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Growth and Organogenesis in Moth Bean Callus Cultures as Influenced by Triazole Growth Regulators and Gibberellic Acid

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Abstract. The triazole plant growth regulators, paclobutrazol and uniconazole, reduced in vitro growth of moth bean callus by 20–25% when added to the culture medium at 1 mg/L (3.4 μM). The addition of 10 mg/L (29 μM) gibberellic acid (GA_3) to the culture medium in combination with the triazoles restored callus growth to a level equivalent to that of the untreated control. GA_3 alone had little effect on callus growth. When added to a regeneration medium at 1 mg/L both paclobutrazol and uniconazole reduced the percentage of cultures that formed roots, as well as the mean number of roots per culture. In contrast, GA_3 increased root formation and counteracted the inhibitory effects of the triazoles on rooting. The addition of triazoles or GA_3 to the regeneration medium reduced the formation of green meristematic nodules, which are precursors of shoots in moth bean callus. When callus was grown in the presence of either paclobutrazol or uniconazole, subsequent root and green meristematic nodule formation were reduced upon transfer to a growth regulator-free regeneration medium. The results of this study indicate that exposure of moth bean callus tissue to micromolar concentrations of triazoles or GA_3 can significantly alter in vitro growth and differentiation.

During the past few years, the biological activities of several triazole derivatives have been studied in detail (reviewed by Davis et al. 1986a, 1988, Fletcher 1985, Koller 1987). The plant growth-regulating properties of these compounds are primarily attributed to the inhibition of gibberellin biosynthesis, although other growth substances, such as ethylene, cytokinins, and abscisic acid might also be affected by the triazoles (Fletcher and Hofstra 1988, Izumi et al. 1988). In general, triazole-induced phenomena, such as reduced shoot elongation, can be reversed by the application of gibberellins. Triazoles also interfere with sterol metabolism, but the significance of this action in relation to plant growth regulation is not yet clear (Henry 1985, Lenton 1987).

In addition to reducing gibberellin biosynthesis and shoot growth, triazoles induce a variety of interesting physiological responses in plants, such as increased chlorophyll content, altered carbohydrate status, increased stress tolerance, and delayed senescence (reviewed by Davis et al. 1986a, 1988). Although the triazoles promote adventitious root formation in stem cuttings of several species (Davis 1986, Davis et al. 1985, 1986b), their effects on growth and organogenesis of tissue cultures have not been studied in detail. Paclobutrazol has been observed to enhance cormlet formation from in vitro cultured gladiolus buds (Ziv 1989). In contrast, Gehlot et al. (1989) reported that paclobutrazol reduced root and shoot formation in vitro. Rajasekaran et al. (1987) reported that treatment of *Pennisetum purpureum* mother plants with paclobutrazol did not effect subsequent embryogenic potential of the explants. Addition of paclobutrazol to the culture medium increased somatic embryogenesis and decreased browning of embryos produced from *Citrus sinensis* callus (Spiegel-Roy and Saad 1986).

The objective of the present investigation was to evaluate the influence of two highly active triazoles, paclobutrazol and uniconazole, on growth and regeneration in moth bean callus cultures. In addition, gibberellic acid (GA_3) was included as a variable to gain insight as to whether the triazole-induced phenomenon in vitro is related to gibberellin biosynthesis inhibition. Moth bean was selected as the test material because of its potential importance as food

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and fodder in arid and semi-arid regions of the tropics. Because this legume is drought-tolerant and is a good protein source, it has been deemed worthy of further study by the US National Academy of Science (1979). Furthermore, the in vitro culture of this plant is of current interest (Gehlot et al. 1989, Shekhawat and Galston 1983).

Materials and Methods

Seeds of moth bean, *Vigna aconitifolia* (Jacq.) Marechal cv. Jaadia, were surface sterilized with 1% sodium hypochlorite for 5 min and then germinated on Murashige and Skoog's (MS) revised medium (Murashige and Skoog 1962). After 8 to 10 days, hypocotyl segments were aseptically excised and transferred to MS medium supplemented with 0.5 mg/L kinetin and 1.5 mg/L 2,4-D (hereafter referred to as "callus medium") to initiate callus formation. After several subcultures, a mass of uniform callus (approximately 200 mg) was transferred to callus medium supplemented with 1 mg/L (3.4 μM) paclobutrazol ([2RS,3RS]-1-[4-chlorophenyl]-4, 4-dimethyl-2-[1,2,4-triazol-1-yl]pentan-3-ol) or uniconazole ([E]-1-(4-chlorophenyl]-4,4-dimethyl-2-[1,2,4-triazol-1-yl]penten-3-ol) alone or in combination with GA_3 at 10 mg/L (29 μM). Callus fresh weights from the various treatments were measured 12 and 24 days after transfer to the callus medium.

To study the effect of the triazoles and GA_3 on organ regeneration from the callus, a homogenous mass (approximately 150 mg) of hypocotyl-derived callus was transferred to a regeneration medium which consisted of MS medium devoid of kinetin and 2,4-D (hereafter referred to as "regeneration medium"). Preliminary studies indicated that moth bean callus placed on such a medium readily regenerates roots within 12 to 24 days and shoot precursors (hereafter referred to as "green meristematic nodules") within 36 to 48 days (Gehlot et al. 1987). The regeneration medium was supplemented with 1 mg/L paclobutrazol or uniconazole alone or in combination with 10 mg/L GA_3. Depending upon the experiment, these growth regulators were either present during the first 24-day subculture period, the second 24-day subculture period, or during both subculture periods (48 days total), following transfer from the callus medium to the regeneration medium. In an additional experiment, the effect of the presence of paclobutrazol, uniconazole, and GA_3 during callus growth on subsequent regeneration upon transfer to a growth regulator-free regeneration medium was studied.

All cultures were maintained at 27 ± 2°C under cool white fluorescent lights (60–80 μmol/m^2/s photosynthetically active radiation) with a photoperiod of 12 h. All experiments were conducted with 12 individual cultures per treatment and each experiment was conducted at least three times.

Results

Fresh weights of moth bean callus decreased by 20–25% compared to the control when paclobutrazol or uniconazole was incorporated into the callus medium at 1 mg/L (Table 1). When 10 mg/L GA_3 was added to the culture medium in combination with paclobutrazol or uniconazole, callus fresh weights were similar to the untreated control. GA_3 alone had little effect on callus fresh weight after 12 and 24 days.

Table 1. Influence of paclobutrazol, uniconazole, and GA_3 on the fresh weight of moth bean callus.

Treatment	Callus fresh weight (mg) after inoculation	
	12 days	24 days
Control	506 ± 13	1160 ± 14
Paclobutrazol (1 mg/L)	391 ± 9	912 ± 14
Uniconazole (1 mg/L)	396 ± 10	891 ± 7
GA_3 (10 mg/L)	522 ± 5	1120 ± 14
Paclobutrazol (1 mg/L) + GA_3 (10 mg/L)	512 ± 11	1180 ± 17
Uniconazole (1 mg/L) + GA_3 (10 mg/L)	491 ± 5	1069 ± 12

Values are given as ± SEM.

Within 48 days after transfer to the regeneration medium, all control cultures exhibited root formation (Table 2). Both paclobutrazol and uniconazole reduced the percentage of cultures exhibiting root formation, as well as the mean number of roots per culture. The inhibitory effect of the triazoles on root formation was more pronounced when the compounds were present during the first 24-day subculture period compared to the second 24-day subculture. The most severe inhibition of rooting occurred when paclobutrazol was present continually in the regeneration medium during both subcultures. In general, the inhibitory effect of uniconazole on root formation was somewhat less pronounced than that of paclobutrazol.

As in the control, all cultures treated with 10 mg/L GA_3 formed roots within 48 days after transfer to the regeneration medium (Table 2). Treatment with GA_3 during the first 24-day subculture period increased the number of roots formed per culture by more than twofold compared to the control. When GA_3 was present only during the second 24-day subculture period, root formation was slightly greater than the control. When GA_3 was continuously present during both subcultures, root number was suppressed compared to the control. The addition of GA_3 in combination with either of the triazoles induced root formation in 85–95% of the cultures. Furthermore, GA_3 also partially reversed the inhibitory effects of paclobutrazol on the mean number of roots formed per culture.

After 48 days, 75% of the control cultures exhibited green meristematic nodules (Table 2). Both paclobutrazol and uniconazole decreased the percentage of cultures that formed these nodules. In addition, the mean number of green nodules per culture also decreased following treatment with triazoles. The most severe inhibition of green meristematic

Table 2. Effect of incorporation of paclobutrazol (1 mg/L), uniconazole (1 mg/L), and/or GA_3 (10 mg/L) into the regeneration medium on root and green meristematic nodule (gmn) formation from moth bean callus.

Regeneration medium treatment		% of cultures exhibiting root formation	Mean no. of roots per culture	% of cultures exhibiting gmn formation	Mean no. of gmn per culture
1st subculture (24 days)	2nd subculture (24 days)				
Untreated	Untreated	100	8.1 ± 0.7	75	16.0 ± 1.9
Paclobutrazol	Untreated	15	1.5 ± 0.3	25	2.8 ± 0.5
Untreated	Paclobutrazol	55	3.0 ± 0.4	35	3.5 ± 0.6
Paclobutrazol	Paclobutrazol	0	—	13	2.3 ± 0.3
Uniconazole	Untreated	45	5.0 ± 1.0	55	4.2 ± 1.0
Untreated	Uniconazole	65	3.2 ± 0.6	35	6.2 ± 1.4
Uniconazole	Uniconazole	43	5.0 ± 1.0	55	5.0 ± 0.8
GA_3	Untreated	100	18.0 ± 2.1	45	4.5 ± 0.6
Untreated	GA_3	100	11.0 ± 1.3	43	3.2 ± 0.2
GA_3	GA_3	100	5.0 ± 1.7	30	4.0 ± 0.9
Paclobutrazol + GA_3	Untreated	85	5.4 ± 1.3	55	6.2 ± 1.3
Paclobutrazol + GA_3	Paclobutrazol + GA_3	85	3.0 ± 0.4	55	6.0 ± 1.3
Uniconazole + GA_3	Untreated	85	3.5 ± 0.3	65	8.2 ± 1.3
Uniconazole + GA_3	Uniconazole + GA_3	95	3.0 ± 0.4	45	4.8 ± 1.0

Observations were made 48 days after transfer to the regeneration medium. Values are given as ± SEM.

nodule formation occurred when paclobutrazol was present for the entire 48-day period in the regeneration medium. Incorporation of GA_3 into the regeneration medium was also inhibitory to the formation of green meristematic nodules (Table 2). Both the percentage of cultures exhibiting nodules and the mean number of nodules per culture were reduced by GA_3. Initially, some unorganized light green tissue did appear in the presence of GA_3, but it did not develop into green meristematic nodules.

An experiment was also conducted wherein the influence of triazole or GA_3 presence during callus growth was studied on subsequent regeneration. When 1 mg/L paclobutrazol or uniconazole was present during callus growth the subsequent formation of roots upon transfer to the regeneration medium was reduced (Table 3). Forty-eight days after transfer to the regeneration medium, only 35% of the cultures which had been in the presence of the triazoles during callus growth formed roots. In addition, the number of roots formed in cultures which had been treated with triazoles during callus growth decreased significantly compared to control cultures. The presence of GA_3 during callus growth reduced subsequent root formation upon transfer to the growth regulator-free medium. Furthermore, the presence of triazoles or GA_3 decreased the subsequent formation of green meristematic nodules.

Discussion

The triazole-induced reduction of callus growth observed in the present study is consistent with the effects of triazoles on the growth of intact plants. Both paclobutrazol and uniconazole are potent inhibitors of shoot growth in a wide range of species (Davis et al. 1986a, 1988). Our current results with moth bean callus indicate that organized tissue is not needed to detect triazole-induced growth retardation. The addition of triazole growth regulators to culture media might be useful in reducing the need for subculturing when callus lines are to be maintained over long periods of time. For example, moth bean callus normally must be subcultured every 2 to 3 weeks, but by adding triazoles to the medium this interval might be extended significantly. When it is desirable to restore rapid callus growth, GA_3 could be added to the medium to overcome the triazole-induced inhibition of growth. In the present study the addition of 10 mg/L GA_3 restored the growth rate of callus treated with 1 mg/L paclobutrazol or uniconazole. More work is needed to fully determine the feasibility of adding triazoles to culture media to reduce the need for subculturing. In particular, it must be determined if calli remain genetically stable in the presence of the growth regulators. Also, specific dosages and protocol would need to be determined. It is interesting to note, however, that Snir (1988) recently concluded that paclobutrazol should facilitate long-term in vitro storage of cherry shoots by reducing undesirable elongation and preserving culture viability.

The triazole-induced reduction of root regeneration in callus tissue contrasts with the action of triazoles on adventitious root formation in stem cuttings. Both paclobutrazol and uniconazole, at micromolar concentrations, have been found to promote root formation in the base of cuttings from various species (Davis 1986, Davis et al. 1985,

Table 3. Effect of incorporation of paclobutrazol, uniconazole, or GA_3 into the callus medium on subsequent formation of roots and green meristematic nodules (gmn) from moth callus upon transfer to a growth regulator-free regeneration medium.

Callus medium treatment	Days after transfer to regeneration medium	% of cultures exhibiting root formation	Mean no. of roots per culture	% of cultures exhibiting gmn	Mean no. of gmn per culture
Untreated	24	85	8.0 ± 1.2	45	6.5 ± 0.8
	48	100	8.1 ± 0.7	75	16.0 ± 1.9
Paclobutrazol (1 mg/L)	24	13	1.5 ± 0.3	15	3.8 ± 0.8
	48	35	2.5 ± 0.3	25	7.0 ± 0.9
Uniconazole (1 mg/L)	24	20	1.5 ± 0.3	30	7.5 ± 1.0
	48	35	1.5 ± 0.3	40	10.4 ± 2.9
GA_3 (10 mg/L)	24	63	3.0 ± 0.5	25	2.5 ± 0.3
	48	65	2.5 ± 0.3	45	3.0 ± 0.3

Values are given as ± SEM.

1986b). Hence it may be that the triazoles have different effects on organogenesis from organized versus undifferentiated tissues. The promotion of root regeneration in vitro by GA_3 in the present study contrasts with the normal inhibition of adventitious rooting in stem cuttings (reviewed by Hansen 1988). However, in a number of in vitro regeneration systems, GA_3 has been shown to promote root formation (Coleman and Greyson 1977, Haddon and Northcote 1976, Negrutiu et al. 1978). Timing of GA_3 exposure is a critical factor in determining rooting response (reviewed by Hansen 1988).

Organogenesis in vitro is a function of a delicate, but complex, interaction between exogenous and endogenous phytohormones (Chandler and Thorpe 1986). The observation that the triazoles, which are rather specific inhibitors of gibberellin biosynthesis (Hedden and Graebe 1985, Izumi et al. 1985), strongly inhibited root formation from moth bean callus suggests that gibberellins may play a role in differentiation of roots in this tissue. This contention is further supported by the observation that GA_3 generally reversed the inhibitory action of the triazoles. The opposing activities of the triazoles and GA_3 on root formation are consistent with previous observations: e.g., triazole-induced phenomena such as reduced shoot elongation (Steffens et al. 1985, Wample and Culver 1983), reduced leaf expansion (Braun and Garth 1986, Steffens et al. 1985, Wample and Culver 1983), enhanced adventitious root formation in cuttings (Davis 1986), increased tolerance to sulfur dioxide (Lee et al. 1985), and reduced stomatal aperture (Santakumari and Fletcher 1987) have all been shown to be reversible by GA_3.

In contrast to their usual opposing activities, the triazoles and GA_3 both reduced shoot formation in moth bean callus. Hence, it does not seem likely that triazole-induced inhibition of shoot formation is strictly related to gibberellin biosynthesis inhibition. The triazoles also inhibit sterol biosynthesis (Henry 1985), and their influence on shoot formation might be related to this activity. More work is needed to assess this possibility.

In the present study the magnitude of the effects of the triazoles on root and green meristematic nodule formation depended upon the timing of treatment, as well as on whether the chemicals were continuously present. Growth of the callus in media containing triazoles also inhibited subsequent root regeneration upon transfer to the growth regulator-free regeneration medium. This indicates that the triazoles need not be continuously present to inhibit root formation in vitro.

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