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BRIEF COMMUNICATION

Thidiazuron induced adventitious shoot regeneration in *Hyoscyamus niger*

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Abstract

A high frequency adventitious shoot regeneration protocol was developed for henbane (*Hyoscyamus niger* L.) using thidiazuron (TDZ). Hypocotyl, cotyledon and stem explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of N^6-benzylaminopurine and TDZ. MS medium supplemented with 16 μM TDZ was the most effective for providing 100 % regeneration frequency associated with a 19.53 shoots per hypocotyl explant. Plantlets were rooted on MS medium supplemented with different concentrations of indole-3-butyric acid (IBA) and α-naphthaleneacetic acid. High rooting and survival was achieved using half strength MS medium supplemented with 8 μM IBA.

Additional key words: N^6-benzylaminopurine, henbane, indole-3-butyric acid, α-naphthaleneacetic acid, rooting, tissue culture.

Henbane (*Hyoscyamus niger* L.) is an important medicinal plant. The biological properties of henbane are associated with the content of tropane alkaloids, hyoscyamine and hyoscine. Although development of *in vitro* propagation systems have already been reported either through organogenesis or somatic embryogenesis for henbane (Cheng and Raghavan 1985, Raghavan and Nagmani 1989), only a limited amount of data was available on shoot regeneration and rooting of shoots. Though there are some reports on the regeneration of henbane, there are still no data concerning regeneration using thidiazuron (TDZ) as a cytokinin source for this species. The aim of the current study was therefore to determine the role of TDZ as compared to N^6-benzyl-aminopurine (BAP) for henbane regeneration.

Seeds of henbane were surface-sterilized in 70 % ethanol for 2 min and then in 25 % commercial bleach (*Axion*) containing 6 % sodium hypochlorite for 10 min. After rinsed with sterile distilled water, they were germinated in Petri dishes containing Murashige and Skoog (1962; MS) medium supplemented with 30 g dm^{-3} sucrose and 7 g dm^{-3} agar. Hypocotyl and cotyledon explants were excised from germinating seeds after 5 d. Hypocotyl segments were dissected by discarding axillary meristems and cut into pieces approximately 0.3 cm long. Cotyledon explants were cut across discarding the petiole and the lower 1 - 2 mm of cotyledon base. Edges of cotyledons were also trimmed off. Stem explants were excised from 15-d-old plantlets and cut into pieces approximately 0.3 mm long. The callus and shoot induction medium was composed of MS basal medium containing myo-inositol (100 mg dm^{-3}), thiamine (0.4 mg dm^{-3}) and 20 g dm^{-3} sucrose. The medium was supplemented with different concentrations of either BAP or TDZ. Growth regulators TDZ and BAP were filter-sterilized using a Milipore filter (0.22 μm pore size) and added to hot autoclaved medium before dispensed into culture tubes. The pH of medium was adjusted to 5.7 with 1 M NaOH or 1 M HCl before autoclaving at 121 ºC, 1.4 kg cm^{-2} for 20 min. All cultures were kept at 24 ± 2 ºC under cool white fluo-rescent tubes (irradiance of 35 μmol m^{-2} s^{-1}) with 16-h photoperiod. After 3 weeks of culture, hypocotyl, cotyledon and stem explants were transferred to

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Abbreviations: BAP - N^6-benzylaminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog medium; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron.

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hormone-free MS medium for shoot elongation. The number of explants producing shoots and the number of shoots per explant were scored after six weeks of culture. Excised shoots (about 1 cm) were separated individually and transferred to ten different rooting medium consisted of half strength MS basal medium supplemented with 20 g dm^{-3} sucrose and various concentrations of indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA). After four weeks, the number of rooted shoots, the number of roots per shoot and mean root length were recorded. For regeneration experiments each treatment had three replicates consisting of Petri dishes each containing 10 explants. Each rooting treatment consisted of 10 explants per *Magenta* (GA-7) vessel replicated three times. Data given in percentages were subjected to arcsine (√X) transformation (Snedecor and Cochran 1967) before statistical analysis using *MSTAT-C*.

Most hypocotyl, stem and cotyledon explants elongated, enlarged and formed green colored callus at the wound sites after 15 - 18 d in culture. Green shoot primordia were visible on range of media containing BAP and TDZ in hypocotyl, cotyledon and stem explants within 3 weeks. These shoot primordia subsequently developed into normal shoots 4 - 5 weeks after culture initiation (Fig. 1*A,B*). Shoot regeneration occurred only from the basal part of cotyledon explants.

It was previously reported that a combined TDZ and BAP treatment evoked no significant stimulation of sycamore maple shoot formation compared to effects of TDZ or BAP alone (Wilhelm 1999). Different concentrations of TDZ or BA alone were tested in this study. Adventitious shoot regeneration was promoted by the two cytokinins tested. It was observed that high TDZ concentrations in henbane promoted massive callus

Fig. 1. Adventitious shoots regeneration from hypocotyl and stem explants of henbane. *A* - Prolific callus and shoot regeneration on hypocotyl explants on a medium with 16 µM TDZ after 5 weeks culture. *B* - Formation of adventitious shoots on stem explants after 4 - 5 weeks culture. *C* - Elongation and rooting of shoots on half strength MS medium with 8 µM IBA. *D* - 4-week-old *in vitro* raised plantlet of henbane after transfer to plastic cup containing *Vermiculite* and *Perlite* (3:1).

Table 1. Effect of various concentrations of BAP and TDZ on adventitious shoot regeneration from different explants in henbane. Values within a column followed by the different uppercase letters and values within a row followed by the different lowercase letters are significantly different at the 0.01 probability level using Duncan's multiple range test.

Growth regulators	[µM]	Explants producing shoots [%] hypocotyl	cotyledon	stem	Number of shoots [explant^{-1}] hypocotyl	cotyledon	stem
BAP	1	63.3 ^{a} B	10.0 ^{b} G	0.0 ^{c} C	10.36 ^{a} E	1.83 ^{b} F	0.00 ^{c} E
	2	36.6 ^{a} C	26.6 ^{a} F	0.0 ^{b} C	4.70 ^{a} H	2.70 ^{b} E	0.00 ^{c} E
	4	46.6 ^{a} C	3.3 ^{b} H	0.0 ^{b} C	7.26 ^{a} G	0.63 ^{b} G	0.00 ^{c} E
	8	40.0 ^{a} C	40.0 ^{a} E	23.3 ^{b} A	7.40 ^{a} G	2.80 ^{b} E	3.23 ^{b} C
	16	70.0 ^{a} B	53.3 ^{b} DE	10.0 ^{c} B	12.76 ^{a} D	4.70 ^{b} D	1.93 ^{c} D
TDZ	1	73.3 ^{a} B	66.6 ^{a} CD	0.0 ^{b} C	8.63 ^{a} F	5.43 ^{b} C	0.00 ^{c} E
	2	100.0 ^{a} A	90.0 ^{b} B	0.0 ^{c} C	10.46 ^{a} E	5.76 ^{b} C	0.00 ^{c} E
	4	100.0 ^{a} A	76.6 ^{b} C	3.3 ^{c} C	14.26 ^{a} C	7.23 ^{b} A	0.73 ^{c} E
	8	100.0 ^{a} A	100.0 ^{a} A	30.0 ^{b} A	15.76 ^{a} B	7.56 ^{b} A	4.70 ^{c} A
	16	100.0 ^{a} A	73.3 ^{b} C	36.6 ^{c} A	19.53 ^{a} A	6.43 ^{b} B	3.86 ^{c} B

production compared to BAP on all explant types. All tested concentrations of TDZ were more callogenic than those of BAP. They also induced more explants producing shoots and higher mean number of shoots per explant as compared to BAP at the same concentrations in all explants tested.

TDZ also played a major and distinctive role in the induction of adventitious shoot regeneration by organogenesis, especially from hypocotyl and cotyledon explants. The highest percentage of regenerated shoots (100 %) occurred on a medium supplemented with 2, 4, 8, 16 µM TDZ in hypocotyl explants and at 8 µM TDZ from cotyledon explants. Cotyledon explants gave a mean number of 7.56 shoots per explant at 8 µM TDZ, while the highest number of shoots from hypocotyl explants (19.53) was obtained on a medium containing 16 µM TDZ (Table 1). Taking both percentage of explants producing shoots and the mean number of shoots per explant into account, it can be summarized that the highest shoot regeneration capacity from hypocotyl explants was achieved on a medium supplemented with 16 µM TDZ. Recent reports are available on the high frequency shoot organogenesis of some crops in tissue culture using TDZ (Malik and Saxena 1992, Lu 1993, Kanyad *et al*. 1994, Kim *et al*. 1997, Hosseini-Nasr and Rashid 2003/4, Thomas 2003). Our results demonstrating high cytokinin activity of TDZ for shoot regeneration partially support these reports.

With respect to the origin of explant cultured, hypocotyl explants showed the best regeneration capacity followed closely by cotyledon and stem explants. These results indicate that the type of explant is highly important is establishing an efficient regeneration system as reported by Koroch *et al*. (2002) and Uranbey *et al*. (2003, 2005). Media supplemented with TDZ promoted shoot regeneration of hypocotyl explants from 73.3 % to 100 %, whereas 36.6 - 70.0 % of hypocotyl explants produced shoots on media supplemented with BAP (Table 1). The highest number of shoots per hypocotyl explant was also achieved on medium supplemented TDZ, ranging between 8.63 and 19.53. The number of shoots per explant from both cotyledon and hypocotyl explants also increased with increasing TDZ concentrations.

Table 2. Effect of NAA and IBA concentrations on rooting of *in vitro* regenerated shoots after 4 weeks of rooting treatment. Values within a column followed by different letters are significantly different at the 0.01 probability level using Duncan's multiple range test.

Growth regulators [µM]		Rooting [%]	Number of roots [shoot^{-1}]	Root length [cm shoot^{-1}]
NAA	0.5	33.3 cd*	1.5 f	2.8 ef
	1.0	13.3 d	1.0 g	2.5 g
	2.0	36.6 cd	2.1 d	2.7 fg
	4.0	56.6 bc	2.8 c	3.4 d
	8.0	76.6 b	3.7 a	3.8 c
IBA	0.5	100.0 a	2.8 c	5.2 a
	1.0	66.6 b	1.7 ef	4.5 b
	2.0	96.6 a	3.1 b	4.3 b
	4.0	66.6 b	1.8 de	3.6 cd
	8.0	100.0 a	3.5 a	3.0 e

Different concentrations of IBA and NAA had various effects on rooting of regenerated shoots. The highest percentage of rooting and number of roots per shoot were achieved with 8 µM IBA (Table 2). Among the different auxins tested, 8 µM IBA was also considered to be the best, because of the absence of the base callusing and the formation thick main roots (Fig. 1*C*). Rooted plantlets were transplanted to pots containing vermiculite and perlite (3:1), and grown in a growth chamber (Fig. 1*D*).

Plant transformation systems were reported for other *Hyoscyamus* species (Sevón *et al*. 1995, Leo *et al*. 2000), however, none has been developed for *Hyoscyamus niger*

up to date. The TDZ induced regeneration system described here provides an efficient method for adventitious shoot regeneration from different explants of *Hyoscyamus niger*. These results will be useful for both genetic transformation studies and for micropropagation of this important medicinal plant.

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