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ORIGINAL PAPER

Elicitation of diosgenin production in *Trigonella foenum-graecum* L. seedlings by heavy metals and signaling molecules

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Abstract Seedlings of *Trigonella foenum-graecum* were treated with four heavy metal salts ($CdCl_2$, $CoCl_2$, $K_2Cr_2O_7$ and $NiCl_2$) to study the effect of heavy metals on growth and diosgenin production. It was found that $CdCl_2$ increased diosgenin production up to 40-fold and $CoCl_2$ increased diosgenin production up to 41-fold at concentrations which did not affect growth significantly. But $K_2Cr_2O_7$ and $NiCl_2$ were toxic to growth and inhibited diosgenin production. Effect of exogenously applied methyl jasmonate (MeJa) and calcium (Ca^{2+}) on diosgenin production in seedlings of *T. foenum-graecum* was also investigated. MeJa enhanced the production of diosgenin. Maximum increase (10.5-fold) was found at 100 μL L^{-1} concentration of MeJa. To study the role of Ca^{2+} on diosgenin production, seedlings of *T. foenum-graecum* were treated with a promoter of Ca^{2+} influx (calcium ionophore A23187), calcium depleted medium, Ca^{2+} channel blocker (verapamil) and antagonist ($LaCl_3$), a divalent cation chelator (EGTA) and modulator of calcium release (caffeine). All the treatments were compared with a control containing 220 mg L^{-1} concentration of $CaCl_2$. The results suggest that the increase in cytosolic Ca^{2+} has an inhibitory role on diosgenin production. However, a calcium chelator or Ca^{2+} channel inhibitors could be used to elicit diosgenin production in this plant.

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Introduction

Elicitation can be an important strategy towards improved production of plant secondary metabolites. A wide variety of substances (biotic and abiotic) are able to act as elicitors which can trigger the production of many secondary metabolites in plants. As the elicitation process is mediated through different signal transduction pathways, many workers have also used the signaling molecules as elicitors (De 2001). Methyl jasmonate (MeJa) and calcium (Ca^{2+}) are the two well-known compounds that affect the signal transduction involved in the elicitation process. Diosgenin is an important steroidal metabolite used as a starting material for the synthesis of steroidal drugs (Evans 1996). It has an estrogenic effect on mammary gland (Aradhana et al. 1992) and plays an important role in the control of cholesterol metabolism (Roman et al. 1995; Sauvaire et al. 1991). Methyl protodioscin, a potent agent with antitumour activity, has been synthesized from diosgenin (Cheng et al. 2003). *Trigonella foenum-graecum* is one of the several plant sources that produce diosgenin (Evans 1996). Seedlings of the plant are also reported to produce diosgenin (Hardman and Fazli 1972; Bhavsar et al. 1980; Ortuno et al. 1999). Previously we have studied the role of ethylene on diosgenin production in *T. foenum-graecum* seedlings (De and De 2003) and in *Dioscorea floribunda* (De and De 2005). In the present work, different concentrations of salts of heavy metals such as Cd, Co, Cr, Ni were used to study their effect on growth and diosgenin production in the seedlings of *T. foenum-graecum*. In an attempt to elicit diosgenin production we have also studied,

in this paper, the effect of exogenously applied MeJa on diosgenin production and the role of Ca^{2+} on the production of the same in *T. foenum-graecum* seedlings.

Materials and methods

Treatment of seedlings

Seeds of *T. foenum-graecum* were surface sterilized and were placed in sterile lidded glass vessels (12 × 6 cm) on the filter paper soaked with sterile water for germination. On day 2 the germinated seeds were treated with different concentrations of heavy metal salts such as $CdCl_2$, $CoCl_2$, $K_2Cr_2O_7$ and $NiCl_2$ after dissolving the compound in MS salt solution (1/2 strength) (Murashige and Skoog 1962). Thirty seedlings (2 days old) were placed on two layers of filter paper soaked with 6–7 ml of salt solution containing different concentrations of above-mentioned substances in each sterile lidded glass vessel and kept for another 5 days at 15–18°C under fluorescent lamp for 8/16 h light/dark period. Controls were exposed to 1/2 strength MS salt solution. After 5 days of treatment, treated and control seedlings were harvested for analysis.

For studying the role of calcium, the seedlings were treated with different concentrations of calcium salt, promoter (calcium ionophore A23187), inhibitor [verapamil, $LaCl_3$, Ethylene glycol bis (β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA)] and modulator (caffeine) of Ca^{2+} influx. Except methyl jasmonate, all other treatments were applied after dissolving the compounds in $^1/_2$ strength MS salt solution as described above. In case of MeJa treatment, germinated seeds were exposed to MeJa vapour by placing MeJa (which is a liquid at room temperature) dissolved in 100% ethanol onto the tip of a cotton swab. The cotton swab was kept in the glass vessel, but not in direct contact with the seedlings. Control set received 100% ethanol only. MeJa was added at concentrations of 50–1,000 µl L^{-1} (of glass vessel volume). After 5 days of treatment, treated and control seedlings were harvested for analysis. Every experiment was repeated in triplicate.

Growth measurement

Seven-day-old seedlings treated with metal salts were harvested and seed coats were removed. Fresh weight and seedling length were measured. The seedlings were then dried at 45°C for 48 h and dry weights were taken.

Analysis of diosgenin

Diosgenin was extracted following the method of Asolkar and Chadha (1979). Powdered dried seedling, 3 N HCl and hexane were refluxed in a glass apparatus on a magnetic stirrer with a hot plate for 2 h at 90–96°C. The mixture was allowed to cool and the aqueous phase was extracted three times with 25 ml hexane each time. The combined organic phase was washed with 1% $NaHCO_3$ solution and subsequently with distilled water and then evaporated to dryness. The extract was then analyzed by HPLC following the method of Indrayanta et al. (1994) optimized for our work conditions (De and De 2003).

Results and discussion

Effect of metal salt solutions

There are several reports where heavy metals elicited different secondary metabolite formation in some plant cell culture system. Manganese induced cardenolide production in *Digitalis thapsi* (Crochete et al. 1991) and *D. lanata* (Ohlsson and Berglund 1989). Copper induced berberine production in *Thallictrum rugosum* suspension culture (Kim et al. 1991), total indole alkaloid production in *Catharanthus roseus* cell culture (Backer-Royer et al. 1990), reserpine production in *Rauwolfia serpentina* culture (Solichatun and Anggarwulan 2008). Gaisser and Heide (1996) reported induction of acetyl shikonin and rosemarinic acid production by arsenate in *Lithospermum erythrorhizon* cell culture. Eu^{3+} increased total anthraquinone content in *Cassia obtusifolia* hairy root culture (Guo et al. 1998). Cobalt chloride induced L-Dopa production in *Stizolobium hassjoo* (Sung and Huang 2000). During the present study, toxic effect on growth of seedlings was observed 5 days after treatment with 500 µM concentration of $CdCl_2$. At this concentration dry and fresh weight remained unaffected, but the length of seedlings decreased significantly (Table 1). At 100 and 300 µM concentrations of $CdCl_2$ growth was unaffected. So these two concentrations showed no toxic effect on growth. In response to different concentrations of $CdCl_2$, diosgenin production increased. At 300 µM concentration of $CdCl_2$, maximum increase of diosgenin (40-fold) ($P < 0.001$) was observed (Fig. 1) without affecting the growth. In 100 and

Table 1 Effect of different concentrations of $CdCl_2$ on seedling growth (5 days after treatment)

Concentration of $CdCl_2$ (µM)	Fresh weight of 30 seedlings (g)	Dry weight of 30 seedlings (g)	Seedling length (mm)
0	1.4 ± 0.17	0.19 ± 0.01	25.54 ± 0.08
100	1.3 ± 0.14[b]	0.19 ± 0.04[b]	24.91 ± 0.51[b]
300	1.3 ± 0.12[b]	0.19 ± 0.04[b]	26.26 ± 0.67[b]
500	1.09 ± 0.11[b]	0.18 ± 0.02[b]	17.38 ± 0.67[a]

Data represent the mean value ± SD ($n = 3$); significance levels: [a] $P < 0.001$, [b] not significant

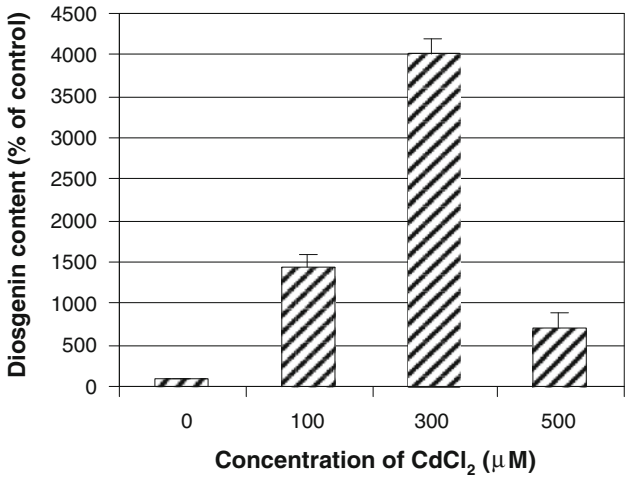

Fig. 1 Effect of $CdCl_2$ on diosgenin content

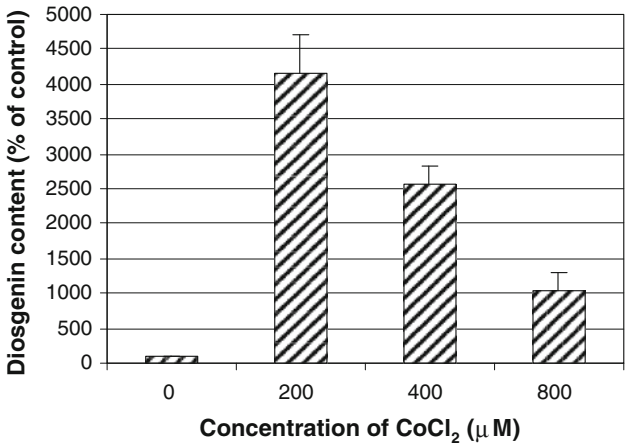

Fig. 2 Effect of $CoCl_2$ on diosgenin content

Table 2 Effect of different concentration of $CoCl_2$ on seedling growth (5 days after treatment)

Concentration of $CoCl_2$ (μM)	Fresh weight of 30 seedlings (g)	Dry weight of 30 seedlings (g)	Seedling length (mm)
0	1.7 ± 1.7	0.20 ± 0.005	21.48 ± 0.31
200	1.7 ± 0.06[b]	0.22 ± 0.02[b]	20.6 ± 0.38[b]
400	1.6 ± 0.26[b]	0.22 ± 0.01[b]	26.3 ± 0.52[a]
800	1.6 ± 0.06[b]	0.21 ± 0.006[b]	36.45 ± 0.29[a]

Data represent the mean value ± SD ($n = 3$); significance levels: [a] $P < 0.001$, [b] not significant

500 μM concentrations of $CdCl_2$ there were about 14-fold and 7-fold increases of diosgenin ($P < 0.001$), respectively. At 5 days after treatment of the seedlings, 400 and 800 μM concentrations of $CoCl_2$ showed stimulatory effect only on seedling length, and at all of the concentrations tested fresh weight and dry weight decreased slightly although the decrease was not statistically significant (Table 2). So, it may be said that the treatment with all the three concentrations had no toxic effect on growth. In all the concentrations of $CoCl_2$ diosgenin production increased significantly (Fig. 2). Maximum increase (about 41-fold) was found at 200 μM concentration ($P < 0.001$) without affecting the growth of the seedlings. In 400 and 800 μM concentrations the content increased about 25-fold ($P < 0.001$) and 10-fold ($P < 0.05$), respectively. But all of the concentrations of $K_2Cr_2O_7$ (300 and 500 μM) and $NiCl_2$ (200 and 400 μM) had inhibitory effect ($P < 0.01$) on diosgenin production (Figs. 3, 4).

Effect of methyl jasmonate

The production of diosgenin was elicited after treatment with methyl jasmonate (Fig. 5). A maximum increase

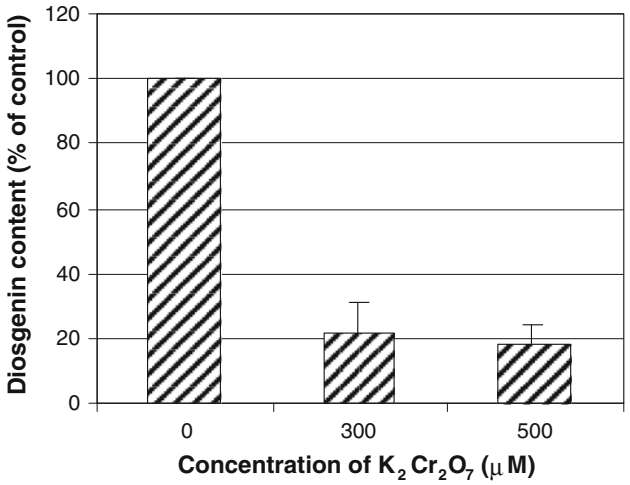

Fig. 3 Effect of $K_2Cr_2O_7$ on diosgenin content

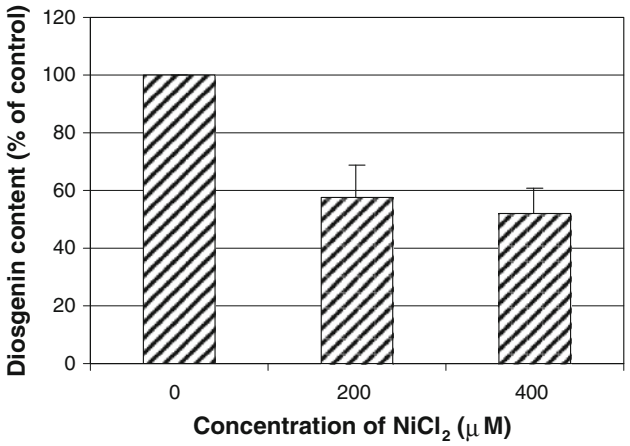

Fig. 4 Effect of $NiCl_2$ on diosgenin content

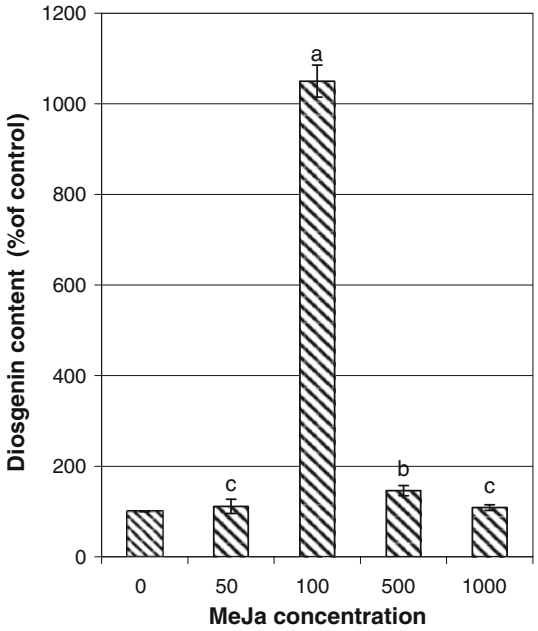

Fig. 5 Effect of different concentrations of methyl jasmonate (50–1,000 μL L^{-1}) on diosgenin content (at 5 days after treatment). The *vertical bars* denote ± SD ($n = 3$); significance level: [a] $P < 0.001$, [b] $P < 0.01$, [c]not significant

(10.5-fold) ($P < 0.001$) was found at 100 μL L^{-1} concentration of MeJa. Then the diosgenin content decreased up to 1,000 μL L^{-1} concentration MeJa after which no significant change in diosgenin content was observed. MeJa has an integral role in the cascade of events that occur in the elicitation process, causing the activation of the genes for secondary metabolism either directly or indirectly. MeJa induces secondary metabolites formation in plants (De 2001). MeJa has been observed to increase anthocyanin accumulation in germinating soybean seedlings (Franceschi and Grimes 1991), *Vitis vinifera* suspension cultures (Curtin et al. 2003), *Vaccinium pahalae* cell cultures (Fang et al. 1999), *Daucus carota* cell culture (Sudha and Ravishankar 2003a, b), MeJa induced glycyrrhizin production in cultured *Glycyrrhiza glabra* cells (Shabani et al. 2009), production of shikonin in *Onosma paniculatum* cells (Ding et al. 2004), silymarin secretion in *Silybum marianum* cultures (Madrid and Corchete 2010), and benzophenanthridine alkaloid accumulation in *Eschscholtzia californica* suspension cultures (Cho et al. 2008). The positive effect of MeJa on ginsenoside production from ginseng cell suspension, hairy root and adventitious root cultures has been documented (Lu et al. 2001; Palazo'n et al. 2003; Choi et al. 2005; Bae et al. 2006; Kim et al. 2009). In addition, external application of MeJa enhanced accumulation of monomeric alkaloids in seedlings of *Catharanthus roseus* (Aerts et al. 1996), triterpene and sterol metabolisms of *Centella asiatica*, *Ruscus aculeatus* and *Galphimia glauca* cultured plants (Mangas et al. 2006). The result of the present study shows that MeJa can be used as an elicitor for the induced production of diosgenin in the seedlings of *T. foenum-graecum*.

Effect of calcium

The process of elicitation activates various Ca^{2+}- and calmodulin-dependent protein kinases by increasing the level of free Ca^{2+} in the cytoplasm and somehow triggers the cellular responses, which may include alterations in gene expression. The multiple Ca^{2+} mobilization pathways and release sites go some way in explaining how stimulus-specific Ca^{2+} signals may be generated. But there is still further complexity: different cell types may have different types of Ca^{2+} channels (Sudha and Ravishankar 2002). The effect of calcium on diosgenin production in seedlings of *T. foenum-graecum* is shown in Table 3. Treatment of seedlings with calcium depleted medium increased diosgenin production by 60% ($P < 0.01$). Twofold increase in the concentration of calcium in the medium (440 mg L^{-1}) resulted in 54% ($P < 0.01$) decrease in diosgenin production. Addition of the calcium ionophore A23187, a compound known to increase the concentration of cytosolic Ca^{2+} (Reed and Lardy 1972) decreased diosgenin production in *T. foenum-graecum* seedlings, as compared to the control. It is reported that EGTA is a Ca^{2+} specific ligand, capable of reducing the availability of extracellular calcium (Mahady and Beecher 1994). Addition of EGTA, a calcium chelator, to the medium resulted in 80%

Table 3 Effect of different concentrations of promoter, inhibitor and modulator of Ca^{2+} influx on diosgenin content (5 days after treatment)

Treatment	Concentration	Diosgenin content (% of control)
Control ($CaCl_2$)	220 mg L^{-1}	100
$CaCl_2$	0 mg L^{-1}	160 ± 12 [b]
	440 mg L^{-1}	46 ± 14.4[b]
Calcium ionophore	200 μg L^{-1}	34 ± 5.29[a]
$LaCl_3$	1 mM	202.7 ± 7.1[a]
	3 mM	702.7 ± 109.2[a]
	5 mM	513.5 ± 27[a]
Verapamil	1 mM	164.9 ± 8.2[a]
	3 mM	259.5 ± 17.8[a]
	5 mM	175.7 ± 34.9[c]
EGTA	6 mM	180.5 ± 14.9[b]
Caffeine	0.001 M	14.2 ± 1.72[a]
	0.01 M	7.7 ± 0.5[a]

Data represent the mean value ± SD ($n = 3$); significance level: [a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$

($P < 0.01$) increase in diosgenin production by the seedlings. To further test the role of extracellular Ca^{2+}, varapamil and $LaCl_3$ which are Ca^{2+} channel inhibitors which inhibit the flow of Ca^{2+} across the plasma membrane (Knight et al. 1992) were used. Treatment with all the concentrations of verapamil and $LaCl_3$ resulted in increased production of diosgenin. Maximum increases in diosgenin production during verapamil and $LaCl_3$ treatment were 159% (about 2.5-fold) ($P < 0.001$) and 602% (7-fold) ($P < 0.001$), respectively, which were seen at 3 mM concentration. Caffeine, a modulator of calcium release (Bolwell et al. 1991) had an inhibitory role on diosgenin production. Calcium ions seem to play a role in the control of the production of secondary metabolites but in a different manner depending on the species. It has stimulatory as well as inhibitory role in production of the secondary metabolites in plants. Calcium stimulated anthocyanin formation in *Vitis vinifera* cells (Vitrac et al. 2000), and *Daucus carota* cells (Sudha and Ravishankar 2003a, b). Production of shikonin from cell suspension cultures of *Onosma paniculatum* was promoted by a fungal elicitor extracted from *Aspergillus oryzae*. This was accompanied by a decrease in the intracellular concentration of Ca^{2+}. Treatments which induce Ca^{2+}-influx were found to suppress the elicitor promoted shikonin formation and agents that decrease the intracellular-free Ca^{2+} level were found to enhance shikonin biosynthesis even in the absence of elicitor (Ning et al. 1998). Calcium acted on *Digitalis thapsi* cultures by inhibiting cardenolide accumulation (Cacho et al. 1995). Calcium deprivation markedly enhanced guggulsterone accumulation in cell cultures of *Commiphora wightii* (Dass and Ramawat 2009). A similar effect has been reported for hecogenin production in callus cultures of *Agave amaniensis* (Indrayanta et al. 1996), and alkaloid production in cell suspensions cultures of *Tabernaemontana divaricata* (Sierra et al. 1991). The results of the present study suggest that production of diosgenin in *T. foenum-graecum* seedlings was inhibited by calcium. However, treatment with calcium chelator or Ca^{2+} channel inhibitors could be used to elicit diosgenin production in this plant.

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