elicitation studies and for the production of secondary metabolites.

In the current study, we implemented some elicitation strategies to increase the amount of phloroglucinol *in vitro*. We likewise studied the different factors such as the elicitor concentration, exposure time, and harvest time in MS medium supplemented with a specific plant growth regulator for optimized biomass production and the enhanced accumulation of phloroglucinol using the two elicitors, MJ and SA, either individually or in combination.

Materials and methods

Induction and maintenance of multiple shoots

Multiple shoot cultures of *A. fruticosus* were induced using explants from the stem and leaf of 28-day-old seedlings that were obtained as we have previously described (Zayed et al., 2022). Full strength MS medium supplemented with 30 g/L sucrose and amended with various concentrations of plant growth regulators were used: 0.5 mg/L of 6-benzylaminopurine (BAP), named "Medium I"; 1.0 mg/L of 6-benzylaminopurine (BAP) named "Medium II:; 0.5 mg/L of thidiazuron (TDZ) named "Medium III"; 1.0 mg/L of kinetin (kn) named "Medium IV: a combination of 1 mg/L of naphthalene acetic acid (NAA) and 0.1 mg/L of BAP named "Medium V". The pH of the medium was adjusted to 5.6–5.8 using 1N NaOH or 1N HCl, then 0.6% agar was added. The media were autoclaved at 121 °C for 15 min, and the culture jars were incubated at 25 °C under a white fluorescent lamp over a 16-/8-h light/dark period. All cultures were sub-cultured to fresh media after 28 days of culture initiation. The most appropriate medium for shoot induction was selected by determining the number of explants that produced shoots and the number of produced shoots per explant after the third subculture. The experiments were repeated three times and data of respective experiments were recorded.

Elicitation of induced shoots by signal compounds

One-month-old healthy shoots (3–4 cm long) grown on MS medium supplemented with 1 mg/L BAP (Medium II) were cut and individually transferred into jars containing sterilized semi-solid MS media consisting of 4.4 g/l MS containing 30 g/L sucrose, 4 g/L agar, and 1 mg/L BAP. The pH of the medium was adjusted to 5.6–5.8. Methyl jasmonate (MJ) and salicylic acid (SA) elicitors were used to evaluate their influence on phloroglucinol accumulation in multiple shoot cultures of *A. fruticosus*. MJ and SA (Sigma- Aldrich, United States of America) stock solutions were prepared in 99.9% ethanol and filter-sterilized using a 0.22-µm bacterial filtration unit (Millipore, Ireland). The multiple shoot cluster was allowed to grow in the presence of various concentrations (50 and 100 µg/mL) of MJ, SA, and a combination of both (50 µg/mL) at different durations (1–3 weeks). A small hole (1 cm in diameter) was made in the sterilized semi-solid MS media around the cultured shoots, where different concentrations (50 and 100 µg/mL) of MJ and SA were aseptically added either separately or combined at concentration of 50 µg/mL of both elicitors on the first day of subculture. The cultures were incubated at 25 °C under a white fluorescent lamp with a 16-/8-h light/dark period then harvested at intervals of 1, 2, and 3 weeks to study growth (number of produced shoots and their dry weight) and phloroglucinol production. All treatments were performed in triplicate. Control experiments were prepared by substituting the elicitor with distilled water.

TABLE 1 Shoot induction percentage and the number of produced shoots per explant after the third subculture in different media.

Medium	% Of explants giving shoots	Number of shoots /Explant
Medium I	63.4%	8–15
Medium II	75.8%	10–25
Medium III	58.9%	5–8
Medium IV	44.7%	3–7
Medium V	37.6%	4–9

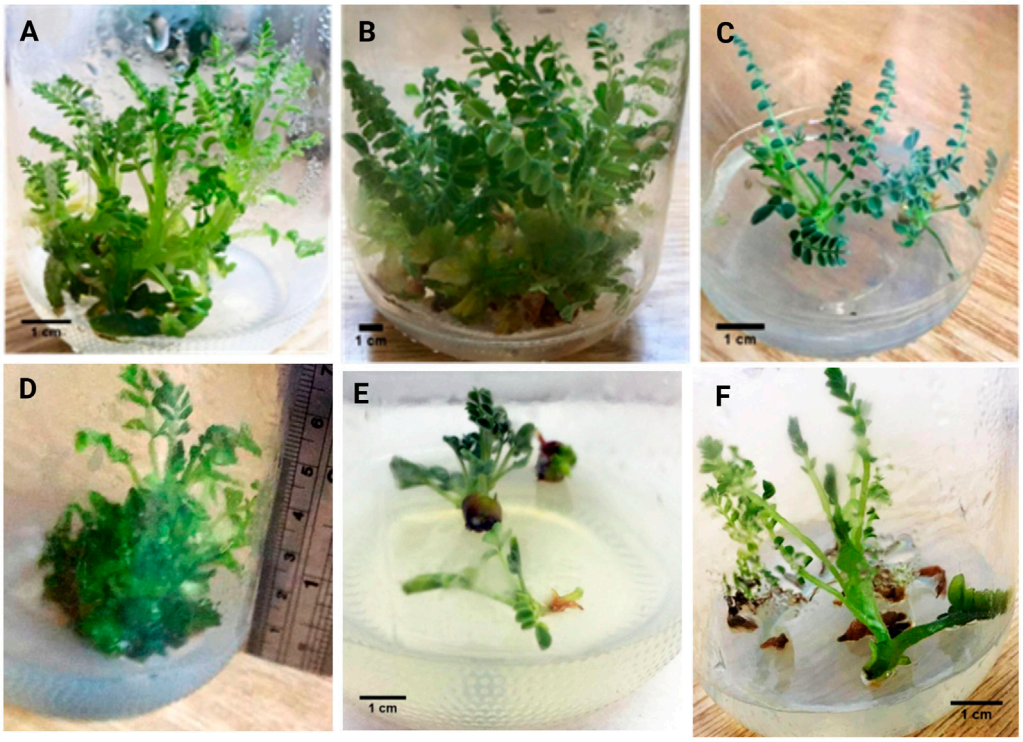

FIGURE 1
Shoot induction in different media: **(A)** 3-month-old shoots in Medium I; **(B)** 3-month-old shoots in Medium II; **(C)** 3-month-old shoots in Medium III; **(D)** 3-month-old shoots in Medium IV; **(E)** 3-week-old shoots in Medium V; **(F)** 6-week-old plantlet in hormonal-free medium.

Growth study for multiple shoot cultures

The growth of the control (untreated) and treated shoot cultures was assessed by recording the mean number of shoots branched after 1, 2, and 3 weeks. Shoot biomass was dried at room temperature and dry weight was recorded.

Extraction and quantitative analysis of phloroglucinol

All elicited and non-elicited shoots were removed from the culture media after 1, 2, and 3 weeks and then washed thoroughly, allowed to dry, and powdered separately. Each dry powder was macerated overnight with the same volume (10 mL) of high-performance liquid chromatography (HPLC)-grade methanol and filtered using Whatman filter paper No. 41. The resulting extracts were concentrated using a rotary evaporator under reduced pressure. The recovered solutions were filtered through a 0.45-µM membrane (MillexHV, Millipore, Ireland) then injected into a HPLC system (Agilent Technology, G1315D) equipped with an autosampler and an Eclipse Plus-C18 column (150 mm × 4.60 mm, 3.5 µm particle size). Isocratic elution was employed with methanol: acetonitrile:water (25: 35: 40, v/v/v) as a mobile phase at 1.0 mL/min flow rate with 20 µL injection volume. The column was maintained at 25 °C. The elution of phloroglucinol was monitored at 256 nm using a photodiode array detector (PDA) at a retention time of 0.9 min. Commercially available authentic standard phlorogluinol (98% purity) was procured from Sigma-Aldrich (United States of America) for analysis. All results were averaged over two consecutive experiments. Phlorogluinol content was expressed as % DW of control and elicited multiple shoot culture samples. The relative

TABLE 2 Effect of different concentrations of elicitors and their combination on the mean number of produced shoots in shoot cultures of *A. fruticosus*.

Elicitor conc (μg/mL)	Mean number of produced shoots		
	After 1 week	After 2 weeks	After 3 weeks
Control	10.67	17.33	24.67
SA 50	8.67	15.33	22.67
SA 100	4.33	10.67	13.33
MJ 50	5.33	9.67	12.67
MJ 100	3.67	7.67	9.33
MJ 50 + SA 50	2.67	6.33	8.33

concentrations of phloroglucinol in different samples were calculated by comparing their peak areas with standard curve generated using different concentrations (1, 2, 3, 4, 5, 6, 7, 8, and 9 μg/mL) of phloroglucinol standard. Phloroglucinol data were expressed as mg/g dry weight. Each sample was run in triplicate.

Results and discussion

Effect of phytohormones on induction of multiple shoot cultures

Cultivation of stem and leaf explants of 28-day-old sterile seedlings on media I-V resulted in the swelling of the explants as an initial response within 8–10 days of incubation. The green protuberances differentiated into direct shoots within 3 weeks. The highest percentage of explants giving shoot (75.8%) was observed in Medium II, with approximately 10–25 shoot/explants with an average length range of 3–8 cm after the third subculture (Table 1; Figure 1). Hasancebi et al. (2011) reported that BAP was the most commonly used cytokinin for the *in vitro* culture of different *Astragalus* species. According to Hill et al. (2015), explant tissues produce a greater amount of undifferentiated mass of cells, which results in viable embryos having the ability to differentiate into healthy shoots in the presence of higher concentrations of cytokinins. Culturing the stem and leaf explants of sterile seedlings on a hormonal-free MS medium resulted in the induction of shoot buds from one cut ending after 2 weeks that gave multiple shoots 3–5 cm long within the 6th week. The other cut ending showed an induction of fine and very tiny white rootlets with average length of 0.1–0.2 cm which showed no further growth (Figure 1F).

Influence of signal compounds (MJ and SA) on growth and phloroglucinol production

Effect of elicitors on shoot culture and biomass accumulation

The effect of MJ and SA, alone or in combination, on *A. fruticosus* shoot growth rates was monitored by determining the mean number of shoots produced and the resultant dry weight. The applied elicitors showed different influences on the biomass accumulation of shoots in culture in addition to the mean number of produced shoots. MJ and SA were added on the first day of the first subculture at different concentrations (50–100 μg/mL) on different exposure times (1–3 weeks).

Table 2 shows the mean number of shoots produced in samples treated with MJ and SA compared to the non-treated ones at different time intervals. The growth of *A. fruticosus* shoots was highly affected by the addition of elicitors. The applied concentrations (50–100 μg/mL) of both elicitors and their combination at a concentration of 50 μg/mL showed various inhibitory actions on shoot growth compared to control. Higher concentrations of SA and MJ displayed more toxic effects on the plant and limited the growth of its shoot. Therefore, the production of shoots was inversely proportional to the elicitor concentration. Additionally, it was noted that SA is less toxic than MJ, which is in accordance with Largia et al. (2015), who used SA and MJ at concentrations similar to those described in our study; they similarly reported that SA was less toxic to the plant than MJ. High MJ concentrations showed inhibitory action on cell growth due to an inhibition of the biosynthesis of photosynthetic pigments resulting in the suppression of the photosynthesis process (Ji et al., 2019).

Elicitors affected the dry weight of the shoots in a manner similar to the mean number of growing shoots, where the elicitors' inhibitory effect was concentration-dependent. Table 3 presents the biomass production after shoot treatment with SA and/or MJ. The biomass of MJ-elicited shoots was dramatically reduced after 3 weeks of treatment at a concentration of 100 μg/mL (2.26 g) compared to control (4.34 g). Biomass reduction was minimal in SA-treated cultures after treatment with a concentration of 50 μg/mL (3.72 g) relative to control (4.34 g). A combination of both elicitors at a concentration of 50 μg/mL showed a synergistic action that caused the highest biomass reduction (2.03 g) relative to control (4.34 g). Similar findings on *Withania somnifera* were likewise reported where elicitation of multiple shoot culture using SA and/or MJ resulted in various effects on biomass reduction. Similarly, SA-treated cultures did not show much variation in biomass accumulation compared with control (Sivanandhan et al., 2013). Previous studies on the effect of jasmonic acid and SA elicitors on biomass production in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum* showed effects similar to those observed in the present study (Coste et al., 2011). A high concentration of SA has been reported to inhibit the multiple shoot culture of *Andrographis paniculata* (Zaheer and Giri, 2015).

TABLE 3 Effect of different concentrations of elicitors and their combination on biomass accumulation in shoot cultures of *A. fruticosus*.

Elicitor conc (µg/mL)	Dry weight (g)		
	After 1 week	After 2 weeks	After 3 weeks
Control	1.63	2.43	4.34
SA 50	1.28	2.05	3.72
SA 100	0.82	1.74	2.84
MJ 50	0.98	1.85	3.19
MJ 100	0.61	1.46	2.26
MJ 50 + SA 50	0.58	1.31	2.03

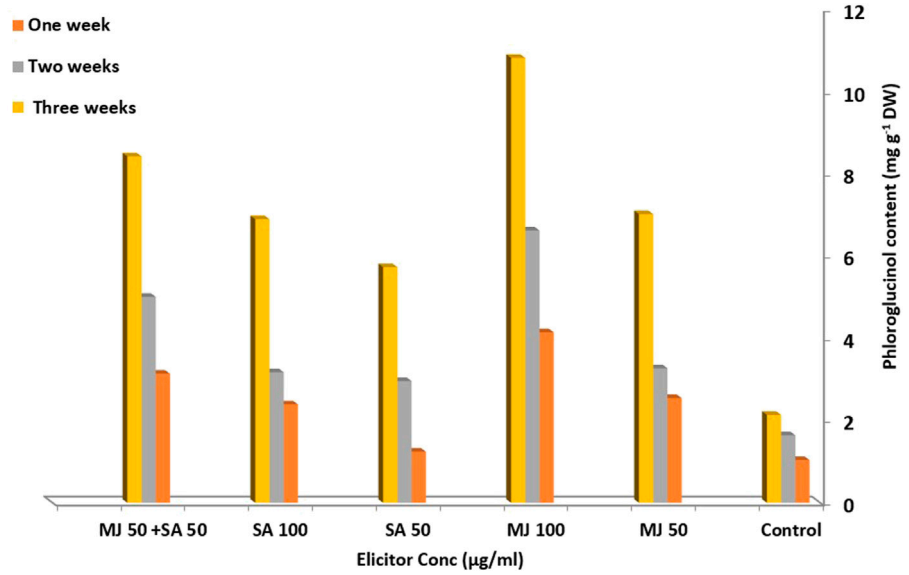

FIGURE 2
Effect of different concentrations of elicitors and their combination on phloroglucinol content in *A. fruticosus* shoot cultures.

Effect of elicitors on phloroglucinol content

Both elicitors (at the studied concentrations) exhibited changeable responses on phloroglucinol content in multiple shoot cultures of *A. fruticosus*. The phloroglucinol content of the untreated shoots showed limited increase over time. Meanwhile, there were different degrees of phloroglucinol enhancement when both elicitors and their combination were applied (Figure 2).

Elicitation by SA (50 µg/mL) showed the least phloroglucinol production enhancement, around 2.7-fold of the control level (2.13 mg/g DW) after 3 weeks. After increasing the SA concentration to 100 µg/mL, phloroglucinol production raised to 3.2-fold compared to control level after 3 weeks. This was in accordance with Beygi et al. (2021) who showed that trigonelline biosynthesis was enhanced in fenugreek callus culture after treatment with 100 µM SA. In contrast, another study revealed that the addition of 50 µg/mL SA to shoot cultures of *Hypericum hirsutum* and *H. maculatum* induced the significant accumulation of hypericin, while the addition of SA at a concentration of 100 µg/mL led to decrease in hypericin production (Coste et al., 2011). MJ highly enhanced phloroglucinol production, and the response depended on the concentration of MJ where 50 µg/mL MJ exhibited a 3.29-fold increase in phloroglucinol content after 3 weeks compared to control. Increasing the concentration of MJ to 100 µg/mL increased the phloroglucinol content to 5.06-fold compared to control. This result was in accordance with previous studies which reported that MJ at concentration of 100 µmol/L showed significant enhancement in the accumulation of polysaccharides in the multiple shoot culture of *Codonopsis pilosula* (Ji et al., 2019). The optimum concentration for the stimulation of secondary metabolites from *in vitro* cultured plant species was 100 µM (Shabani et al., 2009; Zaheer and Giri, 2015). In *Peganum harmala* root cultures, MJ enhanced the production of carboline and quinoline alkaloids 5-fold and 7-8-fold in root and hairy root cultures, respectively, compared to the untreated control (Zayed and Wink, 2005). Additionally, MJ promoted the production of bacoside A, a triterpenoid saponin, from the

in vitro shoot cultures of *Bacopa monnieri* by 1.8-fold (compared to control) after 1 week (Sharma et al., 2013).

The application of both elicitors in combination at a concentration of 50 µg/mL showed an enhancement in phloroglucinol production after 3 weeks of exposure, with the highest induction effect at the third week (four-fold increase compared to control) as shown in Figure 2. It was reported that a combination of MJ and SA at various concentrations exhibited synergistic action for the intensified production of saponins from *Bacopa monnieri* (Largia et al., 2015). In the callus cultures of *Rosa hybrida*, the combination of both elicitors resulted in higher production of anthocyanins than in control (Ram et al., 2013). Overall, the results showed that the enhancement of phloroglucinol accumulation by elicitor treatment is dose-dependent and is accompanied by a notable suppression of biomass production. To the best of our knowledge, this is the first report on the enhanced production of phloroglucinol in multiple shoots of *A. fruticosus* by the influence of elicitors (MJ and SA).

Conclusion

The accumulation of phloroglucinol in multiple shoot culture of *Astragalus fruticosus* was influenced by the concentration of the elicitors (methyl jasmonate and salicylic acid) bringing about enhancement and variation. Our results showed that the application of signal molecules allowed the optimal production of phloroglucinol in multiple shoot cultures of *A. fruticosus*. Synthesis of secondary metabolites requires the plant tissue to perceive and react to various environmental signals in an interactive manner. The outcome of the present study may be exploited for further enhancement of phloroglucinol production through biotechnological interventions using molecular approaches.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding authors.

Author contributions

RZ: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. AE-S: Writing – original draft, Writing – review and editing. AE: Writing – original draft, Writing – review and editing. WI: Writing – original draft, Writing – review and editing. ME-S: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. SF: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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