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BRIEF COMMUNICATION

Thidiazuron induced high frequency axillary shoot multiplication in *Psoralea corylifolia*

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Abstract

The effect of thidiazuron (TDZ) was studied on *in vitro* axillary shoot proliferation from nodal explant of *Psoralea corylifolia* - an endangered medicinal plant. Proliferation of shoots was achieved on Murashige and Skoog (MS) medium supplemented with 0.5, 1, 2, 3, 4 and 5 μM TDZ. The maximum number (13.6 ± 1.4) of shoots per explant were obtained from nodal segment cultured on 2 μM TDZ for 4 weeks and this increased to 29.7 ± 2.1 on hormone free MS medium after 8 weeks. The *in vitro* proliferated and elongated shoots were transferred individually on a root induction medium containing 0.5 μM indole-3-butyric acid (IBA) and within 4 weeks 4.5 ± 0.5 roots per shoot were produced. The regenerated plantlets were transferred to 1:1 soil and vermiculite mixture and acclimatized with 80 % survival rate. Fully acclimatized plants were grown in garden soil in greenhouse and their morphological and physiological parameters were comparable with seedlings.

Additional key words: *ex vitro* transfer, indole-3-butyric acid, *in vitro* cultivation, plant regeneration.

Psoralea corylifolia L. (*Fabaceae*) is an endangered medicinal plant, distributed in tropical and sub-tropical regions of the world. Conventional methods of propagation of *P. corylifolia* through seed is unreliable due to poor seed germination and death of seedlings under natural conditions. Due to destructive harvesting and lack of proper cultivation, the wild population of this medicinally important plant has declined very fast. Therefore, there is an urgent need to develop an appropriate protocol for mass propagation and conservation of this endangered medicinal plant.

Thidiazuron (TDZ), a substituted phenylurea (N-phenyl-1,2,3 thidiazol-5-ylurea) induces high rates of regeneration and axillary shoot proliferation in several plant species (Fiola *et al.* 1990, Malik and Saxena 1992, Huetteman and Preece 1993, Çöçü *et al.* 2004, Faisal *et al.* 2005). Its mode of action may be attributed to its ability to induce cytokinin accumulation (Victor *et al.* 1999) and or to enhance the accumulation and translocation of auxin (Murch and Saxena 2001). Micropropagation of *P. corylifolia* has been reported *via* axillary shoot sprouting (Saxena *et al.* 1998), which yielded very low number of shoots (5 - 9). The present paper describes an efficient *in vitro* method for enhanced axillary shoots multiplication from nodal explants of *P. corylifolia* using TDZ. Subsequently, the micropropagated plants were successfully established to field condition and their performance was evaluated on the basis of some morphological and physiological parameters in comparison to those of *ex vitro* plants of the same age.

Healthy shoots were collected from 2 months old *ex vitro* grown plants of *P. corylifolia* and washed thoroughly in running tap water for 30 min and then treated with a detergent *Teepol* 5 % (v/v) for 5 min. After thorough washing in sterile distilled water, the plant material was surface sterilized with 0.1 % (m/v) $HgCl_2$ for 4 min and then washed at least three times with sterile distilled water. Shoot segments were cut into 0.5 - 1 cm pieces containing one node and cultured on a sterile medium.

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Abbreviations: IBA - indole-3-butyric acid; MS - Murashige and Skoog medium; PFD - photon flux density; PGR - plant growth regulator; P_N - net photosynthetic rate; SFC - shoot forming capacity; TDZ - thidiazuron.

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Murashige and Skoog (1962; MS) medium containing 3 % (m/v) sucrose and 0.8 % (m/v) agar or 2.5 g dm^{-3} *Gelrite* were used in all the experiments. Plant growth regulators were added to the medium as specified below. The pH of the medium was adjusted to 5.8 by 1M NaOH prior to autoclaving at 121 °C for 20 min. All cultures were incubated at 24 ± 2 °C and 16-h photoperiod provided by cool-white fluorescent lamps with a photon flux density (PFD) of 50 μmol m^{-2} s^{-1}.

MS basal medium supplemented with different concentration of TDZ (0.5, 1, 2, 3, 4 and 5 μM) were used for induction and proliferation of shoots. MS medium without a growth regulator served as control. After 4 weeks of culture, frequency of shoots regeneration and number of shoots per nodal explant were recorded and the shoot forming capacity (SFC) index was calculated by using the formula: SFC index = [(% explant with shoot) × (mean number of shoots per explant)]/100.

After an induction period of 2, 4 and 6 weeks on TDZ enriched medium, the responsive explants were transferred to MS basal medium devoid of a growth regulator. Number of shoots per explant was recorded 4 weeks after transfer to growth regulator free MS medium. All cultures were sub-cultured to a fresh medium every 21 d.

Elongated shoots were excised individually and transferred to MS medium supplemented with IAA or IBA (0.1, 0.5, 1 and 2 μM) for rooting. Data on percentage of rooting and number of roots per shoot were recorded four weeks after transfer. The plantlets recovered were washed with distilled water to remove the culture medium and planted in small pots containing 1:1 mixture of soil and vermiculite, and covered with transparent polyethylene bags to ensure high humidity. After one month, hardened-off plants were transferred to pots containing normal garden soil and maintained in greenhouse. Survival percentage was recorded at 8 weeks after the plantlets were removed from tissue culture.

After 3 months, five plants each from micropropagated and control plants were uprooted and data on shoot length, root length, shoot, root fresh and dry mass and leaf area were determined. For the determination of dry mass, plants were kept in an oven at 80 °C till they attained a constant mass. The chlorophyll *a* and *b* contents were determined in 80 % acetone extract reading of absorbance at 663 and 645 nm on spectrophotometer (*SL 171*, *Elico*, Hyderabad, India) (MacKinney 1941). Net photosynthetic rate (P_N) was measured on five upper, fully expanded and healthy leaves with the help of a portable photosynthetic system (*LI-COR 6200*, Lincoln, USA) at PDF of 900 μmol $m^{-2}s^{-1}$ at 11:00 - 12:00.

All experiments were repeated three times with 20 explants for each treatment. The data were analyzed using *SPSS Version 10* (*SPSS Inc*., Chicago, USA) and means were compared using Tukey's test at the 5 % level of significance.

The present study demonstrated that TDZ has potential to inducing shoot buds from nodal explants of *P. corylifolia*. The explants cultured on basal medium failed to produce shoot buds even after 6 weeks and two subcultures. They remained green for 4 weeks, eventually turned brown and finally died. MS medium supplemented with different concentrations of TDZ (0.5, 1, 2, 3, 4, and 5 μM) stimulated axillary shoot sprouting within 4 weeks of inoculation (Fig. 1*A*). The percentage of shoot regenerating explants was 35.4 to 96.2. The data showed significant differences ($P < 0.05$) among the treatments. In this experiment, the maximum percentage (96 %) of shoot regeneration explants was observed on MS medium with 2.0 μM TDZ, which also induced the highest total number (13.6) of shoots (Table 1). This is in accordance with a report by Huetteman and Preece (1993) that high rates of axillary shoot multiplication can be achieved in many species at concentration of TDZ from 0.1 nM to 10 μM.

When the responsive explants were transferred to growth regulator free MS medium, the shoot

Table 1. Effect of TDZ on multiple shoot regeneration from nodal segments of *P. corylifolia* in MS medium after 4 weeks of culture. Values represent means ± SE. Means followed by the same letter within columns are not significantly different by the Tukey's test at 5 % probability level.

TDZ [μM]	Explants with multiple shoots [%]	Number of shoots [explant^{-1}]	SFC
0.5	35.4 ± 2.1^{e}	4.3 ± 0.2^{d}	1.5
1.0	60.0 ± 2.7^{d}	7.2 ± 1.2^{c}	4.3
2.0	96.2 ± 3.1^{a}	13.6 ± 1.4^{a}	13.0
3.0	90.0 ± 2.9^{a}	11.2 ± 0.9^{b}	10.1
4.0	81.3 ± 2.4^{b}	7.9 ± 0.3^{c}	6.4
5.0	70.0 ± 3.0^{c}	3.6 ± 0.4^{d}	2.5

Table 2. Effect of TDZ concentration and duration of exposure on mean number of shoots [explant^{-1}] regenerated from nodal segments of *P. corylifolia.* Data were recorded 4 weeks after transfer to PGR free MS medium. Values represent means ± SE. Means followed by the same letter within columns are not significantly different by the Tukey's test at 5 % probability level.

TDZ [μM]	2 weeks	4 weeks	6 weeks
0.5	4.6 ± 0.4^{f}	6.5 ± 0.5^{d}	8.7 ± 1.5^{d}
1.0	8.4 ± 0.7^{e}	16.2 ± 1.4^{c}	12.1 ± 1.1^{c}
2.0	15.4 ± 1.4^{c}	29.7 ± 2.1^{a}	22.4 ± 1.6^{a}
3.0	23.8 ± 1.7^{a}	22.8 ± 1.7^{b}	19.2 ± 1.2^{b}
4.0	20.0 ± 1.0^{b}	14.9 ± 0.9^{c}	12.5 ± 1.3^{c}
5.0	11.5 ± 0.5^{c}	8.1 ± 0.9^{d}	5.8 ± 0.7^{e}

multiplication rate increased in all treatments. To determine the optimum period for obtaining maximum number of regenerants, the explants were exposed to TDZ for different periods (2, 4 and 6 weeks) (Table 2). There was a significantly greater number of shoots on explants exposed to 2 µM TDZ for 4 weeks, while at high concentrations (3 to 5 µM) shoot numbers were reduced (Table 2). After 4 weeks of incubation on 2 µM TDZ, the number of shoots per explant was 13.6 and this increased to 29.7 on hormone free MS medium after 8 weeks (Fig. 1*B*). However, the cultures continuously grown on TDZ containing media resulted in the formation of fasciated and distorted shoots. The deleterious effect of the continued presence of TDZ has also been reported on the growth and multiplication of *Cicer arietinum* (Murthy *et al.* 1996), *Pisum sativum* (Bohmer *et al.* 1995), *Anoectochilus formosanus* (Ket *et al.* 2004) and *Rauvolfia tetraphylla* (Faisal *et al.* 2005). Our results indicate that an optimum exposure time of explants in TDZ-supplemented medium followed by the withdrawal of PGR effectively triggered shoot multiplication in *P. corylifolia*. The growth regulators may be needed for initiating the multiplication of bud meristems.

Fig. 1. *In vitro* regeneration and plant establishment of *P. corylifolia*. *A* - Induction of shoots on MS + TDZ (0.5 µM); *B* - Shoot multiplication on growth regulator free MS medium; *C* - Rooted plantlet; *D* - Acclimatized plants of *P. corylifolia* maintained in greenhouse.

Table 3. Effect of auxins on root induction from *in vitro* raised microshoots of *P. corylifolia* in MS medium after 4 weeks of culture. Values represent means ± SE. Means followed by the same letter within columns are not significantly different by the Tukey's test at 5 % probability level.

IAA [µM]	IBA [µM]	Rooting [%]	Number of roots [shoot^{-1}]
0.1		74.4 ± 2.3^{d}	2.2 ± 0.6^{cd}
0.5		90.1 ± 3.1^{b}	3.8 ± 0.2^{b}
1.0		70.0 ± 2.7^{d}	2.0 ± 0.1^{de}
2.0		50.3 ± 4.1^{e}	1.5 ± 0.3^{e}
	0.1	75.7 ± 2.5^{d}	2.7 ± 0.7^{c}
	0.5	100.0^{a}	4.5 ± 0.5^{a}
	1.0	80.3 ± 2.6^{c}	2.3 ± 0.3^{cd}
	2.0	60.0 ± 3.9^{e}	1.5 ± 0.5^{e}

For rooting (Fig. 1*C*), individual microshoots were transferred to MS basal medium containing different concentrations of IAA or IBA (Table 3). Two weeks after inoculation, root formation was noticed from the cut portion of the shoot. Of the two auxins used, IBA was found best for root induction. The highest number (4.5) of roots per shoot were recorded at 0.5 µM IBA. The effectiveness of IBA in rooting has been reported for medicinal plants like *Aloe polyphylla* (Abrie and van Staden 2001), *Tylophora indica* (Faisal and Anis 2003), *Sesbania drummondii* (Cheepala *et al.* 2004) and *Rauvolfia tetraphylla* (Faisal *et al.* 2005). Fifty rooted plantlets with 4 - 5 expanded leaves and well developed roots were transferred to pots containing soil and vermiculite mixture for hardening under diffuse light. After one month, they were transferred to pots containing

Table 4. Comparison of some morphological features, chlorophyll contents and photosynthetic rate of micro-propagated plants and seedlings of *P. corylifolia.* Data were recorded in triplicate and five plants were used for each case. Determinations were made on 3-month-old plants.

TDZ [μM]	Regenerants	Seedlings
Root length [cm]	17.51 ± 2.30	19.10 ± 3.44
Shoot length [cm]	42.32 ± 4.31	43.71 ± 4.60
Root fresh mass [g plant^{-1}]	3.21 ± 0.54	3.34 ± 0.71
Shoot fresh mass [g plant^{-1}]	6.65 ± 1.12	6.81 ± 1.30
Root dry mass [g plant^{-1}]	0.93 ± 0.61	0.98 ± 0.43
Shoot dry mass [g plant^{-1}]	1.69 ± 0.13	1.73 ± 0.10
Leaf dry mass [g plant^{-1}]	0.93 ± 0.15	0.91 ± 0.12
Leaf area [cm^2]	158.62 ± 10.15	143.21 ± 12.50
Chlorophyll *a* [mg g^{-1}(f. m.)]	0.91 ± 0.19	0.83 ± 0.31
Chlorophyll *b* [mg g^{-1}(f. m.)]	0.61 ± 0.09	0.53 ± 0.14
P_N [μmol(CO_2) m^{-2}s^{-1}]	9.79 ± 1.64	10.04 ± 1.79

normal garden soil and moved to greenhouse (Fig. 1*D*). Percentage of the plants surviving after transfer to soil was 80 %. The micropropagated plants appeared morphologically more uniform as compared to the respective seedlings of the same age. Slight reduction in the morphology of micropropagated plants in terms of shoot, root length and dry and fresh mass was observed in comparison to seedlings. However, chlorophyll *a* and *b* contents were higher in regenerated plants. Net photosynthetic rate was almost same in both the regenerants and seedlings.

In conclusion, the results presented here clearly reaffirm the efficacy of thidiazuron for the successful regeneration of *Psoralea corylifolia*, an endangered medicinal plant using nodal segment as explants. The described protocol for multiple shoot regeneration with TDZ is simple, efficient and can be applied for the large-scale propagation and conservation of this valuable species.

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