

## EFFECTS OF PLANT GROWTH REGULATORS ON HIGH FREQUENCY SHOOT MULTIPLICATION AND CALLUS REGENERATION OF AN IMPORTANT INDIAN MEDICINAL PLANT, NIRGUNDI (*VITEX NEGUNDO* L.)

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### SUMMARY

A rapid shoot multiplication protocol was established for an important medicinal plant, *Vitex negundo* L., belonging to the family *Verbenaceae*, using Murashige and Skoog medium, achieved by shoot multiplication as well as callus regeneration. Shoot multiplication was induced by different concentrations of 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), Benzyladenine and 6-furfuryl amino purine separately along with 10% (v/v) coconut water. Green organogenetic callus was obtained by the combined effect of 0.5–2.15  $\mu$ M TDZ and 1.7  $\mu$ M indole-3-acetic acid (IAA) along with 1% polyvinylpyrrolidone (PVP), and produced the maximum number of shoots when subcultured onto medium containing 2.7  $\mu$ M TDZ alone. Elongation of *in vitro* shoots was observed in MS medium containing 2.4  $\mu$ M gibberellic acid and rooting was induced by the combined effect of 1.71  $\mu$ M IAA and 1.62  $\mu$ M  $\alpha$ -naphthalene acetic acid.

**Key words:** acclimatization; coconut water; multiple shoots; organogenesis; *Verbenaceae*; *Vitex negundo* L.

### INTRODUCTION

*Vitex negundo* L. (Nirgundi) is a small tree seen in India, Ceylon and China and is common at an altitude of 1500 m in the outer Himalayas. Innumerable properties are attributed to the plant, *viz.* astringent, cephalic, stomachic, anthelmintic and angiogenic (Choi et al., 2002). The entire plant is medicinally significant. Leaves are aromatic and used as a vermifuge (Bhargava, 1986; Nadkarni, 1989). The plant is a rich source of two active compounds, betulinic acid and ursolic acid, which are used as antifeedant and antibacterial compounds that protect plants from insect pests (Chandramu et al., 2003). The snake venom neutralizing activity of the plant extracts of *Vitex negundo* is also reported (Alam and Gomes, 2003). Unrestricted exploitation of this natural resource for pharmaceutical applications along with insufficient attempts for its replenishment has led to dwindling of the wild stock of this rare medicinally important plant species. Conventional propagation with vegetative cuttings is very slow, and the survival rate during planting is very limited (Sahoo and Chand, 1998). Propagation of *V. negundo* through seeds is hindered due to poor germination. However, there have been few reports on the micropropagation of *Vitex negundo* (Sahoo and Chand, 1998; Thiruvengadam and Jayabalan, 2001; Chandramu et al., 2003). No more work has been done on establishing protocols for callus induction and indirect organogenesis even though callus cultures can play an important role in the production of secondary metabolites (Mulabagal and Tsay, 2004). Earlier work on this plant by Sahoo and Chand (1998)

reported the inhibition of shoot multiplication by 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), and they observed seasonal variation for shoot multiplication where no positive result was observed during March–April. The present study was undertaken to establish a rapid propagation protocol for shoot multiplication and callus regeneration as a conservation measure.

### MATERIALS AND METHODS

**Plant materials.** Shoot tip/nodal segments (about 1–1.5 cm) were excised from 6-month old plants during the month of March from the Botany Department campus (Kerala), India. Leaves were separated from the nodal segments carrying dormant axillary buds. Explants were washed well under running tap water for 30 min, were treated with 10% (v/v) labolene (a neutral detergent, Qualigens, India) for 10 min and washed thoroughly with sterile water. The surface sterilization was done with 0.1% (w/v) aqueous mercuric chloride for 5 min and finally washed six to seven times with sterile double-distilled water.

**Preliminary studies.** When preliminary experiments were conducted in the absence of coconut water for shoot multiplication, explants were found to be healthy in all the growth regulator combinations, but the percentage of response was low. Hence, 5–20% (v/v) coconut water (CW) was used as an undefined supplement along with different growth regulators under study and we observed 10% CW to be optimum for shoot multiplication. Likewise, callus regeneration was found to be hindered by the slight browning due to phenolics and hence 0.5–2% (w/v) polyvinyl pyrrolidone (PVP) was provided in the medium along with 3  $\mu$ M IAA or 2,4-D and fresh weight of callus per explant after 18 d of inoculation was calculated.

**Culture conditions for the callus and multiple shoot induction.** Shoot tips (0.5–0.8 cm), leaf segments with midrib (7 × 10 mm) and stem segments (1.0–1.5 cm) were cultured on basal Murashige and Skoog's medium (MS) (Murashige and Skoog, 1962) supplemented with 100 mg l<sup>-1</sup> myo-inositol, 3% (w/v) sucrose and 0.8% (w/v) agar (Hi-media, India). Basal medium was further fortified with 3–19  $\mu$ M 6-benzyladenine (BA), 2.3–11  $\mu$ M 6-furfuryl amino purine (kinetin), or

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TABLE 1

MORPHOGENETIC RESPONSE OF NODAL EXPLANTS OF *V. NEGUNDO* TO DIFFERENT CONCENTRATIONS OF CYTOKININS ALONG WITH 10% (V/V) CW

Cytokinin	Concentration ( $\mu\text{M}$ )	% Explants responding with shoots (mean $\pm$ SE)	Number of shoots/explant (mean $\pm$ SE)	Shoot length (cm) (mean $\pm$ SE)
Kinetin	2.3	34.62 $\pm$ 3.11 ef	1.12 $\pm$ 0.15 ef	3.11 $\pm$ 0.15
	4.6	52.12 $\pm$ 1.87 de	2.58 $\pm$ 0.12 d	3.98 $\pm$ 0.16
	6.0	62.05 $\pm$ 4.12 d	3.21 $\pm$ 0.21 cd	4.32 $\pm$ 0.12
	9.2	50.67 $\pm$ 3.18 de	3.01 $\pm$ 0.13 cd	4.98 $\pm$ 0.15
	11.5	48.59 $\pm$ 4.01 e	2.68 $\pm$ 0.23 d	4.01 $\pm$ 0.07
	13.8	42.59 $\pm$ 2.12 e	2.02 $\pm$ 1.07 de	3.68 $\pm$ 0.13
	16.1	39.09 $\pm$ 2.69 e	1.93 $\pm$ 0.14 e	3.41 $\pm$ 0.11
	18.4	32.51 $\pm$ 1.75 ef	1.20 $\pm$ 0.21 ef	2.96 $\pm$ 0.19
BA	2.2	61.67 $\pm$ 2.32 d	2.31 $\pm$ 0.23 de	3.17 $\pm$ 0.11
	4.4	79.98 $\pm$ 1.35 bc	3.62 $\pm$ 0.12 cd	4.89 $\pm$ 0.23
	6.6	85.95 $\pm$ 4.13 b	4.51 $\pm$ 0.08 c	5.01 $\pm$ 0.12
	8.9	76.52 $\pm$ 2.43 bc	3.38 $\pm$ 0.11 cd	4.72 $\pm$ 0.21
	11.1	73.39 $\pm$ 2.04 c	3.05 $\pm$ 0.25 cd	3.87 $\pm$ 0.09
	13.2	69.25 $\pm$ 2.00 c	2.62 $\pm$ 0.09 d	3.05 $\pm$ 0.18
	15.4	65.87 $\pm$ 1.82 cd	1.98 $\pm$ 0.08 e	2.11 $\pm$ 0.20
TDZ	0.5	68.06 $\pm$ 1.62 c	2.12 $\pm$ 0.16 de	
	1.0	73.39 $\pm$ 1.54 c	4.31 $\pm$ 0.12 c	
	1.5	85.32 $\pm$ 1.91 b	9.59 $\pm$ 0.23 b	
	1.8	96.59 $\pm$ 2.52 a	14.62 $\pm$ 0.26 a	
	2.0	89.95 $\pm$ 2.19 b	12.57 $\pm$ 0.24 ab	ND <sup>z</sup>
	2.5	72.32 $\pm$ 3.10 c	7.39 $\pm$ 0.19 bc	
	2.75	69.68 $\pm$ 2.15 c	3.87 $\pm$ 0.16 cd	
	3.0	67.1 $\pm$ 1.90 c	2.64 $\pm$ 0.09 d	

Means of the number of shoots followed by the same letter within a column are not significantly different according to Tukey test at 5% level.

<sup>z</sup> ND, not determined because of their stunted nature.

0.225–5  $\mu\text{M}$  1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) separately for shoot multiplication along with 10% (v/v) coconut water (shoot tips/nodal segments placed vertically). Leaf (adaxial surface towards the medium) explants and stem segments were cultured horizontally on media containing 1% (w/v) PVP (polyvinyl pyrrolidone), supplemented with 0.1–5  $\mu\text{M}$  indole-3-acetic acid (IAA) and 0.1–6  $\mu\text{M}$  2,4-dichlorophenoxy acetic acid (2,4-D) individually or either in conjunction with TDZ (0.1–4  $\mu\text{M}$ ) or BA (1–25  $\mu\text{M}$ ) for callus induction. pH of the medium was adjusted to 5.8 before adding agar. Media (20 ml) were dispensed in test tubes of 150  $\times$  25 mm size and autoclaving was done at 121°C for 15 min. After inoculation, cultures were incubated at 25  $\pm$  2°C and 70–90% relative humidity under cool white fluorescent light (Philips, India) with a total irradiance of 36  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for a photo period of 12/12 h. Further subculturing for callus proliferation as well as shoot multiplication were done onto 70 ml media in 250 ml conical flasks (Borosil, India). Each treatment consisted of 10 explants and was replicated 10 times, representing a total of 100 explants per treatment. The rate of shoot multiplication as well as callus formation was determined after four weeks.

**Elongation medium.** In order to study the effect of gibberellic acid ( $\text{GA}_3$ ) on elongating stunted shoots, shoots induced by 1.8  $\mu\text{M}$  TDZ were excised and placed onto media containing different concentrations of  $\text{GA}_3$  (1.2–4.8  $\mu\text{M}$ ). The results were observed after 14 d using 24 replicates per treatment.

**Multiplication of cultures.** *In vitro* shoots (28 d old) were cut into single nodes carrying dormant axillary buds and cultured on MS medium supplemented with 10% (v/v) CW and 1.8  $\mu\text{M}$  TDZ for the induction of multiple shoots. Subcultures were done at 4-week intervals to study the effect of culture passages on the explant response for shoot induction and multiple shoot formation. Each time, the stunted shoots were grown for 14 d in elongation medium containing 2.4  $\mu\text{M}$  gibberellic acid ( $\text{GA}_3$ ).

**Callus regeneration.** Dark green calluses proliferated by the combined effect of 1.7  $\mu\text{M}$  IAA and 0.5–2.15  $\mu\text{M}$  TDZ were continuously subcultured onto fresh medium or to media containing different concentrations of TDZ or BA alone or in conjunction with 0.5–4.5  $\mu\text{M}$  IAA. In all these cases, the medium was provided with 1% (w/v) PVP.

**Rooting of shoots.** The effect of auxins on root induction was studied by placing excised shoots measuring 1.5–2.0 cm into 25–150 mm test tubes (Borosil, India Ltd.), each containing 15 ml  $\frac{1}{2}$ -strength MS basal medium with various concentrations of  $\alpha$ -naphthalene acetic acid (NAA) (0.54–2.7  $\mu\text{M}$ ), IAA (0.57–2.85  $\mu\text{M}$ ) and combinations of both. A set of 15 excised shoots with four replicates cultured on  $\frac{1}{2}$ -strength MS medium without growth regulators served as a control.

**Acclimatization and transfer to field.** Rooted plantlets from the culture medium were gently washed with tap water, rinsed with sterile water and transferred to plastic pots containing sterile vermiculite (local made), nourished with 1/10-strength Hoagland's solution (Epstein, 1972) on every seventh day. The pots were covered with polythene bags containing a few pores to allow gas exchange. After about 25 d, the plants were watered for 3 weeks and were transferred to sterilized garden soil and were exposed to sunlight 3 h d<sup>-1</sup>. After another 10 d, plantlets were transferred to a glasshouse at 25  $\pm$  3°C and 80–85% relative humidity and were successfully transferred outside.

**Statistical analysis.** All experiments were repeated three times with 10 replicates unless otherwise stated and the data were analysed statistically by analysis of variance followed by the Tukey test, with the level of significance set at 5%.

## RESULTS

**Initiation and proliferation of nodal cultures.** Nodal segments were found to be better than shoot tip explants in all growth regulator combinations tested. Significant differences (by Tukey test at 5% level) were observed for shoot multiplication when the type of cytokinin used was varied. Of the three cytokinins tested, TDZ was found to yield the maximum number of shoots from a single nodal explant (14.6  $\pm$  0.26) at a concentration of 1.8  $\mu\text{M}$

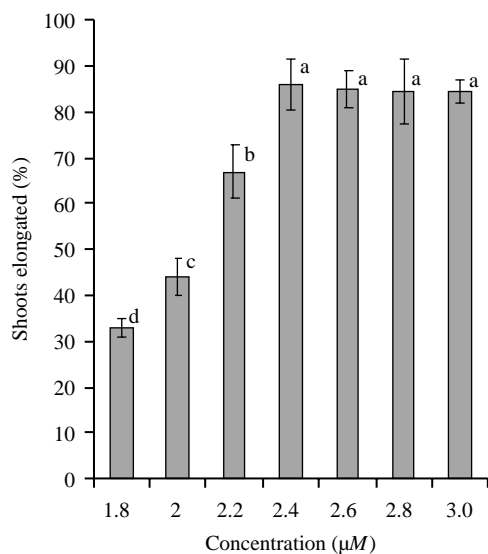


FIG. 1. Effect of GA<sub>3</sub> on elongation of stunted shoots of *V. negundo* induced by TDZ. Bars with the same letter are not significantly different according to Tukey test at 5% level.

in the medium (Table 1). MS containing 1.8 μM TDZ induced bud break in 96% of the nodal explants within 12–15 d. Unlike adenine-type cytokinins (BA and KN), TDZ was more effective at lower concentrations.

**Elongation of stunted shoots.** Though TDZ was found to stunt the shoots (Fig. 2A), elongation was induced by growing the stunted shoots in MS containing GA<sub>3</sub>. A concentration of 2.4 μM was found to induce elongation of 86.8 ± 2.14% stunted shoots formed by 1.8 μM TDZ (Fig. 1) and the individual plantlets grew normally (Fig. 2B).

**Multiplication of shoots.** The highest response of nodal explants (96–100%) and maximum number of shoots per explant (an average of 14) were observed in the first eight culture passages and then there was a gradual decline followed by necrosis of the shoots at the 14th culture passage (data not shown).

**Callus induction and regeneration.** Explants showed an expansion after one week of culture and developed callus within

12 d. Callus was initially developed at the cut regions, then spreading to other regions. The frequency of explants developing callus varied from 71–100% for both explants depending on the PGR used. Of the different types/combination of PGRs used, the best results were obtained (in terms of fresh weight and nature of callus) with 1.7 μM IAA and 0.5–2.15 μM TDZ (data not shown). When the above callus was subcultured onto different PGRs, after a period of 25 d, maximum number of shoot buds were observed in medium containing 2.7 μM TDZ alone. Better regeneration was observed when cytokinins were provided in the medium alone than when combined with an auxin (Table 2). TDZ was found to be better than BA, as was the case in the study of shoot multiplication. The leaf-derived callus showed better regeneration compared to that derived from stem explants. Highest frequency of proliferation was observed from both explants in the presence of TDZ. The *in vitro* developed shoots were treated in the same way as the multiple shoots for further elongation, rooting and acclimatization.

**Rooting of micro shoots.** No rooting was observed on half-strength or full-strength MS medium without growth regulators even after 45 d of culture. The best rooting was obtained using the combined effect of 1.71 μM IAA and 1.62 μM NAA (Table 3) (Fig. 2C).

**Acclimatization.** Plants showed very good survival rates when transferred to the natural soil and grew normally without any detectable variation among the potted plants with respect to morphological and growth characteristics and were free from external defects (Fig. 2D).

## DISCUSSION

Treatment with 1.8 μM TDZ was found to be better than other growth regulators for shoot multiplication. Though this is contradictory to the report by Sahoo and Chand (1998) where they report the least number of shoots in the presence of TDZ, high shoot multiplication in our result might be due to the synergistic effect of coconut water. Comparing BA and kinetin, the former was found to be more effective than the latter. This is in concordance with the results of Sahoo and Chand (1998) and Chandramu et al. (2003). The optimum concentration of BA required in our study (6.6 μM)

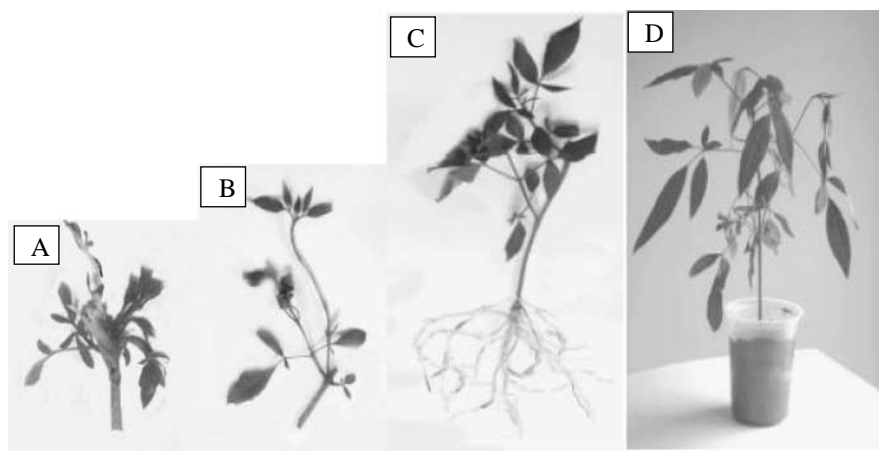


FIG. 2. Different stages in the micropropagation of *V. negundo*. A, Stunted multiple shoots from single nodal explants of *V. negundo* on MS medium supplemented with 1.8 μM TDZ. B, Elongation of stunted shoots on MS medium supplemented with 2.4 μM GA<sub>3</sub>. C, Rooting of microshoots by the combined effect of 1.7 μM IAA and 1.62 μM NAA. D, Acclimatized micropropagated plants after 12 d.

TABLE 2  
EFFECT OF PGRs ON *V. NEGUNDO* CALLUS REGENERATION DURING SUBCULTURING<sup>x</sup>

PGRs ( $\mu M$ )			Percentage of callus regeneration <sup>y,z</sup>		Number of shoots per explant	
IAA	BA	TDZ	Leaf	Stem	Leaf	Stem
1.7	–	1.0	54 cd	36 d	8.5 $\pm$ 0.17 b	6.39 $\pm$ 0.72 b
1.7	–	1.3	55 cd	39 d	9.9 $\pm$ 1.01 b	7.06 $\pm$ 1.02 b
1.7	–	1.5	79 b	60 c	14.17 $\pm$ 0.92 a	10.14 $\pm$ 0.87 ab
1.7	–	1.8	85 b	72 bc	15.11 $\pm$ 0.72 a	12.78 $\pm$ 0.31 ab
1.7	–	2.0	84 b	70 bc	15.32 $\pm$ 0.69 a	12.61 $\pm$ 0.46 ab
1.7	–	2.15	83 b	69 bc	14.87 $\pm$ 0.39 a	11.39 $\pm$ 0.42 ab
–	–	1.0	20 e	19 e	3.12 $\pm$ 0.07 c	3.03 $\pm$ 0.09 c
–	–	1.5	41 d	31 de	7.14 $\pm$ 0.06 b	5.09 $\pm$ 0.1 bc
–	–	2.0	73 bc	65 c	13.05 $\pm$ 0.13 ab	10.98 $\pm$ 0.06 ab
–	–	2.5	94 ab	89 ab	16.31 $\pm$ 0.03 a	16.12 $\pm$ 0.05 a
–	–	2.7	100 a	83 b	18.1 $\pm$ 0.1 a	14.31 $\pm$ 0.02 a
–	–	2.9	100 a	81 b	18.3 $\pm$ 0.05 a	14.53 $\pm$ 0.09 a
–	–	3.1	98 a	80 b	17.24 $\pm$ 0.07 a	14.04 $\pm$ 0.4 a
–	–	3.3	98 a	80 b	17.24 $\pm$ 0.02 a	14.05 $\pm$ 0.05 a
–	–	3.5	97 a	79 b	16.19 $\pm$ 0.3 a	13.01 $\pm$ 0.13 ab
–	1.3	–	11 ef	10 ef	1.97 $\pm$ 0.7 c	1.8 $\pm$ 0.3 c
–	2.2	–	23 e	29 de	4.12 $\pm$ 0.03 bc	5.31 $\pm$ 0.32 bc
–	4.4	–	49 cd	32 de	8.79 $\pm$ 0.1 b	5.67 $\pm$ 0.6 bc
–	6.6	–	60 c	51 cd	10.67 $\pm$ 0.7 ab	9.12 $\pm$ 0.1 b
–	8.9	–	73 bc	65 c	13.21 $\pm$ 0.06 ab	11.65 $\pm$ 0.43 ab
–	11.1	–	72 bc	63 c	12.92 $\pm$ 0.09 ab	11.32 $\pm$ 0.1 ab
–	13.2	–	69 bc	63 c	12.41 $\pm$ 0.07 ab	11.31 $\pm$ 0.02 ab
–	15.4	–	68 c	60 c	11.92 $\pm$ 0.07 ab	10.02 $\pm$ 0.15 ab
–	22	–	68 c	60 c	11.92 $\pm$ 0.1 ab	10.02 $\pm$ 0.32 ab
0.5	–	1.5	56 cd	40 d	9.96 $\pm$ 0.23 b	7.12 $\pm$ 0.78 b
1.5	–	1.5	78 b	57 cd	14.95 $\pm$ 0.05 a	9.96 $\pm$ 0.06 b
2.5	–	1.5	80 b	62 c	14.17 $\pm$ 0.02 a	11.25 $\pm$ 0.32 ab
3.5	–	1.5	73 bc	58 cd	13.19 $\pm$ 0.32 ab	10.03 $\pm$ 0.09 ab
4.5	–	1.5	65 c	41 d	11.93 $\pm$ 0.31 ab	7.05 $\pm$ 0.07 b
0.5	4.4	–	46 cd	32 de	8.19 $\pm$ 0.26 b	5.69 $\pm$ 0.05 bc
1.5	4.4	–	48 cd	36 d	8.61 $\pm$ 0.09 b	6.37 $\pm$ 0.08 b
2.5	4.4	–	54 cd	41 d	9.67 $\pm$ 0.13 b	7.35 $\pm$ 0.14 b
3.5	4.4	–	46 cd	37 d	8.27 $\pm$ 0.06 b	6.72 $\pm$ 0.32 b
4.5	4.4	–	42 d	31 de	7.54 $\pm$ 0.11 b	5.61 $\pm$ 0.21 bc

<sup>x</sup> Callus was induced by the combined effect of 1.7  $\mu M$  IAA and 0.5–2.15  $\mu M$  TDZ.

<sup>y</sup> In terms of shoot bud induction.

<sup>z</sup> Values followed by the same *letter* within a *column* are not significantly different according to Tukey test at 5% level.

was much lower than that reported by Sahoo and Chand (8.9  $\mu M$ ) and Chandramu et al. (17.3  $\mu M$ ). This difference again may be due to the presence in our medium of CW (10%) which contains a cytokinin, ribosyl zeatin (Letham, 1974). The organogenetic nature of CW can be attributed to the presence of cytokinin along with other undefined supplements. There are a number of reports showing the influence of TDZ on shoot organogenesis (Pelah et al., 2002; Ganeshan et al., 2003), but often the shoots induced by TDZ are stunted, as has been reported in several plant species such as apple (Van Nieuwkerk et al., 1986) and *Rhododendron* (Preece and Imel, 1991). The inhibition of shoot elongation may be due to the high cytokinin activity of TDZ, and cytokinins commonly stimulate shoot proliferation while inhibiting their elongation (Huetteman and Preece, 1993). While studying the influence of explanting period on culture establishment, Sahoo and Chand (1998) had reported that the collection periods of December to May

were not desirable for bud break. But in our study, good results were obtained even though we collected the explants during the month of March.

TDZ was found to be significant for callus regeneration also. It was observed that, though an auxin was required for the callus induction, further proliferation and shoot bud formation was more favoured by the cytokinin activity alone. Leaf derived callus showed more regeneration capacity than the stem derived ones. This difference between leaf and stem explant response to different growth regulators in media could be a reflection of probable differences of endogenous growth regulator levels in the explant sources or different tissue sensitivities to these plant growth regulators (Lisowska and Wysonkowska, 2000). PVP also might have contributed to the callus proliferation as no browning was observed during regeneration, compared to the browning we observed during our preliminary studies without PVP. The significant effect of PVP on callus regeneration

TABLE 3  
EFFECT OF DIFFERENT AUXINS ON THE ROOTING OF *IN-VITRO* SHOOTS OF *V. NEGUNDO*

Growth regulators ( $\mu\text{M}$ )		% Shoots with roots (mean $\pm$ SE)	Number of roots/shoot (mean $\pm$ SE)	Root length (cm) (mean $\pm$ SE)	% Plants surviving after hardening (mean $\pm$ SE)
IAA	NAA				
0.57	–	30.22 $\pm$ 3.12 <sup>y</sup>	2.25 $\pm$ 0.32 <sup>z</sup>	2.96 $\pm$ 0.07 <sup>z</sup>	75.8 $\pm$ 2.06 <sup>y</sup>
1.14	–	49.08 $\pm$ 1.33	3.32 $\pm$ 0.28	4.47 $\pm$ 0.08	78.7 $\pm$ 1.68
1.71	–	60.13 $\pm$ 0.69	4.04 $\pm$ 0.17	4.98 $\pm$ 0.24	83.54 $\pm$ 1.12
2.28	–	32.34 $\pm$ 1.69	2.18 $\pm$ 0.05	4.05 $\pm$ 0.17	75.8 $\pm$ 2.31
2.85	–	13.18 $\pm$ 2.35	1.27 $\pm$ 0.03	2.56 $\pm$ 0.19	72.65 $\pm$ 1.03
–	0.54	19.95 $\pm$ 2.98	1.49 $\pm$ 0.15	2.85 $\pm$ 0.21	72.27 $\pm$ 1.32
–	1.08	26.09 $\pm$ 2.11	2.36 $\pm$ 0.02	3.08 $\pm$ 0.14	75.39 $\pm$ 1.65
–	1.62	40.85 $\pm$ 1.93	3.65 $\pm$ 0.16	4.96 $\pm$ 0.18	77.16 $\pm$ 1.06
–	2.16	23.42 $\pm$ 2.54	1.48 $\pm$ 0.08	2.36 $\pm$ 0.07	70.33 $\pm$ 2.45
–	2.70	13.36 $\pm$ 2.27	1.22 $\pm$ 0.05	2.34 $\pm$ 0.15	70.25 $\pm$ 1.98
0.57	0.54	40.75 $\pm$ 1.94	3.17 $\pm$ 0.21	4.62 $\pm$ 0.15	78.45 $\pm$ 2.01
1.14	1.08	51.66 $\pm$ 2.08	4.38 $\pm$ 0.01	5.05 $\pm$ 0.08	80.29 $\pm$ 0.09
1.71	1.62	95.09 $\pm$ 0.09	8.86 $\pm$ 0.08	7.39 $\pm$ 0.06	88.71 $\pm$ 1.08
2.28	2.16	67.26 $\pm$ 1.22	5.39 $\pm$ 0.12	4.2 $\pm$ 0.16	86.69 $\pm$ 2.56
2.85	2.70	32.44 $\pm$ 2.17	2.96 $\pm$ 0.25	4.32 $\pm$ 0.11	83.71 $\pm$ 1.10

<sup>y</sup> Values shown were obtained from 4 replicates of 15 microcuttings each.

<sup>z</sup> Values are means of 15 microcuttings.

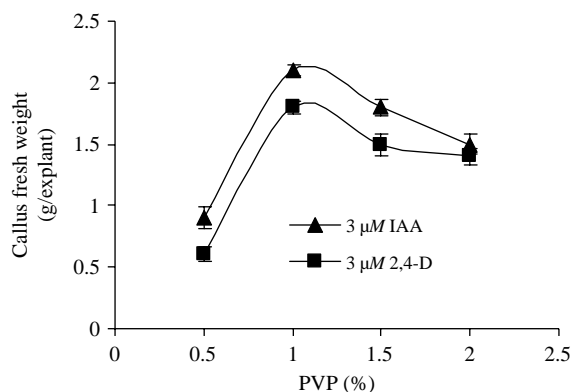


FIG. 3. Effect of PVP on callus induction (callus fresh weight after 18d of inoculation).

(Fig. 3) might be due to its ability to bind phenolics and some toxins thereby protecting the cultures from turning brown (Chang and Yang, 1996). The protocol established can serve as a very efficient method for the conservation of this endangered medicinal plant for the extraction of secondary metabolites.

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