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Effect of ethephon on antioxidant enzymes and diosgenin production in seedlings of *Trigonella foenum-graecum*

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Abstract

To study the effect of ethylene on growth, diosgenin production and activities of the two antioxidant enzymes catalase and peroxidase, the seedlings of *Trigonella foenum-graecum* were treated with different concentrations of ethephon (as ethylene generating agent). The fresh weight and the length of seedlings decreased significantly and the dry weight of seedlings increased with increase in concentration of ethephon. At 100 mg/l concentration of ethephon, enzyme activities and diosgenin content decreased significantly. Subsequently, enzyme activities and diosgenin content increased with increase in concentration of ethephon. But diosgenin content did not increase over that of the control. The results suggest that although there is a relation between ethephon concentration, ethephon-induced catalase and peroxidase activities and diosgenin content, ethylene is not required for induction of diosgenin content in *T. foenum-graecum* seedlings.

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Keywords: *Trigonella foenum-graecum* seedlings; Diosgenin; Ethephon; Catalase; Peroxidase

1. Introduction

The simple gas ethylene serves as a plant hormone and has a profound effect on plant growth and developmental processes, including senescence, abscission, flowering, fruit ripening, germination and morphogenic response in seedlings, called 'triple response' (Kieber, 1997). Environmental stresses, such as wounding, temperature and pathogenic attack, enhance ethylene biosynthesis (Chang, Knok, Blecker, & Meyerowitz, 1993). Ethylene is perceived by receptor and transduction of the ethylene signal leads to changes in gene expression (Chang & Meyerowitz, 1995). Stimulation of ethylene synthesis has been reported to involve the generation of reactive oxygen species (Wang, Li, & Ecker, 2002). Ethylene is involved in the oxidative responses of barley plants induced by aphids infestation and induces peroxidase activity (Argandona, Chaman, Cardemil, Munoz, Zuniga, & Corcuera, 2001) Peroxidase and catalase are involved in many physiological processes, including abiotic and biotic stress responses (Kocsy, Owttrim, Brander, & Brunold, 1997; Medina, Botella, Quesada, & Valpuesta, 1997; Rao, Paliyath, & Ormrod, 1996). They scavenge the reactive oxygen intermediates produced in response to stress. Peroxidases are also involved in plant growth, differentiation and development processes (Aouas, Baaziz, & Mergoum, 2000).

Exogenously applied gaseous ethylene has been closely related to part of the signal transduction pathway initiated by some, but not all elicitors (Raj & Fluhr, 1992; Roby, Broglic, Gayner, & Broglic, 1992) of secondary metabolite formation. There is limited information regarding the effect of ethylene on secondary metabolism. Ethylene enhances production of chrysophanol and emodin in rhubarb cell culture (Kurosaki, Nagase, & Nishi, 1992), caffein and theobromine in *Coffea arabica* cell culture and berberine in *Thalictrum rugosum* cell culture (Cho, Kim, Pederson, & Chin, 1988). But ethylene does not seem to influence alkaloid production in tissue culture of *Papaver somniferum* (Songstad et al., 1989), *Eschscholtzia californica* (Piatti, Boller, & Brodelius, 1991) or *Catharanthus roseus* (Lee & Shuler, 1991; Schlatmann, Fonck, Hoopen, & Heijen, 1994). Endogenous ethylene release decreases berberine accumulation in bioreactor-grown tissue culture of *Thalictrum*

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minus (Kobayashi, Fukui, & Tabata, 1991). Negative effects of ethylene on accumulation of cardenolide production in *Digitalis lanata* (Berglund & Ohlsson, 1992) and L-dopa production in hairy root culture of *Stizolobium hassjoo* (Sung & Huang, 2000) were also reported. The effects of ethylene on secondary metabolism depend on the biochemical pathways involved. There may also be variations in the response to ethylene due to the level of tissue organization (Berglund & Ohlsson, 1992).

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, Rao, & Kale, 1992) and plays an important role in the control of cholesterol metabolism (Roman, Thewles, & Coleman, 1995; Sauvaire, Ribes, Baccou, & Loubatieeres Mariani, 1991). *Trigonella foenum-graecum* is one of several plant sources which produce diosgenin (Evans, 1996). Seedlings of the plant are also reported to produce diosgenin (Bhavsar, Kapadia, & Patel, 1980; Hardman & Fazli, 1972; Ortuno, Oncina, Botia, & Del Rio, 1999). In the present work, different concentrations of ethylene were used to study its effect on growth, production of diosgenin and the activities of the two antioxidant enzymes catalase and peroxidase in the seedlings of *T. foenum-graecum*.

2. Materials and methods

2.1. Growth and treatment of seedlings

Seeds of *T. foenum-graecum* were surface-sterilized and placed in sterile lidded glass vessels (12×6 cm) on filter paper wetted with sterile water for germination. On day 2 the germinated seeds were treated with different concentrations of ethephon (as the ethylene generating agent) in MS salt solution (half-strength). 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 ethephon in each sterile lidded glass vessel, and kept for another 5 days at 15–18 °C under a fluorescent lamp for 8/16 h light/dark period. Controls were exposed to half strength MS salt solution. After 5 days of treatment, treated and contol seedlings were harvested for analysis Seed coats were removed and the fresh weight and seedling length were measured. The seedlings were then dried at 45 °C for 48 h.

2.2. Estimation of total protein content and enzyme activity

At 1 day and 5 days after treatment, 1 g fresh seedlings were homogenized in 15 ml of 0.067 M cold phosphate buffer (pH7). Homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was used for assay.

The total protein content in the homogenate was determined following the method of Lowry, Rosebrough, Farr, and Randall (1951).

Catalase activity was determined by using a slightly modified method of Aebi (1984). The activity was assayed by measuring the initial rate of disappearance of H_2O_2 in the reaction mixture containing 0.1 ml enzyme extract in 1 ml 0.067 M buffer (pH 7). The reaction was started by addition of 2 ml 5 mM buffered H_2O_2 and its consumption was measured after 1 min at 240 nm by UV–visible spectrophotometer (Varian 634). The activity was calculated using the extinction coefficient (40 mM^{-1} cm^{-1} at 240 nm) for H_2O_2 (Kato & Shimizu, 1987). The activity was expressed as unit/g fresh weight (1 unit being defined as the amount of enzyme that decomposed 1 mM of H_2O_2 per min).

Peroxidase activity was estimated following the method of Kim and Yoo (1996). The activity was determined at 470 nm by UV–visible spectrophotometer, following the formation of tetraguaiacol in a 3 ml reaction mixture containing 1 ml of 0.067 M phosphate buffer (pH 7), 1 ml of 15 mM 2-methoxyphenol (guaiacol), 1 ml of 3 mM H_2O_2 and 0.1 ml of enzyme extract. The activity was expressed as change in OD per gramme fresh weight per minute (ΔOD/g fresh wt./min).

2.3. Isolation and measurement of diosgenin

Diosgenin was extracted, following the method of Asolkar and Chadha (1979). Powdered dried seedlings, 3 N HCl and hexane were refluxed in a glass apparatus on a magnetic stirrer hot plate for 2 h at 90–96 °C. The mixture was allowed to cool and the aqueous phase was given three shakings 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, Utani, Santosa, and Syarani (1994) optimized for our work conditions. The analyses were performed with a Varian 9010 solvent delivery system, Varian 9050 variable wavelength UV–vis detector and Varian 4400 integrator. Chromatographic separation was carried out on Waters Symmetry C_{18} 5 μm (150×4.6 mm i.d.) column using methanol as the solvent at a flow rate of 0.6 ml/min. Changes in absorbance were recorded at 205 nm. The diosgenin was identified by comparing its retention time with that of authentic diosgenin and by co-elution with authentic diosgenin (Sigma). Identification of diosgenin from fractions collected after repeated injections was also carried out, by comparing the UV spectrum with that of authentic diosgenin using photo diode array (SPD-MIOA VP Shimadzu, Diode Array Detector, LC-10AT Shimadzu Liquid Chromatography). Concentration of diosgenin in samples was calculated

from a regression equation prepared from peak area and known concentration of authentic diosgenin.

3. Results and discussion

3.1. Effect on growth

The 7-day-old seedlings of *T. foenum-graecum* were comprised of root, hypocotyl and the two green cotyledonary leaves. The fresh weight and the length of the seedlings decreased significantly with increase in concentration of ethephon (Table 1). Maximum decrease in fresh weight (41.7%) was found at 1600 mg/l concentration and maximum decrease in seedling length (46.3%) was observed at a 2500 mg/l concentration of ethephon. Except at 100 and 200 mg/l concentrations, in all other concentrations of ethephon, dry weight of seedlings significantly increased over that of control. Maximum increases (22.7%) were found at 2000 and 2500 mg/l concentrations. Seedlings of many dicotyledonous plant species, in the presence of ethylene, adopt a striking morphology referred to as 'triple response'. In pea, the triple response consists of an inhibition of elongation, radial swelling of the epicotyl and diageotropism (Geoschi, Rappaport, & Pratt, 1966; Knight, Rose, & Carter, 1910). In *Arabidopsis*, the triple response consists of an inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and an exaggeration of the curvation of the apical hook (Kieber, 1997). In *T. foenum graecum*, in addition to the decrease in the length of the seedlings and increase in dry weight, swelling of the hypocotyl was also observed with increase in concentration of ethephon.

Ethylene is classically considered to be an inhibitor of growth because of its ability to reduce cell elongation (Smalle & Van der Straeten, 1997). According to Konings and Jackson (1979) low ethylene concentration stimulates growth whereas higher concentration inhibits growth. Stimulatory effects of low ethylene levels have been reported for first internode elongation in wheat seedlings (Suge, Nishizawa, Takahashi, & Takeda, 1997) and hypocotyl elongation of *Arabidopsis* seedling growth in the light (Smalle, Haegman, Kurepa, Van Montagu, & Van Der Straeten, 1997) or in the dark (Hua & Mayerowitz, 1998). Stem and petiole growth is stimulated by ethylene in flood-tolerant species such as deep water rice, which grow vigorously under flooded conditions (Kende, Van Der Knaap, & Hyung Taeg Cho, 1998). In *T. foenum-graecum*, the functional significance of the effect of ethylene on growth is yet to be clarified.

Table 1
Effects of different concentrations of ethephon on seedling growth (5 days after treatment)

Concentration of ethephon (mg/l)	Fresh weight of 30 seedlings (g)	Dry weight of 30 seedlings (g)	Seedling length (mm)
0	2.4±0.18	0.22±0.001	25.5±0.06
100	2.0±0.08 ns	0.24±0.01 ns	23.0±2.00 ns
200	1.7±0.09***	0.22±0.02 ns	20.8±1.40**
800	1.7±0.07***	0.25±0.01***	17.9±0.40*
1200	1.7±0.03***	0.26±0.004*	16.6±0.69*
1600	1.4±0.18**	0.26±0.003*	16.7±1.00*
2000	1.9±0.05***	0.27±0.01*	14.3±0.80*
2500	1.9±0.03***	0.27±0.02***	13.7±0.30

Data represent the mean value±S.D., $n=3$. ns, Not significant.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

3.2. Effect on diosgenin content

At 100 mg/l concentration of ethephon, diosgenin content decreased 73.3% below that of the control. Then the production increased gradually with the increase in ethephon concentration up to 1600 mg/l concentration. Then the production decreased again (Table 2). The inhibitory effect of ethephon on diosgenin production at 100 mg/l concentration might be due to induction of some other metabolic pathways whose activity gradually decreases with increase in concentration of ethephon and results in general increase in diosgenin production. But diosgenin production did not increase over that of the control. While our work was in progress, Ortuno et al. (1999) reported that the growth and diosgenin content in stem and root of 15 day and 30 day-old seedlings decreased in *T. foenum-graecum* when the seeds were soaked in 50 mg/l ethephon for 24 h and then germinated in sterile peat in a greenhouse. We report here, for the first time, the direct effect of ethylene, generated by ethephon, on diosgenin content in *T. fonum-graecum* seedlings.

3.3. Effect on catalase activity

At 1 day after treatment with 100 mg/l of ethephon, the catalase activity decreased 35.3% below that of the control. Then the activity gradually increased with increase in concentration of ethephon up to 1200 mg/l, reaching the control level. Then the activity again decreased. At 1 day, at different concentrations of ethephon (except 100 mg/l), catalase activity was significantly below the control level. It was found that control seedlings showed decrease in catalase activity at 5 days after treatment in comparison to that at 1 day after treatment. But, in the case of treated seedlings, the activity increased significantly with increase in concentration of ethephon up to 1200 mg/l concentration (except at 100 mg/l concentration) and then decreased again (Table 2).

Table 2
Effects of different concentrations of ethephon on diosgenin content, catalase and peroxidase activities

Ethephon conc. (mg/l)	Diosgenin content (% of control)	Catalase activity (unit/g fresh wt.)		Peroxidase activity (Δ OD/g fresh wt.)		Total protein content (mg/g fresh wt.)	
		At 1 day after treatment	At 5 days after treatment	At 1 day after treatment	At 5 days after treatment	At 1 day after treatment	At 5 days after treatment
0	100	0.00224±0.00018	0.00147±0.00006	1018±3.74	931±1.41	103.1±0.37	49.2±0.88
100	26.7±1.67* †	0.00145±0.00006***	0.00127±0.00006*	931±9.90***	853±13.5**	98.6±0.56***	50.3±0.25 ns
200	40.00±20.00†	0.00156±0.00069**	0.00143±0.00007 ns	948±8.83***	1036±13.49***	96.1±0.92***	60.2±2.02**
800	66.67±2.84**	0.00174±0.00014*	0.00469±0.00046***	976±10.19**	1780±6.16***	105±1.26 ns	69.7±1.78***
1200	85.00±15.85 †	0.00226±0.00022*	0.00713±0.00028***	1046±8.60*	1931±22.76***	127±1.72***	72.9±1.74***
1600	97.50±12.5 ns †	0.00171±0.00014*	0.00331±0.00007***	1119±13.64***	2926±74.71***	135±2.15***	80.0±2.70***
2000	43.3±3.60**	0.00142±0.00010**	0.00245±0.00009***	1060±11.05**	1894±110.45***	129±1.43***	72.6±1.29***
2500	23.33±0* †	0.00132±0.00010***	0.00127±0.00003*	1013±12.57 ns	1439±69.9***	132±2.15***	78.8±1.96***

Data represent means±S.D., $n=3$ or †2. ns, Not significant.
* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

3.4. Effect on peroxidase activity

At 1 day after treatment with 100, 200 and 800 mg/l of ethephon, peroxidase activity decreased. At 1200, 1600 and 2000 mg/l of ethephon, peroxidase activity increased over that of the control. At 5 days after treatment, in all the concentrations of ethephon (except 100 mg/l), the peroxidase activity increased over that of control. Maximum increase (approximately 3-fold) was observed at 1600 mg/l concentration (Table 2). Ethylene has previously been reported to increase peroxidase activity in sweet potato, cucumber, and pea seedlings (Abeles, Dunn, Morgens, Callahan, Dinterman, & Schmidt, 1988; Cassab, Lin, Lin, & Varner, 1988). Kim et al. (2000) showed that, in sweet potato, the four peroxidase genes were expressed differently after ethephon treatment.

3.5. Effect on total protein content

At 1 day after ethephon treatment, at 100 mg/l concentration, the protein content decreased. Then the content increased with increase in concentration of ethephon. Significant increases over that of control were observed at 1200, 1600, 2000 and 2500 mg/l concentrations. However, the total protein content decreased in seedlings at 5 days after treatment in comparison to that at one day after treatment. But, in comparison to the control, the protein content gradually increased with increase in concentration of ethephon (Table 2).

3.6. Correlation between ethephon content, enzyme activities and diosgenin content

In response to ethylene-induced stress, catalase and peroxidase activities increased over that of the control at 5 days after treatment with the stress-related hormone ethephon, an ethylene-generating chemical (except at 100 mg/l concentration). At 1 day after treatment, catalase activity decreased but peroxidase activity increased with higher concentrations of ethephon. At 100 mg/l concentration of ethephon enzyme activities and diosgenin content decreased significantly. Subsequently, enzyme activities and diosgenin content increased with increase in concentration of ethephon. But diosgenin content did not increase over that of the control. The results suggest that, although there is a relationship between ethephon concentration, ethephon-induced catalase and peroxidase activities and diosgenin content, ethylene is not required for induction of diosgenin content.

Ethylene is perceived by a receptor and transduction of ethylene signal leads to changes in gene expression (Chang & Mayerowitz, 1995). Whether or not a target gene will be affected by ethylene depends on the availability of a free ethylene sensor and a functional signal transduction chain in the cell (Grichko & Glick, 2001). Ethylene enhances production of a number of secondary metabolites, e.g. chrysophanol and emodin, in rhubarb cell culture (Kurosaki et al., 1992), caffeine and theobromine in *Coffea arabica* cell culture, and berberine in *Thalictrum rugosum* cell culture (Cho et al., 1988). However Piatti et al. (1991) and Songstad et al. (1989) concluded that ethylene is not needed for secondary metabolism in *Eschcholtzia* or *Papaver somniferum* cell cultures. Negative/negligible effect of ethylene on accumulation of secondary metabolites was also reported *in C. roseus* (Lee & Shuler,1991; Schlatmann et al., 1994), *Digitalis lanata* (Berglund & Ohlsson, 1992) and *Stizolobium hassjoo* (Sung & Huang, 2000). Ethylene suppressed methyl jasmonate-activated nicotine biosynthesis genes in *Nicotiana sylvestris* (Shoji, Nakajima, & Hashimoto, 2000). Attack by nicotine-tolerant insect causes *N. attenuata* to produce ethylene, which

directly suppresses the biosynthesis of nicotine (Winz & Baldwin, 2001). In *Cucurbita maxima*, wound-induced ethylene synthesis is associated with increase in peroxidase activity (Kato, Hayakawa, Hyodo, Ikoma, & Yano, 2000). The last step in the synthesis of lignin and suberin has been proposed to be catalysed by peroxidases (Quiroga et al., 2000). In the present study the inhibitory effect of ethephon on diosgenin production at 100 mg/l concentration might be due to induction of some other metabolic pathways whose activity gradually decreases with increase in concentration of ethephon and results in general increase in diosgenin production reaching the control level.

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