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Original Research Article

In vitro alteration of artemisinin biosynthesis in *Artemisia annua* L during treatment with methyl jasmonate

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Abstract

Purpose: *To investigate the in vitro effect of methyl jasmonate (MeJA) on artemisinin biosynthesis in Artemisia annua.*
Method: *The effect of two concentrations of MeJA i.e. 2 µm (T2) and 5 µm (T5) on biomass, plant height and artemisinin content of Artemisia annua L. was investigated in vitro in MS medium.*
Results: *Plant height (6.5 ± 1.8 cm), biomass (0.18 ± 0.02 g/plant) and artemisinin content (0.035 ± 0.002 % dry weight) were higher in T5 and T2 treatment groups than in non-treated control plants (C). Artemisinin content, biomass and plant height were positively correlated after 2 - 8 days of treatment, but remained unchanged on the 12th day of treatment.*
Conclusion: *The observed effects of exogenous MeJA on the biosynthesis of artemisinin and other secondary metabolites may lead to elucidation of promising targets for further studies on metabolic engineering of Artemisia annua L.*

Keywords: *Artemisia annua L. Methyl jasmonate, HPLC, Artemisinin*

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INTRODUCTION

Artemisinin is extracted from the Chinese medicinal plant *Artemisia annua.* Due to artemisinin combined therapy (ACTs), the demand for artemisinin for malaria treatment has increased tremendously. However, the natural level of artemisinin in *A. annua* L. (0.01 – 1.0 %), the only known source of the drug, is too low to satisfy the huge demand for ACTs [1,2]. Different attempts have been made by different investigators to increase artemisinin biosynthesis in *A. annua* L. through various cutting-edge technologies such as biochemical and genetic engineering, breeding, as well as chemical synthesis [1-6]. Due to the complexicity of its synthesis in plants, attempts at artemisinin production either through genetic engineering or through breeding technology has not met with much success. Researchers all over the world have tried to elucidate and target the specific genes involved so as to regulate the down-stream genes responsible for artemisinin biosynthesis. Chemical synthesis of artemisinin is expensive due its complex nature.

Artemisinin accumulates in the leaves of *A. annua* L. at concentrations dependent on climatic

conditions, planting period and stage of harvesting [7-9]. Environmental stress is a very critical factor that results in lower yield and low biomass. Environmental stress affects the survival and growth of the plant, and also alters the flux of primary assimilation processes e.g. mineral uptake and indogenous level of plant growth regulators. *Artemisia annua* L. cultivated in low temperature regions is structurally diverse, with tightly regulated secondary metabolites.

Moreover, the accumulation of artemisinin in these plants is also directly or indirectly regulated by plant growth regulators. Salicylic acid (SA), jasmonate (JA) and abscisic acid (ABA) are well-known endogenous plant growth regulators that play major roles in plant signaling and also in the growth of *A. annua* L plant. Under biotic and abiotic stress conditions, these endogenous regulators trigger the production of cellular pool of metabolites [10]. One of these metabolites is jasmonate. It directly regulates secondary metabolism and enhances the cellular pool of active metabolites in many plant species. The effect of jasmonate on the production of vitamin C and terpenoids in *Cathranthus roeus* and tobacco plant has been reported earlier [10,11]. In the present study, the effect of exogenous methyl jasmonate on biomass, plant height and artemisinin content of *Artemisia annua* were investigated *in vitro*.

EXPERIMENTAL

Plant material and growth conditions

Seeds of *A. annua* (n = 100) were sown on MS basal medium containing sucrose (2 % w/v) [12] and agar (0.8 %) under 16 h/8 h light/dark cycle.

Methyl jasmonate (MeJA) treatment

When the seedlings reached a height of about 2 cm (after 15 days of germination), they were transplanted into another MS basal medium containing MeJA at two different concentrations T2 and T5 (2 and 5 µM, respectively) for 2, 4, 8, and 12 days. Three individual plants at each level of MeJA treatment (2 and 5 µM), and control (C) plants without MeJA treatment, were selected randomly for determination of plant height, biomass and artemisinin content.

Artemisinin extraction

Artemisinin estimation was carried out using the method of Zhao and Zeng [13]. The leaves of the treated (T2 and T5) and non-treated (C) plants were taken and dried to constant weight in an oven at 50 ^{o}C. Then, 100 mg of the weighed powder was added to an extraction bottle containing 20 mL of petroleum ether and extracted overnight. The residue was dissolved in 1mL methanol and centrifuged at 12000 rpm for 10 min to remove the undissolved components. The artemisinin extract (200 µL) was mixed with methanol (800 µL) and sodium hydroxide (0.2 %), and incubated for 30 min. The mixture was cooled at room tempreture and the reaction mixture (500 µL) was transferred to an Eppendorf tube containing methanol (100 µL) and 0.05 M acetic acid (400 µL). The resultant solution was subjected to filtration through a millipore filter (0.25 µm).

HPLC analysis of artemisinin

The concentration of artemisinin was analyzed from derivatized T2, T5 and C samples using HPLC (Waters Alliance 2695, USA). Artemisinin was quantified using reverse phase column (C18, 5 µm, 4.6 × 250 mm) with phosphate buffer i.e., 100 mM K-phosphate buffer (60:40; pH 6.5) at a constant flow rate of 1 mL/min. Absorbance was read at 260 nm under UV detector. The exact concentration of artemisinin was calculated with Waters HPLC software system from a calibration curve prepared with standard artemisinin solution.

Statistical analysis

The data were assessed in terms of the plant height, biomass and artemisinin content, and were measured in triplicate. The values were presented in each bar signifies ± SE (n = 3) using SPSS 16.0 statistical software.

RESULTS

In this study, the two concentrations of MeJA (2 and 5µm) positively regulated the height, biomass and artemisinin content of *A. annua* after 2 days of treatment. The two concentrations were selected randomly on the basis of previous studies, and were found to staisfactorily boost the artemisinin content of the plant.

The highest shoot length (6.51 ± 0.2 cm) was recorded in T5 plantlets on the 8th day of treatment with MeJA, relative to those of T2 treatment (4.6 ± 0.2 cm) and control (3.0 ± 0.18 cm). These values were 1.5 and 1.2 folds higher than that of control plantlets, respectively (Figure 1). Significant differences in shoot biomass accumulation per plant were observed in MeJA-treated *A. annua* L. plantlets after 2, 4, 8 and 12 days of treatment on MS basal medium. The maximum biomass (0.23 ± 0.02 g dw/plant) was recorded in the treatment T5, followed by T2

plants (0.18 ± 0.01 g dw/plant), when compared with non-treated *A. annua* L. plants (0.15 ± 0.002 g dw/plant) on 8th day of the treatment. These values were 1.5 and 1.2 folds higher than that of the non-treated *A. annua* L. plantlet, respectively. These results are shown in Figure 2. On day 8, the height and biomass obtained with 2µM MeJA (T2) were lower than corresponding values with T5 treatment, indicating that T2 was less effective than T5. This may be due to the positive regulation of MeJA on the biosynthesis and carbon flux of metabolites. It could be also due to the excess gene expression related to artemisinin biosynthesis.

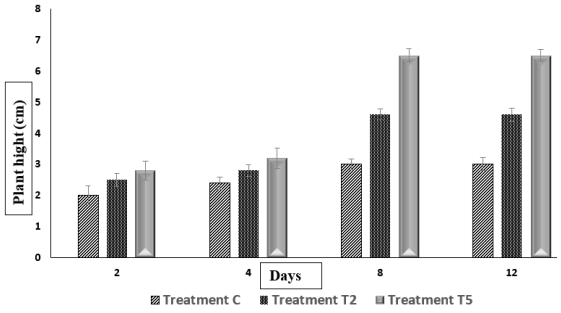

Figure 1: Effect of MeJA treatment on plant height (cm) on different days (2, 4, 8 and 12 days). Control, T2 (2µm) and T5 (5µm) treatments *in vitro*. Each value is mean± standard error (n = 3)

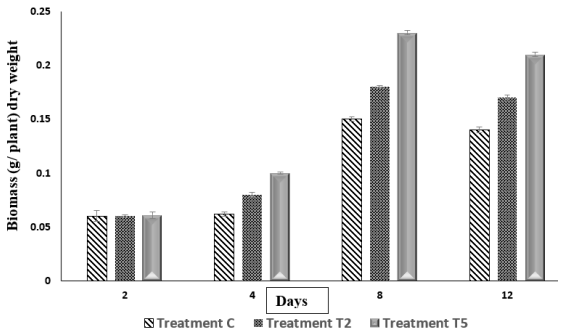

Figure 2: Effect of methyl jasmonate (MeJA) on biomass accumulation (g/plant) on different days (2, 4, 8 and 12 days). Control, T2 (2 µm) and T5 (5 µm) treatments *in vitro*. Each value is mean ± standard error (n = 3)

The content of artemisinin in *A. annua* was determined using HPLC. For each concentration of MeJA treatment, three randomly selected individual plants were analyzed. It was found that the plants treated with MeJA had higher contents of artemisinin (0.022 – 0.035 % on dry weight), when compared to the control (0.013 % dry weight basis). These results are shown in Figure 3 A - C. The highest average content of artemisinin (0.035 % dry weight), which was 2.5 times higher than the control value (0.014 %) was obtained in the T5 treatment (5 µM MeJA), followed by that from T2 treatment.

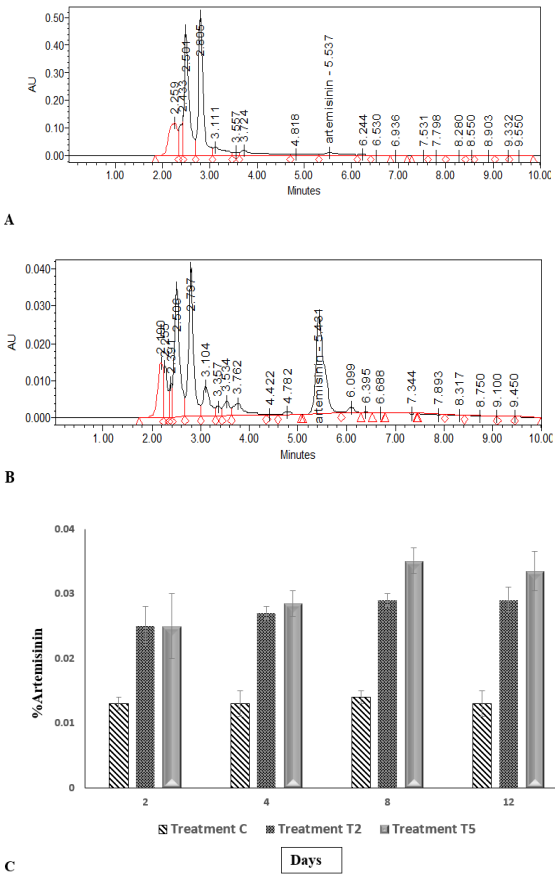

Figure 3: Artemisinin estimation through HPLC. (A). Artemisnin chromatogram of control (C) non-treated plant of *A. annua* L. after 8 days. (B). *Artemisia annua* plant exposed to 5µM MeJA after for 8 days showed highest concentration of artemisinin, relative to control. (C). Determination of artemisinin content on different days (2, 4, 8 and 12 days) *in vitro* under MeJA treatment. Control, T2 (2 µm) and T5 (5µm) treatments *in vitro*. Each value is mean± standard error (n = 3)

DISCUSSION

In this study, it was observed that plants exposed to methyl jasmonate (MeJA) showed higher concentration of artemisinin than the non-treated plants (C). These findings indicate that jasmonic acid is a good elicitor in that it induced increases in artemisinin content within a short time frame. Thus, the level of artemisnin produced with 5 µM jasmonic acid within 8 days was very significant, when compared to the control.

Enhancement of levels of bioactive compounds by many biotic and abiotic elicitors such as taxol, terpenoids, alkaloids, and phenylpropanoids production has been reported in different studies [14,15]. MeJA is a well-known signalosome that induces gene expression and affects

transcriptional repeats of pathways in plant cells, leading to enhanced concentration of bioactive compounds [16-19]. In the present study, the observed significant increase in artemisinin levels after the addition of 5µm MeJA indicates a rapid stimulation artemisinin production after 8th days of treatment. It may be due to substantial changes in, or increased fold gene expression by internal MeJA responsive factors present in *A. annua* L. at the transcriptional level. Similar findings were also reported in cell suspension culture of *A. annua* L. [20]

CONCLUSION

The findings of the study show that MeJA stress at *in vitro* level results in 2.5-fold enhancement of artemisinin production and positively affects the growth and development of *A. annua* plants after 8 days of treatment. This may be due to the positive regulation of the transcriptional factors on particular time by upregulation the enzymes in artemisinin biosynthetic pathway. Although different kinds of transcription factors have been reported by many investigators, these are not enough to boost the production of artemisinin on a commercial scale. Therefore, further studies are needed to understand the cellular mechanism of artemisinin biosynthesis and transcriptional factors' role in regulating the cellular pool of enzymes in artemisinin production.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. PA and TB designed the work, performed the experiments, and evaluated the data presented in this article.

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