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Effect of certain elicitors on production of pyrrolizidine alkaloids in hairy root cultures of *Echium rauwolfii*

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Hairy root cultures of *Echium rauwolfii* were obtained by infection of sterile apical shoots with *Agrobacterium rhizogenes*. The linear increase in fresh weight was found to be parallel to the alkaloids production. The transformed cultures were exposed to different elicitors, such as methyl jasmonate (MJ), quercetin and salicylic acid in order to increase their productivity. Pyrrolizidine alkaloids were quantitatively determined by HPLC. Estimation of total alkaloids was achieved by peak area calculations. MJ at a concentration of 100 μ M induced the accumulation of total alkaloids about 19-fold compared to the untreated control. The flavonoid quercetin (Q) at a concentration of 50 μ M enhanced the pyrrolizidine accumulation approximately 6-fold. The induction effect of both MJ and Q can be suppressed by pre-incubation of hairy root cultures with salicylic acid.

1. Introduction

About 40 species of the genus *Echium* (Family Boraginaceae) are known to be distributed in the Mediterranean region, southern Europe and western Asia (Feinbrun-Dothan 1978; Jafri and El-Gadi 1979). In Egypt the genus is represented by about 7 species (Taekholm 1974). *Echium rauwolfii* Del. is an erect annual herb with narrow leaves and a 12–15 mm long, white-or flesh-colored corolla.

The plant families Boraginaceae, Compositae and Leguminosae have been reported to contain pyrrolizidine alkaloids (Robins 1982; Mattocks 1986; Rizk 1990; Hartmann and Witte 1995). Alkaloid composition of *Echium rauwolfii* has been studied and their antimicrobial activity was analyzed (El-Shazly et al. 1999). In the present work we report the establishment of stable hairy root cultures of *Echium rauwolfii* via transformation with *Agrobacterium rhizogenes*. These transformed root cultures are valuable and versatile systems for the study of secondary metabolism. Furthermore, the effect of certain elicitors on production of pyrrolizidine alkaloids was studied.

2. Investigations, results and discussion

2.1. Alkaloid accumulation and growth curve

A linear increase in fresh weight was observed between week 1 and week 6 after inoculation of hairy roots of *E. rauwolfii* into fresh B5 medium. HPLC analysis of alkaloids content of hairy roots showed that alkaloid accumulation paralleled cell growth. The highest content of alkaloids was observed between week 2 and 3 (Fig. 1).

2.2. Effect of elicitors on alkaloid accumulation

Methyl jasmonate (MJ) and salicylic acid are involved in signal transduction and induce the transcription of biosynthetic

enzymes involved in the formation of defence compounds in plants (Baldwin 1999). Since MJ can induce the formation of secondary metabolites in other systems, we have tried to stimulate the accumulation of pyrrolizidine alkaloids in hairy root cultures of *E. rauwolfii* by adding MJ and salicylic acid (SA).

The hairy root cultures of *E. rauwolfii* (2 weeks old) were treated with MJ. After 24 h of incubation the alkaloids were extracted and determined quantitatively by HPLC. Estimation of total alkaloids was done by peak area calculations. MJ at a concentration of 100 μ M induced the accumulation of total alkaloids about 19-fold compared to the untreated control (Table 1). The flavonoid quercetin (Q) at a concentration of 50 μ M enhanced the pyrrolizidine accumulation approximately 6-fold (Table 1). The induction effect of both MJ and Q can be suppressed by pre-incubation of hairy root cultures with SA (Fig. 2). SA (100 μ M) was added to the culture 1 h before the addition of MJ and Q. Incubation of cells with salicylic acid alone at different concentrations decreased the content of pyrrolizidine alkaloids (Table 1). Treatment with EtOH had a negative effect on the accumulation of alkaloids.

Elicitation with MJ and Q showed a small positive effect on diffusion of pyrrolizidine alkaloids into the medium (Table 2). The most important effect was induced by MJ at a concentration of 100 μ M and by Q at a concentration of 50 μ M.

Although pyrrolizidine alkaloids are toxic compounds, some of them e.g. indicine N-oxide are being tested as antitumour drugs in human beings. Some are marketed commercially as fine chemicals for research purposes (Mattocks 1986; Hartmann and Witte 1995; Roeder 1995; Schmeller et al. 1997; Roberts and Wink 1998). In addition, alkaloid extracts of both *E. rauwolfii* and *E. horridum* exhibited antibacterial effects (El-Shazly et al. 1999). This encouraged us to improve their yields by elicitation via certain elicitors. Plant cell and organ cultures present several advantages in comparison to field-grown plants, such as independence from variations induced by climatic and seasonal

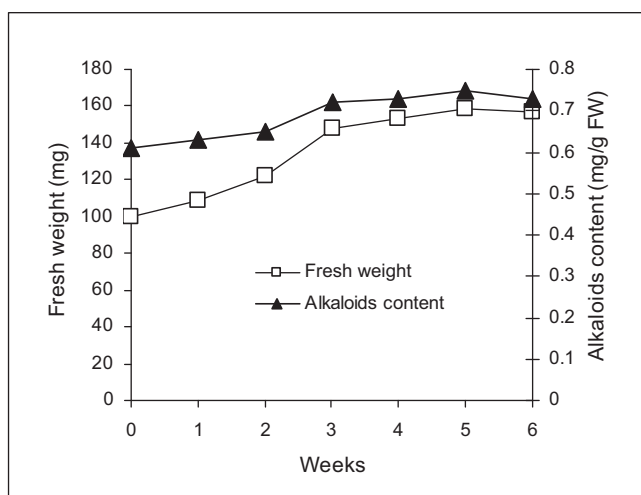


Fig. 1: Changes in fresh weight and alkaloids content of *E. rauwolfii* hairy root cultures. The data are man value of two independent experiments

Table 1: Pyrrolizidine alkaloids concentration in hairy root cultures of *E. rauwolfii* after incubation for 24 h with different concentrations of MJ, Q and SA

Elicitor concentration (μM)	Alkaloids content (mg/g fresh weight), ±SD		
	MJ	Q	SA
Control	0.69 ± 0.04	0.71 ± 0.03	0.68 ± 0.05
10	8.7 ± 0.38	0.54 ± 0.02	0.66 ± 0.06
20	10.1 ± 0.50	1.5 ± 0.04	0.031 ± 0.003
50	9.8 ± 0.49	4.3 ± 0.15	0.025 ± 0.002
100	13.26 ± 0.73	3.2 ± 0.11	0.029 ± 0.002
150	12.6 ± 0.64	1.1 ± 0.04	0.033 ± 0.004
200	10.3 ± 0.52	0.27 ± 0.01	0.041 ± 0.003

*The data are mean values of two independent experiments

changes and losses due to plagues. Also, they allow the possibility of working under aseptic conditions. Elicitors, which affect positively the accumulation of secondary metabolites, represent a valuable biotechnological strategy. For example, in large scale fermentation designed to obtain compounds from plant cultures, this approach could simplify and reduce the costs of downstream processes and at the same time allow biomass re-utilization.

3. Experimental

3.1. General procedure

TLC was performed on silica gel 60 F₂₅₄-coated aluminum sheets (Merck, Darmstadt, Germany). Preparative TLC was carried out on silica gel 60 F₂₅₄-

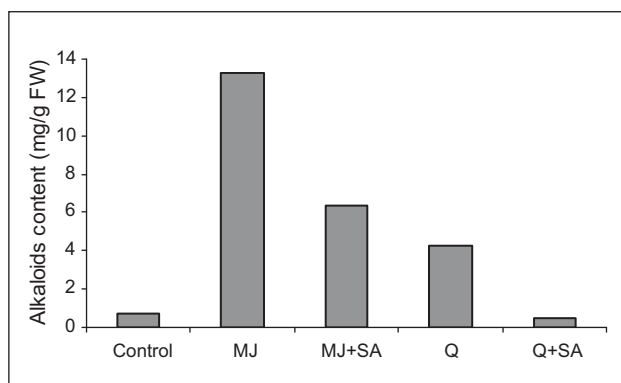


Fig. 2: Effect of salicylic acid on the elicitation of pyrrolizidine alkaloids by MJ and Q. The data are mean values of two independent experiments

Table 2: The concentration of pyrrolizidine alkaloids diffused into the medium of *E. rauwolfii* hairy root cultures after incubation for 24 h with different concentrations of MJ, Q and SA

Elicitor concentration (μM)	Alkaloids content (mg/g fresh weight), ±SD	
	MJ	Q
Control	0.023 ± 0.003	0.022 ± 0.001
10	0.059 ± 0.004	0.024 ± 0.002
20	0.13 ± 0.01	0.066 ± 0.003
50	0.33 ± 0.03	0.13 ± 0.01
100	0.56 ± 0.02	0.09 ± 0.01
150	0.41 ± 0.03	0.029 ± 0.002
200	0.22 ± 0.02	0.017 ± 0.001

*The data are mean values of two independent experiments

coated glass sheets (Merck, Darmstadt, Germany). Pyrrolizidine alkaloids were visualized by spraying with Dragendorff reagent (Fluka, Germany). EI-MS spectrum was carried out on JEOL JMS 600 Hz (Japan). HPLC analysis was carried on L-6200A intelligent pump and L-4000 UV detector (Merck, Germany).

3.2. Plant material

Echium rauwolfii plant was collected in the flowering stage from New vale region, Egypt. Identification of the plant was confirmed by Prof. Abdel Aziz Fayed, Faculty of Science, Assiut University, Assiut, Egypt.

3.3. Chemicals

All the media components were purchased from E.Merck (Darmstadt, Germany). The authentic echimidine alkaloid was obtained from the Pharmacognosy Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

3.4. Establishment and maintenance of hairy root cultures

Transformed roots were obtained by infection of sterile apical shoots of *E. rauwolfii* with *Agrobacterium rhizogenes* LBA 9402 according to a procedure reported previously (Pitta-Alvarez et al. 1995). The transformed roots were maintained in hormone-free B5 liquid-medium (Gamborg et al. 1969). The roots were subcultured every 2–3 weeks and incubated at 25 ± 2 °C on a gyratory shaker at 100 rpm in an illuminated culture room.

3.5. Growth curve

Ten flasks (100 ml) with 20 ml fresh B5 medium were prepared and inoculated with 100 mg of hairy roots of *E. rauwolfii* (week zero). The cultures were incubated as described above. Fresh weight (FW) was determined from week zero to week 6 at one week intervals. Hairy root cultures were harvested by vacuum filtration and weighed.

3.6. Effect of elicitors

Hairy roots (100 mg FW) were treated in 20 ml B5 medium by different inducers such as methyl jasmonate (MJ), quercetin (Q) and salicylic acid (SA) to stimulate the accumulation of pyrrolizidine alkaloids. Dose-dependent induction experiments were repeated with different concentrations (10, 50, 100, and 200 μM) of MJ in EtOH, Q in DMSO and SA in H₂O (Sigma, Germany). Hairy roots of *E. rauwolfii* in the exponential phase (2 weeks-old cultures) were exposed to the elicitors for 24 h. Hairy root cultures were harvested by vacuum filtration, weighed and kept at –20 °C until the extraction and analysis. Growth (FW) and alkaloids accumulation in the hairy roots were determined.

3.7. Alkaloid extraction, isolation and identification

Hairy roots (100 g) were homogenized in 10 M HCl, filtered, and the acid aqueous solution was then stirred with zinc dust overnight, filtered and extracted with CH₂Cl₂ to yield the total alkaloids (El-Shazly et al. 1999). The CH₂Cl₂ extract was evaporated to dryness. The residue was re-dissolved in 10 ml acidulated methanol for phytochemical study. TLC screening revealed 11 spots, one of them was major. It was isolated as colorless oily compound (12 mg, R_f 0.43) on a preparative TLC using CH₂Cl₂ - MeOH - NH₄OH (25%) (85: 15: 2 v/v) as a solving system. Its EI-MS spectrum

agreed with published data for echimidine alkaloid (Roeder et al. 1991; Sarg et al. 1992; El-Shazly et al. 1999). Furthermore, co-chromatography (TLC & HPLC) with authentic echimidine was performed.

3.8. HPLC analysis

One gram of hairy roots was extracted as mentioned above and the obtained residue was re-dissolved in 1 ml acidulated methanol and filtered. The filtrate was used for quantitative determination of the total pyrrolizidine alkaloids using HPLC (30 μ l was injected). This was performed on a reversed phase (styrene-divinylbenzene resin) column (150 $\times$ 4.1 mm, PRP-1, 10 μ m) using 0.1 M ammonium hydroxide (A) and acetonitrile (B) as solvents. The following gradient was employed: 10–30% (B) in 20 min. The flow rate was 1 ml/min and the detection wavelength set to 220 nm. Echimidine alkaloid (Fig. 3) in a concentration of 1 μ g/100 μ l was used as

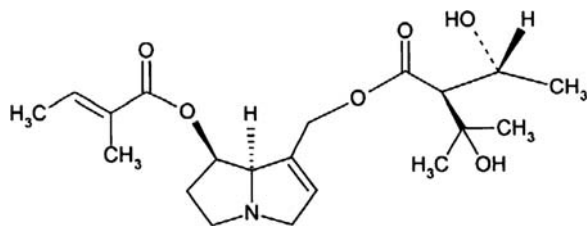


Fig. 3: Chemical structure of echimidine

a reference compound. The quantity of total pyrrolizidine alkaloid was estimated on the bases of their area with respective to the area of echimidine (0.3 μ g) as external standard.

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