

Enhancement of bioactive compound production in *Convolvulus arvensis* callus cultures using elicitors

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Abstract

Callus cultures offer a reproducible technique for increasing the production of secondary metabolites (SMs) in medicinal plants. *Convolvulus arvensis* as well as other wild plants are great source of bioactive compounds, but their production is inconsistent and insufficient. A potential approach to overcome this limitation and enhance specific phytochemicals is through elicitation *in-vitro*. This study aimed to investigate the impact of phenylalanine and salicylic acid (SA) as elicitors in production of bioactive compounds in *C. arvensis* callus cultures. Leaf explants of *C. arvensis* were cultured on MS (Murashige and Skoog) medium fortified with 0.3 mg/L Benzyl adenine (BA) and 2.5 mg/L 2,4-D to induct callus. The calli were then treated with different concentrations of 15 mg/L, 30 mg/L, 45 mg/L and 60 mg/L phenylalanine and 30 mg/L, 60 mg/L, 90 mg/L and 120 mg/L salicylic acid, separately. High-performance liquid chromatography (HPLC) data interpretation indicated that the untreated calli produce phenolic substances like chlorogenic acid, ferulic acid, rutin, quercetin and kaempferol, as well as alkaloids such as convolvine, convolamine, and wiansonine. The elicitors treatment significantly increased the secondary metabolites accumulation in the calli, 60 mg/L phenylalanine and 120 mg/L SA, accordantly showed the height level of secondary metabolite production. The findings of this research indicate the targeted elicitations with bioactive compounds can significantly increase the secondary metabolite production in *C. arvensis* callus, offering a potential strategy for enhance secondary metabolites in medicinal plants.

Keywords: alkaloids, callus, *Convolvulus arvensis*, elicitors, phenolics

Introduction

Convolvulus arvensis or field bindweed, is a hardy, rhizomatous perennial from the Convolvulaceae family that is normally grown in Europe and Asia. It is well-known for its specialized flowers, but it is also a well-known invasive weed in many areas, spreading quickly through deep roots and seeds. Since the 1730s, this species has been utilized in traditional medicine for many years (Alwan *et al.*, 2020). Because of their laxative, anti-spasmodic, anti-hemorrhagic, and anti-parasitic qualities, the aerial portions of *C. arvensis* have been used. It is also used to treat a number of skin diseases, such as furunculosis, dandruff, and spider bites, as well as jaundice and as a diuretic (Ali *et al.*, 2013). *Convolvulus* species are widely distributed worldwide and has long traditional use, which indicates their importance as a source of therapeutic compounds. *C. arvensis* morphologically include trailing/scrambling unarmed stems, petiolate

leaves with rounded or truncated bases, axillary flower cymes, prominent peduncles (**Figure 1**) (Carine & Robba, 2010). *Convolvulus* species contain many bioactive compounds like flavonoids, alkaloids, carbohydrates, phenolic acids, unsaturated sterols, resins, tannins, and proteins. Numerous biological roles, such as antibacterial, antioxidant, anticancer, and therapeutic properties are displayed by these compounds. As a result, each portion of the plant have pharmacological worth, and previous investigations have demonstrated that the *C. arvensis* is effective in traditional treatments (Salehi *et al.*, 2020).

Phenolic compounds that are most prevalent among the secondary metabolites found in the aerial parts of *C. arvensis* like quercetin, rutin, and kaempferol. These compounds have important anti-inflammatory, anticancer, and antioxidant properties (Lin *et al.*, 2006; Khare, 2015) and phenolic acids like, ferulic acid, caffeic acid and chlorogenic acid support the plant's anti-



Figure 1. Leaves of *Convolvulus arvensis* (collected from Al-mustansiriyah University Garden)

inflammatory, anti-cancer, antidiabetic, and antioxidant properties (Aoudia *et al.*, 2013; Kılıçkaya Selvi, 2025). *C. arvensis* is also contain alkaloids such as convolvine, convolamine, and wiansonine, which increases its medicinal importance. Thus, the plant became a choice for biotechnological research for targeted increment of the secondary metabolites production. Encountering the biotic and abiotic stresses, such as pathogen attack, wounding, and environmental stresses, plants naturally produce secondary metabolites (SMs). These SMs are essential for plant adaptation to the environment (Biswas *et al.*, 2022). Since long times, people have used these metabolites as a therapeutic agent in addition to their ecological contribution. Plant based SMs are still important for drug discovery in pharmaceutical sectors, for maximizing the production of specific SMs. The artificial environment like plant tissue culture techniques make it possible to manipulate the production of SMs. Callus, cell suspension, and organ cultures are frequently used to increase the output of bioactive compounds from plant sources (Fritz *et al.*, 2022). The use of elicitors that promote the accumulation of SMs has become a potential approach among other methods. For example, amino acids can act as elicitors to promote metabolite accumulation and as precursors in important metabolic pathways. In the phenylpropanoid pathway that produces flavonoids, lignins, and other phenolic compounds, starts with the aromatic amino acid phenylalanine (PA). It has been observed that supplementation PA to culture

media greatly increases the yield of advantageous SMs (Bashir *et al.*, 2018; Szewczyk *et al.*, 2023). Salicylic acid (SA), a naturally occurring phenolic phytohormone, is another well-studied elicitor used in plant tissue culture. SA regulates numerous physiological and biochemical processes, including seed germination, stomatal closure, pigment production, photosynthesis, ethylene production, enzymes, flower induction, nutrient intake, and overall plant growth (Ali, 2021). On the other hand, SA plays an important role in plant defense mechanism by triggering the biosynthesis of SMs. It has been reported that the SA treatment in tissue culture enhance the accumulation of phenolics, flavonoids, and other bioactive compounds different plant species. Controlled application and supplementation of elicitors in culture media can stimulate the SMs synthesis pathways which leads the higher yields than the untreated culture (Singh & Dwivedi, 2018). *C. arvensis* rich in phenolics and alkaloids which earns a special interest in enhancing the production of these bioactive substances. *In-vitro* callus culture is a promising system for controlled metabolite production, and the application of elicitors like phenylalanine or SA enhance the SMs yields. It has been reported that the elicitors are effectively regulate the metabolic pathways in plant but their specific roles are remained unexploited. That's why the objectives of the current research to investigate the effect of diverse concentrations of elicitors (phenylalanine and salicylic acid) in the accumulation of phenolic compounds and alkaloids in *C. arvensis* callus. Optimization of elicitor concentrations to enhance the SMs production may provide a foundation for future application in the field of pharmaceuticals and plant biotechnology which help in sustainable applications of medicinal plants in the production of medicinally important SMs.

Materials and Methods

Explant Collection and Preparation

Leaves of *C. arvensis* were collected from the Al-Mustansiriyah University Gardens, Baghdad, Iraq. The explants were initially rinsed under running tap water for 30 minutes to remove surface waste. After that, leaves were transferred to a laminar airflow cabinet, briefly immersed in 75% ethanol for 5 minutes, and washed with sterile distilled water (DW) for 5 minutes. Surface sterilization was then performed using 2% sodium MS medium (Taha *et al.*, 2020; Murashige & Skoog, 1962). For HPLC analysis of the mother plant, approximately 2 g of air-dried leaves were collected and stored for phenolic and alkaloid quantification.

Callus Induction

Leaf explants were dissected and cultured on MS media mixed with 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.3 mg/L benzyl adenine (BA). The inoculated cultures were then incubated in the dark condition at $25 \pm 1^\circ\text{C}$ for 30 days, with three replicates per treatment (Twaij, Taha, & Hasan, 2016). To obtain sufficient callus for subsequent experiments, sub-culture was done in every 21 days using the same callus-inducing medium.

Preparation and Application of Elicitors

To treat the calli with the elicitors, 300 mg of calli were transferred in the MS media fortified with different doses of phenylalanine (15 mg/L, 30 mg/L, 45 mg/L, and 60 mg/L) and 30 mg/L, 60 mg/L, 90 mg/L, and 120 mg/L SA separately. And then the treated samples were kept in dark condition at $25 \pm 1^\circ\text{C}$ for 30 days (Rahimi Khonakdari *et al.*, 2020).

Determination of Callus Fresh and Dry Weight

After treatment period, fresh weight (FW) of calli were weighted using precision balance. The calli were dried in an incubator at 68°C for 72 hours and calculated the dry weight (DW) (Twaij *et al.*, 2022).

Extraction and HPLC Analysis of Phenolics

For the measurement of phenolic compounds, 0.5 g of dried callus powder was added in 10 ml of methanol and water (80:20 ratio). Then the mixture was sonicated for proper mixing using a Branson sonifier (USA) at 60% duty cycle for 25 minutes at 25°C . After centrifugation at 7,500 rpm for 15 minutes, using a syringe filter (0.45 μm), the supernatant was filtered and further filtered using a 2.5 μm disposable filter. Finally, the samples were then preserved at 4°C temperature until further analysis. HPLC (High performance liquid chromatography) was conducted on a Phenomenex C-18 column (3 μm and 50×2.0 mm I.D.) system under a linear gradient program. Solvent-A was 0.1% phosphoric acid and solvent-B was acetonitrile: methanol (60:40, v/v) with phosphoric acid (0.1%). The gradient of solvent B ranged from 10% to 100% over 10 min maintaining the flow rate of 1.5 mL/min and detected at 280 nm wavelength. The analysis was conducted on a 10AV-LC system (Shimadzu) facilitated with a binary pump (10AV-LC) and UV vis Detector (SPD). Quantification was calculated using the equation:

$$\text{Conc. mg/mL} = \frac{\text{area of sample peak}}{\text{area of standard peak}} \times \text{conc. standard} \times \text{Dilution de factor}$$

Extraction and HPLC Analysis of Alkaloids

For the extraction and measurement of alkaloids, 0.5 g of callus from each treatment was homogenized in 3% H_2SO_4 for 2 hours at room temperature. Using the 2.5 μm filter paper, whole mixtures were filtered. After filtration, the filtrates were alkalinized to pH 9.5 using 25% NH_4OH . Alkaloids were then applied to Extrelut (Merck) columns and eluted with CH_2Cl_2 (6 mL per 1 g of Extrelut). The eluted fractions were evaporated to dry using the stream of liquid nitrogen. The mixtures were diluted in 1 mL HPLC-grade acetonitrile and analyzed under optimized HPLC conditions. Then the HPLC separation was conducted on a Phenomenex C-18 column (3 μm , 50×4.6 mm I.D.) with solvent-A (0.1% triethylamine in water, pH 3 adjusted with phosphoric acid) and solvent-B (HPLC-grade acetonitrile). A linear gradient from 0% to 100% B was applied over 5 minutes, at a flow rate of 1.4 mL/min, and UV detection was done at 254 nm at 30°C . Calculations were performed by calculating the peak areas of samples and compared with authentic standards.

Statistical Analysis

All of the experiments were performed randomly with three samples per treatment. The findings were evaluated using one-way ANOVA, and mean comparisons were calculated using Fisher's LSD test. Mean values were taken statistically significant at $P \leq 0.01$ and marked with different letters.

Results and Discussion

Effect of Phenylalanine on Callus Growth

The impact of phenylalanine on callus growth of *C. arvensis* is summarized in **Table 1**. Supplementation of MS medium with phenylalanine at different concentrations generally enhanced callus fresh weight (FW) and dry weight (DW) compared to the control. The maximum fresh weight (1884.3 mg) and dry weight (85.3 mg) were observed at 30 mg/L phenylalanine. Lower concentrations (15 and 45 mg/L) also increased biomass significantly, whereas the highest concentration (60 mg/L) reduced both FW (650.7 mg) and DW (45 mg) compared to the control (677.7 mg FW, 31.3 mg DW) which indicates that moderate concentrations of phenylalanine promote callus growth, while excessive levels may inhibit the accumulation of SMs in plants. (**Figure 2A**).

Salicylic Acid on Callus Growth

The impact of salicylic acid (SA) on callus biomass is presented in **Table 2**. Fresh weight increased at highest value (1288 mg) at 30 mg/L SA supplementation,

while other concentrations provided different responses. Dry weight did not impact significantly response to the SA treatments ($P > 0.01$). The findings indicate that SA moderately promotes biomass accumulation at reduced concentrations (Figure 2B).

Table 1. Effect of varying concentrations of PA on fresh weight (FW) and dry weight (DW) of *C. arvensis* callus after 30 days of incubation

Phenylalanine (mg/L)	FW (mg)	DW (mg)
Control	677.7 b	31.3 b
15	1222.3 ab	59.7 a
30	1884.3 a	85.3 a
45	1813.7 ab	76.0 a
60	650.7 b	45.0 b
LSD (0.01)	1167	27

Data were interpreted using one-way ANOVA, and mean value comparisons were analyzed using Fisher's LSD test. Mean value differences were taken significant at P value ≤ 0.01 and marked with different letters.

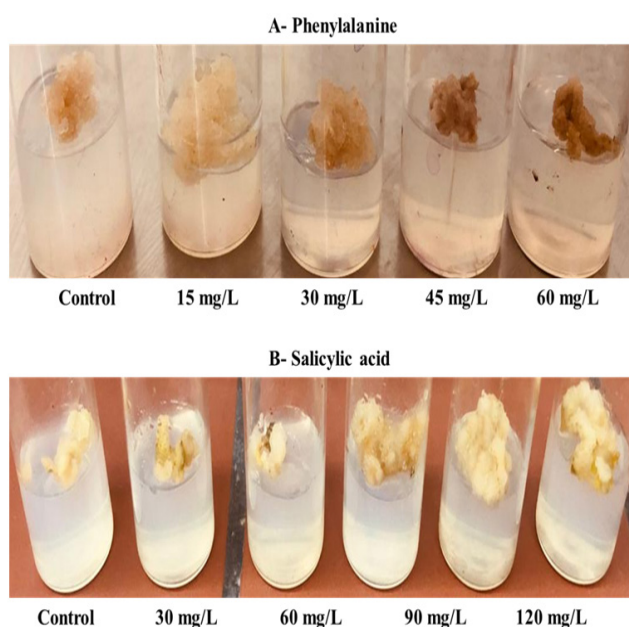


Figure 2. Different concentrations of A) phenylalanine (cont., 15, 30, 45, and 60) mg/l and B) salicylic acid (cont., 30, 60, 90, and 120 mg/L) were mixed to MS media to induce phenolic and alkaloid compounds in *Convolvulus arvensis* callus cultures after four weeks of incubation.

Table 2. Effect of varying concentrations of SA on fresh weight (FW) and dry weight (DW) of *C. arvensis* callus after 30 days of incubation

SA (mg/L)	FW (mg)	DW (mg)
Control	677.7 ab	31.3
30	1288.0 a	53.3
60	1106.3 ab	60.0
90	469.7 b	35.0
120	863.0 ab	46.0
LSD (0.01)	734	N.S.

Data were interpreted using one-way ANOVA, and mean value comparisons were analyzed using Fisher's LSD test. Mean value differences were taken significant at P value ≤ 0.01 and marked with different letters.

Impact of Phenylalanine on Phenolic Compound Accumulation

Table 3 shows the impact of phenylalanine on phenolic compound production in callus cultures of *C. arvensis*. All the measured phenolic compounds were increased significantly in response to the Phenylalanine supplementation on MS media compared to the control. Caffeic acid (4.01 mg/mL), chlorogenic acid (4.70 mg/mL), ferulic acid (2.95 mg/mL), rutin (5.98 mg/mL), quercetin (4.53 mg/mL), and kaempferol (4.03 mg/mL) were found at highest level with 60 mg/L phenylalanine supplementation. It has been also observed that lower concentrations increase the SMs accumulations in callus indicating its dose dependent response (Supp. Figure 1).

Effect of Phenylalanine on Alkaloid Production

Phenylalanine supplementation in MS medium also increased alkaloid production which is presented in **Table 4**. The highest concentrations of convolvine (1.68 mg/mL), convolamine (1.47 mg/mL), and wiansonine (2.39 mg/mL) were found at 60 mg/L phenylalanine supplementation which is significantly higher compared to the lower concentration and the controls (Supp. Figure 2).

Impact of Salicylic Acid on Phenolic and Alkaloid Accumulation

Tables 5(a) and **5(b)** represent the findings of SA treatment where SA significantly increased phenolic and alkaloid content in response to the treatment. The maximum accumulation of phenolic compounds was observed at 120 mg/L SA supplementation. Caffeic acid (5.14 mg/mL), chlorogenic acid (5.74 mg/mL), ferulic acid (3.22 mg/mL), rutin (6.56 mg/mL), quercetin (5.99 mg/mL), and kaempferol (4.53 mg/mL) all the measured compound increased sufficiently compared to the treatment. Again, alkaloid production increased at height level with supplementation of 120 mg/L SA: convolvine (1.84 mg/mL), convolamine (1.64 mg/mL), and wiansonine (2.95 mg/mL). The findings indicate that SA effectively stimulates secondary metabolite biosynthesis in callus cultures, particularly at higher concentrations.

In this research, effect of amino acids such as PA and SA on *C. arvensis* callus on the biomass development and the accumulation of bioactive compounds have been investigated. The findings demonstrate that supplementation of the amino acids (phenylalanine and salicylic acid) in culture media significantly increase the callus biomass and secondary metabolites. The increment of SMs was dose (concentration of amino acid)

Table 3. Effect of phenylalanine on phenolic compound accumulation in *C. arvensis* callus

Phenolic Compounds	Control	15	30	45	60	Mother Plant	LSD (0.01)
Caffeic Acid	1.47d	1.87d	2.79c	3.21b	4.01a	1.43e	0.41
Chlorogenic Acid	1.62e	2.21d	3.16c	3.73b	4.70a	1.74e	0.21
Ferulic Acid	1.47d	1.31d	1.83c	2.23b	2.95a	1.70d	0.30
Rutin	2.20d	2.75d	4.12c	4.92b	5.98a	2.58d	0.68
Quercetin	1.66c	2.16c	2.86b	4.11a	4.53a	1.90c	0.50
Kaempferol	1.54d	1.53d	2.28c	3.42b	4.03a	1.43 d	0.31

Data were interpreted following one-way ANOVA, and mean value comparisons were analyzed using Fisher's LSD (Least Significant Difference) test. Mean value differences were taken significant at P value ≤ 0.01 and marked with different letters.

Table 4. Effect of phenylalanine on alkaloid accumulation in *C. arvensis* callus

Alkaloids	Control	15	30	45	60	Mother Plant	LSD (0.01)
Convolvine	1.017 c	0.79d	1.08c	1.42b	1.68a	0.89 d	0.14
Convolamine	0.92 c	0.92c	1.16b	1.37a	1.47a	1.06 bc	0.15
Wiansonine	1.18 f	1.52e	1.95c	2.18b	2.39a	1.78 d	0.10

Data were interpreted following the one-way ANOVA, and mean value comparisons were analyzed using Fisher's LSD test. Mean value differences were taken significant at P value ≤ 0.01 and marked with different letters.

Table 5(a). Effect of SA on phenolic compounds in *C. arvensis* callus

Phenolic Compounds	Control	30	60	90	120	Mother Plant	LSD (0.01)
Caffeic Acid	2.24d	2.56cd	2.69c	3.29b	5.14a	1.43e	0.42
Chlorogenic Acid	1.61e	3.07d	3.58c	4.96b	5.74a	1.74e	0.34
Ferulic Acid	1.49e	1.93d	2.10c	2.52b	3.22a	1.70de	0.31
Rutin	2.44d	3.78c	4.85b	5.92a	6.56a	2.58d	0.71
Quercetin	1.93e	3.03d	4.07c	5.18b	5.99a	1.89e	0.43
Kaempferol	1.59e	2.29d	2.76c	3.70b	4.53a	1.43e	0.34

Data were interpreted using one-way ANOVA, and mean value comparisons were calculated using Fisher's LSD test. Mean value differences were taken significant at P value ≤ 0.01 and marked with different letters.

Table 5(b). Effect of SA on alkaloid accumulation in *C. arvensis* callus

Alkaloids	Control	30	60	90	120	Mother Plant	LSD (0.01)
Convolvine	1.017c	0.95c	1.27b	1.40b	1.84a	0.89c	0.25
Convolamine	0.91d	0.91d	1.17c	1.46b	1.64a	1.06c	0.13
Wiansonine	1.18d	1.78c	2.40b	2.79a	2.95a	1.78c	0.23

Data were interpreted using one-way ANOVA, and mean value comparisons were calculated using Fisher's LSD test. Mean value differences were taken significant at P value ≤ 0.01 and marked with different letters.

dependent. Supplementation of Murashige and Skoog (MS) medium with phenylalanine yielded increased fresh and dry weights in callus tissues of *C. arvensis*, with the most substantial accumulation found at a dose of 30 mg/L. In this concentration, the fresh weight reached at 1884.3 mg, and the dry weight was 85.3 mg, both were significantly higher than that of the controls (677.7 mg and 31.3 mg, respectively). Conversely, concentrations over 30 mg/L, especially at 60 mg/L, indicated in a reduction in both fresh and dry weights which demonstrate the presence of a potential inhibitory effect at elevated levels. These findings align with prior research that describe phenylalanine's capacity to enhance biomass accumulation in plant cultures (Szewczyk et al., 2021). On the other hand, the SA treatment also affected the callus growth, the highest FW (1288 mg) was observed at a dose of 30 mg/L. No significant changes in dry weight were found in the salicylic acid concentrations used in this study. Previous studies also indicated the similar results, where salicylic acid influenced callus growth (Sharifi et al., 2019). The supplementation of the PA at a dose of 60 mg/L led

to the maximum accumulation of phenolic compounds, encompassing caffeic acid, chlorogenic acid, ferulic acid, rutin, quercetin, and kaempferol. This particular concentration resulted in 4.01 mg/ml of caffeic acid, 4.70 mg/ml of chlorogenic acid, and other compounds at notable levels. The observations found in this research are similar with the existing research were indicated that the supplementation of phenylalanine in the medium stimulates the biosynthesis of phenolic compounds in plant cultures (Szewczyk et al., 2021; Autajamsripon et al., 2023). Similarly, the salicylic acid treatment at 120 mg/L showed the highest levels of phenolic compounds, with caffeic acid 5.14 mg/ml and chlorogenic acid 5.74 mg/ml. This indicates the role of salicylic acid as an elicitor in the stimulation of biosynthesis of SMs (Ali et al., 2021; Jeyasri et al., 2023; Mahalakshmi, Eganathan, & Parida, 2013; Shabbir et al., 2025; Golkar, Taghizadeh, & Yousefian, 2019; Ehsani, Talebi, & Tabatabaei, 2025; and Golkar, Moradi, & Garousi, 2019). The alkaloid accumulation in callus cultures was significantly enhanced by the treatments with phenylalanine and SA separately. Specifically,

phenylalanine at a concentration of 60 mg/L yielded 1.68 mg/ml of convolvine, 1.47 mg/ml of convolamine, and 2.39 mg/ml of wiansonine. Likewise, salicylic acid at 120 mg/L resulted in 1.84 mg/mL of convolvine, 1.64 mg/mL of convolamine, and 2.95 mg/mL of wiansonine. These results are corroborated with the previous findings that have indicated the potentiality of the phenylalanine and the salicylic acid in the stimulation of alkaloid production within plant callus cultures.

Conclusion

The current study reveals that both PA and SA act as potent elicitors, thereby increasing biomass and the synthesis of bioactive substances within *C. arvensis* callus cultures. Specifically, the admision of PA at the concentrations ranging from 30 to 60 mg/L stimulated callus proliferation and elevated the percentage of phenolic compounds and alkaloids. On the other hand, salicylic acid, at the dose of 120 mg/L, significantly increase the synthesis of the SMs, with a particular emphasis on phenolics and alkaloids. These results suggest that precursor feeding and elicitor application represent practical methodologies for optimizing the in vitro production of compounds with medicinal significance, thus offering a sustainable alternative to the collection of these substances from natural populations. Consequently, the study underscores the potential of tissue culture techniques, when coupled with elicitation, to maximize the yield of therapeutically valuable phytochemicals for pharmaceutical purposes.

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