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ORIGINAL PAPER

Pretreatment in thidiazuron improves the in vitro shoot induction from leaves in *Curculigo orchioides* Gaertn., an endangered medicinal plant

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Abstract Leaf regeneration via direct induction of adventitious shoots obtained from an endangered medicinal plant, *Curculigo orchioides* Gaertn. by pretreating with thidiazuron. *C. orchioides* is an endangered medicinal herb belonging to the family Hypoxidaceae. Direct inoculation of leaf pieces on MS medium supplemented with various concentrations of BAP (2–8 μM) or TDZ (2–8 μM) alone or in combination with NAA (0.5 and 1.0 μM) produced low shoot induction both in terms of % response and number of shoots per explant. Hence, leaf explants were pretreated with 15, 25 or 50 μM thidiazuron (TDZ), for 6, 24 or 48 h with the aim of improving shoot regeneration from cultured explants. After pretreatment, explants were transferred to an agar solidified MS medium that was supplemented with BAP (4 μM), TDZ (6 μM), BAP (4 μM) + NAA (1.0 μM), TDZ (6 μM) + NAA (0.5 μM). Control explants were incubated directly on the medium without any pretreatment. The pretreatment of explants with 15 μM TDZ for 24 h significantly promoted the formation of adventitious shoots and the maximum response was observed on MS medium supplemented with 6 μM TDZ. In this medium, 96 % cultures responded with an average number of 16.2 adventitious shoots per explant. The percentage of leaf explants producing shoots and the average number of shoots per explant were significantly improved when TDZ pretreated leaves were cultured onto MS medium supplemented with BAP or TDZ alone or in combination with NAA. The rooted plantlets were successfully transplanted to soil with 90% success. The present investigation indicated the stimulatory role of TDZ pretreatment in regulating shoot regeneration from leaf explants of *C. orchioides*.

Keywords *Curculigo orchioides* · Endangered · Leaf regeneration · Medicinal plant · Pretreatment

Abbreviations

BA	6-Benzylaminopurine
Kn	Kinetin
MS	Murashige and Skoog (1962) medium
TDZ	Thidiazuron (N-phenyl N′ 1,2,3-thidiazol-5-yl urea)

Introduction

Curculigo orchioides Gaertn. is an endangered, medicinally important perennial herb belonging to the family Hypoxidaceae (Dhar et al. 1968). This stemless herb is seen in Western Ghats and appears in the forests immediately after monsoon rains. The leaves, root and rhizome of *Curculigo* are medicinally useful (Bhamare 1998; Jain 1991). The entire plant has hypoglycaemic, spasmolytic, aphrodisiac, diuretic, antipyretic, antiinflammatory and anticancer principles (Chandel 1996; Kurma and Mishra 1995). In Chinese medicine *Curculigo* is used as a tonic for the treatment of decline in physical strength (Anonymous 1979). The rhizome is used against piles, asthma, jaundice, diarrhoea, etc (Kiritkar and Basu 1935). The rhizome extract of this plant consists of a number of useful compounds like flavonone glycoside I, steroids, saponins and triterpenoids including curculigol, corchioside A, curculigoside,

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curculigenin A-C, curculigo saponin A-F (Garg et al. 1989, Xu and Xu 1992, Xu et al. 1992, Tandon and Shukla 1995). The anticarcinogenic activity of this plant against sarcoma 180 in mouse is also reported (Dhar et al. 1968). Flavanone glycoside I possesses powerful uterine stimulant activity and *C.orchioides* has been used along with some other plants in a number of pharmaceutical formulations in the Indian system of medicine as a metabolic enhancer and aphrodisiac.

This subterranean herb is naturally available only during rainy season and due to excessive exploitation, the species has become endangered. *C. orchioides* propagates naturally through seeds but poor seed set and germination are the limiting factors for natural propagation (Gupta and Chadha 1995). Plant tissue culture can enable the large-scale propagation of this endangered plant and thus save this precious medicinal plant from extinction.

There are some reports on the micropropagation of *C. orchioides* (Suri et al. 1998, 1999; Prajapati et al. 2003, 2004; Thomas and Jacob 2004). The rate of shoot induction from leaf explants in *C. orchioides* is very low in all the previous reports. In an earlier study, the effect of BAP and 2, 4-D on shoot regeneration from leaf explants were investigated. MS medium supplemented with BAP at 0.88 μM gave maximum response of 1.44 shoots per explant among the various BAP concentrations tried (Prajapati et al. 2003). In another report on leaf regeneration, B_5 medium containing BAP at 4.4 μM concentration gave maximum response of 4.0 shoots per explant (Suri et al. 1999). Prajapathi et al. (2004) reported in vitro callus regeneration in *C. orchioides*. Here also the regeneration frequency was moderate (8.36 shoots per culture). Hence, the present investigation aims to improve the shoot regeneration capacity from leaf explants of *C. orchioides* by pretreating the explant with various concentrations of TDZ at different time periods. Recently, thidiazuron (TDZ–N-phenyl N'–1,2,3-thidiazol-5-yl urea), a substituted urea with cytokinin activity, has been extensively used for plant regeneration. TDZ is found to be as or more efficient than 6-Benzyl amino purine (BAP) for shoot induction (Van Nieuwkirk et. al. 1987) and adventitious shoot regeneration from leaves, cotyledons and hypocotyls of several plant species (Fasolo et. al. 1989; Bretague et. al. 1994; Prathanturarug et al. 2003; Passey et al. 2003; Gu and Zhang 2005; Zhang et al. 2005).

Materials and methods

The mature young leaf explants were taken from in vitro maintained healthy plantlets on MS (Murashige and Skoog 1962) basal medium. The leaves were fully expanded and green at the time of inoculation. The mature leaves (about 40 days old) were excised from the parent plant and cut into pieces with each piece having a size of 1× 1 cm (Length × width). The leaf pieces were first cultured directly on MS semisolid medium supplemented with BAP (2–8 μM), TDZ (2–8 μM) alone and in the second step, the best medium from these concentrations were selected (here 4 μM BAP and 6 μM TDZ) and treated with NAA (0.5 and 1.0 μM). The advantage of using in vitro raised leaves is that it can avoid treating the tender leaves with harmful surface sterilizing agents.

In the second set of experiments, the excised leaves were soaked in MS liquid medium supplemented with three different concentrations of TDZ (15, 25 and 50 μM) and maintained on a shaker for three different time periods (6, 24 and 48 h).

Following the initial pretreatment, the leaves were cut into pieces (1 × 1 cm; length × width) using a surgical blade. The excised leaves were cultured with its abaxial side down on MS semisolid medium with 0.8% agar and supplemented with 3% sucrose and either BAP (4 μM) or TDZ (6 μM) alone or in combination with NAA (4 μM BAP + 1 μM NAA; 6 μM TDZ + 0.5 μM NAA) in flasks and test tubes.

When the shoots had reached 2.3 cm in height, they were transferred onto half strength MS medium supplemented with IBA or NAA (1–5 μM) to induce root formation. Rooted plantlets were then separated and planted on autoclaved garden soil in plastic cups after washing off the medium with tap water. The plastic cups were covered with polythene bags with small holes, placed in a greenhouse under a maximum photosynthetically photon flux density of 200 μmol m^{-2} s^{-1}, temperature range of 20–28°C, and relative humidity of 70–100% and irrigated with a solution of 1/2-strength MS inorganic salts at 2- to 3-day intervals. The hardened plants were ultimately transplanted to field for evaluation.

Culture conditions

The media were steam sterilized in an autoclave under 1.5 kg/cm^2 and 121°C for 15 min. All the cultures were grown at 25 ± 2°C under 16 h photoperiod, (two Philips TL 40 W fluorescent tubes—irradiance of 200 μmol $m^{-2}s^{-1}$). At least 24 cultures were raised for each treatment and all experiments were repeated three times. Analysis of variance and Duncan's multiple range test was used for comparison among treatment means.

Results and discussion

The shoot induction from leaf explants was reported previously in *C. orchioides*. However, the direct inoculation of

untreated leaf explants on media often produce low shoot number (Suri et al. 1999; Prajapati et al. 2003, 2004). The present investigation describes high frequency shoot induction from TDZ pretreated leaf explants in *C. orchioides*.

Initially, the efficiency of shoot induction from leaf explants without pretreatment were analysed on MS medium supplemented with different concentrations of BAP (2–8 μM) and TDZ (2–8 μM) alone or in combination with 0.5 and 1.0 μM NAA (Table 1). Of the different concentrations of BAP tried, 4 μM gave optimum result. On this medium a maximum of 42% cultures responded with an average number of 4.8 shoots per explant (Fig. 1a). TDZ at 6 μM gave a maximum of 46% cultures responded with an average number of 5.1 shoots per explant, 45 days after culture. In order to improve shoot induction further, NAA (0.5 and 1 μM) was added to the medium. The presence of NAA along with BAP or TDZ in the medium further improved the shoot induction both in terms of percent cultures responding as well as number of shoots per explant. Among the two NAA concentrations tried along with 4 μM BAP, 1.0 μM gave optimum result. Here a maximum of 56% cultures responded with an average number of 5.7 shoots per explant. Similarly, among the two NAA concentrations employed along with 6 μM TDZ, 0.5 μM NAA showed optimum result. On this medium, 51% cultures responded with an average number of 5.4 shoots per culture (Fig. 1b). Even though there are several reports on high frequency shoot induction from BAP and TDZ alone or in combination with NAA from different explants (Herve et al. 2001; Yancheva et al. 2003; Lee et al. 2003; Geneve 2005; Guo et al. 2005; Raghu et al. 2006; Landi and Mezzetti 2006; Espinosa et al. 2006), in the present study, the shoot induction was poor and the time required for shoot induction was also more. Comparatively, the presence of NAA along with BAP or TDZ improved shoot induction as compared to BAP or TDZ alone.

Table 1 Effect of different concentrations of BAP and TDZ alone or in combination with 0.5 and 1.0 μM NAA on shoot regeneration from the leaf explants of *C. orchioides*

Plant growth regulator concentrations (μM)			Percent response	No. of shoots/ Culture[a]	Average shoot length (cm)[a]
BAP	TDZ	NAA			
0	0	0	0	0	0
2			35b	2.4 ± 0.5a	0.6 ± 0.2a
4			42d	4.8 ± 0.7c	0.9 ± 0.1b
6			38c	3.7 ± 0.6b	0.8 ± 0.2a
8			33b	2.9 ± 0.5a	0.7 ± 0.1a
	2		25a	3.3 ± 0.4b	0.7 ± 0.3a
	4		33b	4.1 ± 0.4b	0.6 ± 0.2a
	6		46d	5.1 ± 0.7c	0.9 ± 0.3b
	8		40c	4.0 ± 0.8b	0.6 ± 0.1a
4		0.5	40c	4.3 ± 0.5b	0.6 ± 0.2a
4		1.0	56g	5.7 ± 0.8d	1.4 ± 0.2b
	6	0.5	51f	5.4 ± 0.9d	1.3 ± 0.3b
	6	1.0	48e	4.4 ± 0.6c	0.8 ± 0.2a

The culture period was 45days. Each treatment consisted of 24 cultures each. All experiments were repeated three times

Means within a column followed by the same letter are not significantly different using Duncan's multiple range test ($P \geq 0.05$)

[a] The values represent the mean (±SE) of three independent experiments

Generally, the development of shoots from the explant was slow in all the plant growth regulator combinations. The emergence of small granular out growths from the cut ends of leaf explants were noticed 1 week after culture. These shoots enlarged in size and reached an average length of about 0.9 cm in 45 days of culture. The adventitious shoot formation was observed only on the two cut ends of the explant. The induction frequency as well as number of shoots per explant was low in all the plant growth regulator combinations employed. Hence, a new strategy of pretreatment of explants was tried to improve the shoot induction further. The control treatment, i.e. MS medium without any growth regulators, did not induce any shoot induction.

Explant pretreatment with different plant growth regulators is a common technique generally employed for getting maximum results (Kintzios et al. 2002; Shan et al. 2005; D'Onofrio and Morini 2006). Recently, it was reported that the pretreatment of explants in TDZ is more effective than other plant growth regulators including *Curcuma longa* (Prathanturarug et al. 2003, 2005) and rose (Singh and Syamal 2001). In the present investigation, explant pretreatment with various concentrations of TDZ (15, 25 and 50 μM) for different time periods (6, 24 and 48 h) had a very significant effect on shoot induction as presented in Figs. 2, 3, 4, 5.

The pretreated leaves did not produce any shoots on growth regulator free medium. Figure 2 shows the effect of 4 μM BAP on induction of shoots from leaf explants after pretreating with TDZ (15, 25 and 50 μM) for three different time periods (6, 24 and 48 h). Pretreatment of explants with TDZ at 25 μM for 6 and 24 h significantly improved the shoot induction. An average of 87% cultures responded and 11.1 shoots per explant emerged when the explants were pretreated with 25 μM TDZ for 6 h and cultured on 4 μM BAP. The maximum response was observed when the explant was pretreated with TDZ at 25 μM for 24 h and subsequently cultured on MS medium supplemented with BAP (4 μM). Here an average of 90% cultures responded with 12.5 shoots per explant. The shoots reached an average length of 2.3 cm in 45 days. When

Fig. 1 Direct shoot regeneration from leaf regeneration explants of *C. orchioides*. **a**, **b** shows leaf regeneration from untreated leaf explants, and **c, d** from TDZ pretreated leaf explants. Culture period: 45 days. **a** Leaf regeneration on MS medium supplemented with BAP (4 μM) after 45 days of culture. Two large shoots and some small shoots have emerged from the leaf. **b** Shoot regeneration on MS medium supplemented with 6 μM TDZ + 0.5 μM NAA. Five shoots have already emerged from the explant. **c** Shoot regeneration from leaf explants pretreated with 15 μM TDZ for 24 h and cultured on MS medium supplemented with 6 μM TDZ. About 15 shoots have emerged from one leaf explant. **d** A bunch of five shoots taken out from the above culture (i.e. 6 μM TDZ). Each shoot will be separated and subcultured for rooting

pretreated leaf explants were cultured on BAP supplemented medium, shoot initials were found emerging from all over the leaf areas in 10 days of culture. There was no callus formation in any of the cultures.

Figure 3 indicates the effect of 6 μM TDZ on induction of shoots from leaf explants after pretreatment with TDZ. Here the optimum response was observed on 15 μM TDZ pretreated cultures for 24 and 48 h. When 24-h pretreated leaves were subcultured on 6 μM TDZ, 96% cultures responded with an average number of 16.2 shoots per explant (Fig. 1c, d). The pretreatment of TDZ at 15 μM for 48 h also showed a significantly higher shoot induction efficiency of the explant. Here an average of 94% cultures responded with 14.6 shoots per explant after 45 days of inoculation.

Comparatively 6 μM TDZ gave better response than 4 μM BAP. But the growth rate and average length of the shoots were same in both cases. The superiority of TDZ over BAP for shoot induction has been reported in several systems (Visser et al. 1992; Huetteman and Preece 1993; Lu 1993; Geneve 2005).

The pretreated explants were cultured on MS medium supplemented with BAP (4 μM) + NAA (1 μM) and TDZ

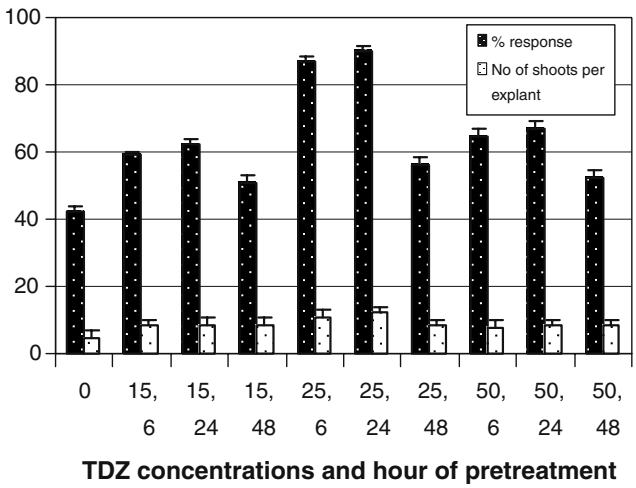

Fig. 2 Effect of MS medium supplemented with 4 μM BAP on induction of shoots from leaf explants after pretreating with TDZ at three different concentrations (15, 25 and 50 μM) for three different time periods (6, 24 and 48 h). The number 0 indicates the control treatment (i.e. 4 μM BAP without pretreatment) and the other numbers (15, 25 and 50) indicate the concentrations of TDZ and the hour of pretreatment (6, 24 and 48). Results expressed as means of three replicates ±SD

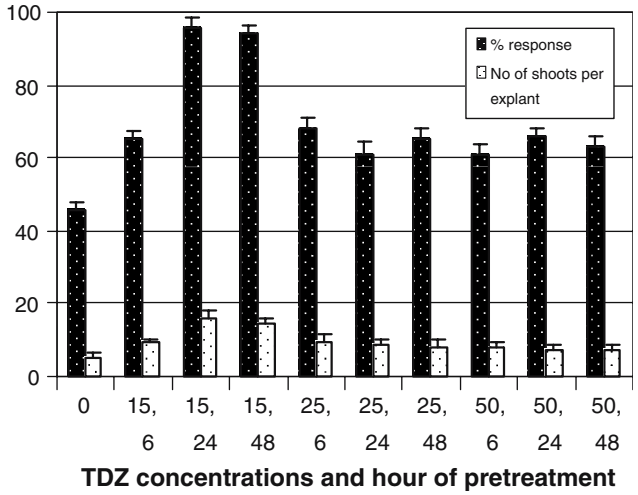

Fig. 3 Effect of MS medium supplemented with 6 μM TDZ on induction of shoots from leaf explants after pretreating with TDZ at three different concentrations (15, 25 and 50 μM) for three different time periods (6, 24 and 48 h). The number 0 indicates the control treatment (i.e. 6 μM TDZ without pretreatment) and the other numbers (15, 25 and 50) indicate the concentrations of TDZ and the hour of pretreatment (6, 24 and 48). Results expressed as means of three replicates ±SD

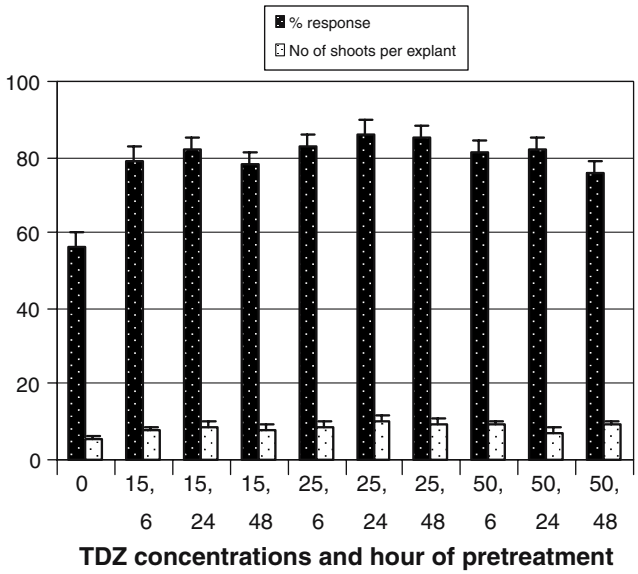

Fig. 4 Effect of MS medium supplemented with 4 μM BAP and 1 μM NAA on induction of shoots from leaf explants after pretreating with TDZ at three different concentrations (15, 25 and 50 μM) for three different time periods (6, 24 and 48 h). The number 0 indicates the control treatment (i.e. 4 μM BAP and 1 μM NAA without pretreatment) and the other numbers (15, 25 and 50) indicate the concentrations of TDZ and the hour of pretreatment (6, 24 and 48). Results expressed as means of three replicates ±SD

(6 μM) + NAA (0.5 μM). As shown in Fig. 4, the maximum response was observed when explants were pretreated with 25 μM TDZ for 24 h and cultured on BAP (4 μM) +

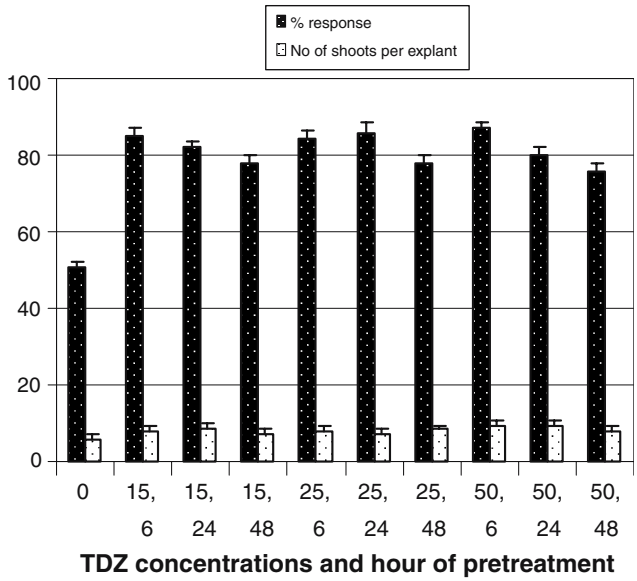

Fig. 5 Effect of MS medium supplemented with 6 μM TDZ and 0.5 μM NAA on induction of shoots from leaf explants after pretreating with TDZ at three different concentrations (15, 25 and 50 μM) for three different time periods (6, 24 and 48 h). The number 0 indicates the control treatment (i.e. 6 μM TDZ and 0.5 μM NAA without pretreatment) and the other numbers (15, 25 and 50) indicate the concentrations of TDZ and the hour of pretreatment (6, 24 and 48). Results expressed as means of three replicates ±SD

NAA (1 μM). On this medium, a maximum of 86% cultures responded with an average number of 10.4 shoots. Similarly, pretreated explants were cultured on TDZ (6 μM) + NAA (0.5 μM). Maximum response was observed when leaf explants were pretreated with 50 μM TDZ for 6 h and cultured on TDZ (6 μM) + NAA (0.5 μM). Here a maximum response of 87% cultures responded with an average number of 9.4 shoots per explant (Fig. 5). Even though the addition of NAA along with BAP or TDZ decreases the percent response and shoot number as compared to BAP and TDZ alone, it was significantly better than the control treatment.

Generally, low range of TDZ concentrations are used for shoot induction i.e. from 1 nM to 10 μM. In higher concentrations of TDZ, the shoots might be susceptible to hyperhydricity and fasciation (Huetteman and Preece 1993). TDZ promotes the synthesis and accumulation of purine and also alters cytokinin metabolism to increase the levels of endogenous cytokinins by inhibiting the action of cytokinin oxidase (Hare and Van Staden 1994; Murthy et al. 1998). But for quick pretreatment, higher concentrations of TDZ is employed i.e. up to 200 μM (Singh and Syamal 2001). In the present study, three different concentrations (i.e. 15, 25 and 50 μM) of TDZ were employed for pretreatment and of these three concentrations, 15 μM TDZ pretreated leaf explants gave highest frequency of regenerating leaves when cultured on 6 μM TDZ.

Table 2 Effect of half strength MS medium supplemented with various concentrations of IBA and NAA on rooting of the shoots in *Curculigo orchioides* after 45 days in culture

Auxin treatment (μM)	Shoots rooted (%)	No. of roots per shoot	Mean root length (cm)	Time required for root initiation (days)
0.0	0	0	0	0
IBA				
1.0	52b	1.2 ± 0.3a	2.8 ± 0.3a	9 ± 1.9c
2.0	68c	2.6 ± 0.4b	2.9 ± 0.8a	8 ± 2.1c
3.0	92d	3.4 ± 0.2c	3.1 ± 0.2b	7 ± 2.2b
4.0	100e	4.8 ± 0.2d	3.5 ± 0.5c	7 ± 1.8b
5.0	90d	3.6 ± 0.6c	2.3 ± 1.2a	5 ± 1.4a
NAA				
1.0	40a	1.4 ± 0.3a	2.1 ± 0.3a	13 ± 2.3e
2.0	47a	1.6 ± 0.4a	2.3 ± 0.1a	11 ± 2.2d
3.0	64c	1.8 ± 0.2a	2.5 ± 0.3a	11 ± 2.5d
4.0	58b	1.7 ± 0.2a	2.2 ± 0.4a	9 ± 1.9c
5.0	51b	1.5 ± 0.3a	1.9 ± 0.2a	9 ± 2.2c

The values represent the mean (±SE) of three independent experiments. At least 24 cultures were raised for each experiment

Means within a column followed by the same letter are not significantly different by Duncans's multiple range test ($P > 0.05$)

The shoots derived from pretreated cultures were superior to untreated cultures in terms of shoot length, leaf size and chlorophyll content. The average shoot length of the pretreated cultures were 2.3 cm as against 0.8 cm of the untreated cultures. Similarly, the average leaf width of the pretreated cultures were 0.8 cm as against 0.6 cm in untreated cultures. Also the chlorophyll content in the leaves of microshoots was high in pretreated cultures as it remains greener than untreated cultures (data not shown).

When transferred to half-strength, MS medium supplemented with various concentrations of IBA or NAA, the shoots produced roots. IBA was comparatively better than NAA. IBA (4 μM) was found optimum for root induction. On this medium a maximum of 100% cultures responded with an average number of 4.8 roots per shoots (Table 2; Fig. 6a). The rooted plantlets were successfully transplanted to soil in plastic cups (Fig. 6b) with 90% success and grew normally in the field. The transplanted plants were similar to parental plants in their morphology.

In conclusion, an efficient and reproducible protocol for adventitious shoot regeneration from *C. orchioides* leaf explants have been demonstrated. The data presented here suggest that there was a marked difference between the response of pretreated cultures and the untreated cultures. All the pretreated cultures produced significantly higher adventitious shoot induction than the untreated ones. Hence, a pretreatment of the leaf explant in TDZ before culture is recommended for maximum shoot induction efficiency in *C. orchioides*.

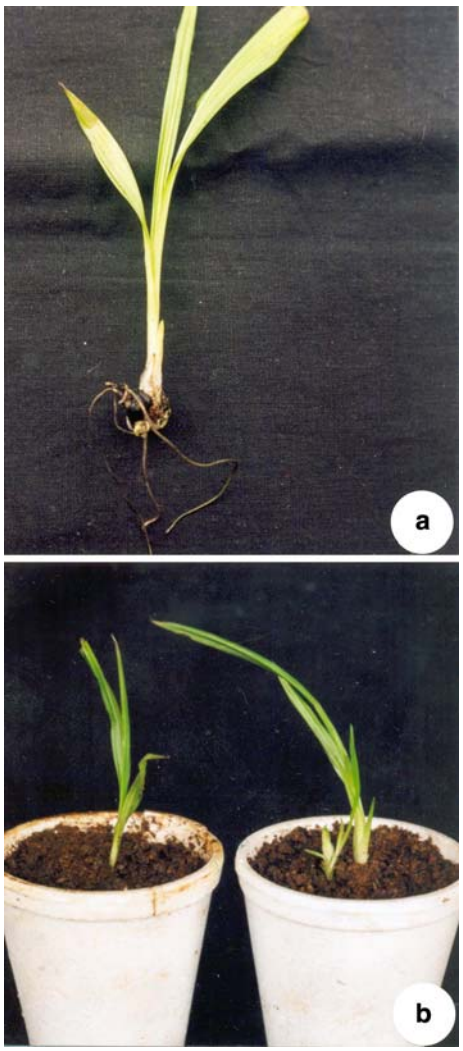

Fig. 6 Rooting and transfer of rooted plantlets to soil. **a** Rooted plantlets 45 days after culture. The plantlets were taken out from test tubes, removed agar and ready for transfer to soil. **b** Plants of *C. orchioides* 4 months after transplanting in the soil. One of the plants has developed a small bulblet

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