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Full Length Research Paper

Plant regeneration through organogenesis and shoot proliferation in *Trichodesma indicum* (Linn) R. Br. – a medicinal herb

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***Adhahpuspi* (*Trichodesma indicum*, Family – Boraginaceae) is a cross-pollinated species. Its complete regeneration was accomplished through *in vitro* techniques. The zygotic embryos placed on MS (Murashige and Skoog) medium fortified either with kinetin, BA (N^6-benzyl Aminopurine) or NAA (α-naphthalene acetic acid) produced callus and adventitious shoots; whereas those placed on MS medium supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid) formed callus. On subculture, the nodal pieces produced axillary shoots that were suitable for further propagule proliferation. Rhizogenesis occurred in 60% micro shoots treated with IBA (indole-3-butyric acid) pulse. The regenerated plants successfully acclimatized and started flowering in green house maintained at 30 ± 2°C temperature and 70% RH.**

Key words: Acclimatization, callus, caulogenesis, micropropagation, pulse treatment.

INTRODUCTION

Trichodesma indicum (family – Boraginaceae), commonly known as *adhahpuspi*, *Andhahuli*, *Chotta-kulpha* (Prajapati et al., 2006), is a medicinal plant species prescribed for abortion (Tarafder, 1983) and expulsion of dead fetus (C.S.I.R., 1986). Plant is used to cure eczema, stomachache and as a brain tonic (Jain, 1991). It inhibits diarrhea (Perianayagam et al., 2005) and sulphur dioxide induced cough reflex in mice (Srikanth et al., 2002). Tribal peoples use it for treatment of breast cancer (Tirky, 2002).

Two species of *Anthophora* carry out buzz-type pollination in a*dhahpuspi* (Ahmed et al., 1995). The cross-pollinated populations being heterogeneous influence the medicinal properties of the plants. Plant tissue culture methods are useful tools for propagation of superior genotypes without the risk of recombination that takes place in sexual reproduction. Earlier, plant regeneration through *in vitro* propagation has been reported in some members of the family, Boraginaceae including *Arnebia euchroma* (Manjkhola et al., 2005), *Cordea verbenacea* (Lameira and Pinto, 2006), *Heliotropium indicum* (Datta et al., 2003), *Rotula aquatica* (Martin, 2003; Chitra et al., 2005) and *Tournefortia cf paniculata* (Bertoluci et al., 2001).

The present paper reports for the first time initiation of callus from zygotic embryos, plantlet regeneration through differentiation of callus, shoot-proliferation from nodal segments, and acclimatization of rooted plantlets of *T. indicum*.

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **BA**, N^6-benzyl aminopurine; **IBA**, indole-3-butyric acid; **MS**, Murashige and Skoog medium; **NAA**, α-naphthalene acetic acid; **SH**, Schenk and Hildebrandt medium; **WPM**, woody plant medium.

MATERIALS AND METHODS

Plant material and sterilization

The immature fruits of *T. indicum*, collected from plants grown in the Ravishankar University Garden, Raipur, served as source of explants. The dust particles were removed from the fruits under running tap water. Healthy fruits were surface sterilized with 0.2% mercuric chloride solution for 15 min, rinsed 4 - 5 times with sterile water and embryos were extracted from fruits.Medium and culture conditionThe embryos were inoculated aseptically onto MS (Murashige and Skoog, 1962), SH (Schenk and Hiderbrandt, 1972)

Table 1. *Trichodesma indicum* shoot bud and shoot differentiation response on different media and levels of kinetin.

Medium + kinetin (µM)	Shoot no.	Average shoot length (mm)	Shoot bud no
MS			
0.00	1.17 ± 0.19	20.97 ± 4.91	0.0
0.23	1.17 ± 0.09	21.08 ± 4.05	2.24 ± 0.75
2.32	2.5 ± 0.18	41.65 ± 3.83	4.30 ± 0.24
23.26	2.13 ± 0.38	22.39 ± 2.69	0.0
SH			
0.00	1.11 ± 0.17	31.28 ± 4.29	0.0
0.23	1.17 ± 0.17	21.08 ± 4.05	5.33 ± 0.91
2.32	1.67 ± 0.27	19.72 ± 0.65	3.0 ± 0.71
23.26	1.34 ± 0.18	8.17 ± 1.68	4.1 ± 0.78
WPM			
0.00	1.38 ± 0.30	24.17 ± 5.29	0.0
0.23	1.0 ± 0.09	15.0 ± 3.20	0.0
2.32	2.06 ± 0.34	17.35 ± 2.49	0.0
23.26	1.50 ± 0.67	7.50 ± 3.53	5.0 ± 0.93

Values are mean ± SE (standard error).

and WPM (Lloyd and Mc Cown, 1980) medium solidified with 0.8% agar and supplemented with different concentrations of kinetin. The pH of the medium was adjusted to 5.7, 5.6 and 5.8, respectively, before autoclaving at 121ºC for 20 min. Similarly, the embryos were inoculated on MS medium supplemented with different concentrations of 2,4-D (2,4-dichlorophenoxy acetic acid), BA (6-benzyl aminopurine), or NAA (α-nNapthaline acetic acid). All the cultures were maintained by sub-culturing every four weeks in a growth room maintained at 25 ± 2ºC under a photoperiod of 16 h supplied by cool, white fluorescent tubes. The shoots regenerated *in vitro* were sub cultured for shoot proliferation as well as rooting.

Subculture and shoot proliferation

The shoots differentiated from callus on MS medium supplemented with 2.32 µM kinetin were excised and used for subculture. The nodal segments cut from micro shoots were placed on MS medium supplemented with 0.23 µM kinetin for four weeks. Data for shoot growth were recorded for three consecutive subcultures.

Shoot differentiation and rooting

The shoots that differentiated from callus on MS, SH and WPM containing different concentrations of kinetin were cut and placed on rooting medium. Rooting of micro shoots was attempted *in vitro* on half strength MS medium with IBA. The micro shoots approximately 10 – 15 mm long were placed on half-MS medium containing 2.46 µM IBA for 24, 48 and 72 h pulse before their transfer to hormone free medium.

Acclimatization and field transfer

The plantlets were washed in sterilized water, treated with bavistin and transferred to root trainers filled with soil rite. The root trainers were placed on holes at the top of the table in green house at 30 ± 2°C and 70% RH.

In the present investigation, each experiment included 10 replicates (except in case of sub culture where > 10 nodes were inoculated), and each experiment was repeated three times. The observations were recorded every 4 weeks.

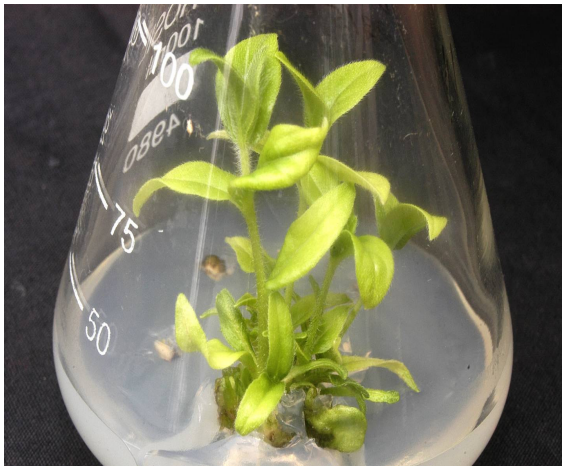

Figure 1. *Trichodesma indicum* microshoots differentiated from callus on MS + 2.32 µM kinetin after 4 weeks.

RESULTS

Effect of basal medium and kinetin

The basal media MS, SH and WPM containing kinetin induced callus in embryo explants and caused differentiation of shoot buds and shoots (Table 1 and Figure 1). Analysis of variance revealed that the explants placed on various media did not exhibit significant difference in shoot number or shoot length. However, the shoot-buds per calls differed significantly. Different levels of kinetin

Table 2. Effect of 2,4-D, NAA and BA (in MS medium) on *Trichodesma indicum* callus induction and shoot differentiation.

MS + PGR (µM)	Callus induction (%)	Shoot no.	Shoot length (mm)
Control			
0.00	00	2.50 ± 2.0	23.30 ± 8.33
2,4-D			
2.26	100	-	-
4.52	100	-	-
9.05	100	-	-
18.09	100	-	-
NAA			
0.27	56.0	3.0 ± 0.53	18.59 ± 2.31
2.69	86.0	00	0.0
26.89	70.0	0.0	0.0
BA			
0.22	00	2.83 ± 0.88	23.97 ± 4.53
2.22	50	2.74 ± 0.57	16.18 ± 3.12
22.22	50	0.00	0.00

Values are mean ± SE (standard error).

Figure 2. *Trichodesma indicum* callus on MS + 18.09 µM 2,4-D after 4 weeks of culture.

exhibited significant influence on shoot number, shoot length and shoot buds. In general, kinetin at 2.32 µM concentration induced optimum callus induction and shoot differentiation responses. MS medium with 2.32 µM kinetin induced differentiation of maximum (2.5 ± 0.31) shoots per callus and best shoot length (41.65 ± 3.83 mm) per shoot, respectively. The control explants placed on basal MS, SH or WPM showed shoot elongation from apical shoot meristem.

Effect of 2,4-D

Callus induction occurred in the explants placed on MS medium supplemented with different concentrations of 2,4-D (Table 2). Best callus growth occurred on MS medium supplemented with 184.09 µM 2,4-D (Figure 2). However, the callus failed to differentiate either on the first medium or on modified medium during subculture (Data not given).

Effect of NAA

NAA induced callus at 0.27, 2.69 and 26.89 µM concentrations. However, shoot buds differentiation and shoots elongation was noticed in callus grown only on medium containing 0.27 µM NAA. On this medium, the shoots per callus were 3.0 ± 0.53 with 18.59 ± 2.31 mm length per shoot (Table 2).

Effect of BA

The explants exhibited interesting response when placed on MS medium supplemented with BA. The MS medium with 0.22 µM BA showed shoot bud differentiation and formed shoots. The mean shoot number and average shoot length were 2.83 ± 0.88 and 23.97 ± 4.53 mm, respectively, while 2.22 µM BA showed 50% callusing with 2.74 ± 0.57 shoots number and 16.18 ± 3.12 mm shoot length, respectively.

Sub culture and shoot proliferation

The nodal segments cut from micro shoots differentiated from callus grown on MS medium supplemented with 2.32 µM kinetin, were inoculated on MS medium with re-

Table 3. *Trichodesma indicum* shoot multiplication during sub culture of nodal segments on MS + 0.23 µM.

S + KIN (0.23 µM) subculture	Shoot no.	Shoot length (mm)	Shoot bud no.
1	3.1 ± 0.91a	36.67 ± 7.78a	0.0b
2	2.8 ± 0.76a	13.65 ± 4.52b	5.38 ± 4.31a
3	1.25 ± 0.16b	6.5 ± 2.16b	7.5 ± 0.91a

Values are mean ± SE (standard error).
Values followed by similar letters on the same column do not differ significantly at 5% level (ANOVA).

Table 4. Effect of 2.46 µM IBA pulse treatment on rooting in micro-shoots of *Trichodesma indicum* after 4 weeks.

S/N	Pulse period(h)	Rooting response (%)	Roots/micro shoots	Shootlength/root length
1	24	40	1.25 ± 0.22	17.50 ± 4.92
2	48	60	5.71 ± 1.13	14.83 ± 1.92
3	72	60	8.0 ± 5.26	10.0 ± 2.20

Values are mean ± SE (standard error).

Figure 3. *Trichodesma indicum* microshoots elongation on MS + 0.23 µM kinetin after 4 weeks.

Figure 4. Rooting in *Trichodesma indicum* microshoot placed on half-MS + 2.46 µM IBA for 72 h and 4 weeks on hormone free half-MS medium

duced level of kinetin (0.23 µM). Shoot elongation occurred from the axillary buds (Table 3 and Figure 3). The data revealed decrease in shoot number and average shoot length during second and third subculture. However, several shoot buds (< 5 mm in length) appeared at second and third subcultures.

Rooting of micro shoots

The micro shoots supplemented with 2.46 µM IBA for 72 h pulse and for 4 weeks on hormone free half-MS medium showed root initiation. It decreased from 60 to 40% with lowering of pulse period to 24 h (Table 4 and Figure 4).

Acclimatization of regenerated plants

The plantlets were successfully acclimatized in green house adjusted at 30 ± 2ºC temperature and 70% RH. The new leaves and axillary shoots appeared within 15 days, and 25% of plantlets started flowering that continu-

Figure 5. Acclimatized *Trichodesma indicum* plants with flowers in green house.

ed during secondary hardening (Figure 5).

DISCUSSION

Pyrrolizidine alkaloids (PA) represent a class of secondary metabolites that occur primarily in members of families Compositae, Leguminoseae and Boraginaceae (Roeder, 1995). Recent reports suggest their role as a part of the plant defense against predators (Hartmann and Witte, 1995). Pyrrolizidine alkaloids are strongly feeding deterrents for most herbivorous organisms; they are toxic to the liver for vertebrates (Mattock, 1986) and mutagenic for insects (Frei et al., 1992). Some specialized herbivorous insects also contain pyrrolizidine alkaloids and use it for defense against predators. Its non-toxic form is N-oxides that convert into toxic tertiary amines (Hartmann and Toppel, 1987). The pyrrolizidine alkaloids vary in different plant parts and the level varies with age. Datta et al. (2003) reported enhanced yield of N-oxides in callus. Occurrence of (PA) has been reported in *Trichodesma incanum* and *T. africana*, but reports are lacking on *T. indicum*. It would be useful to know variation in PA content in callus and clones of *T. indicum*.

The establishment medium for *T. indicum* is flexible as the callus growth and shoot differentiation occurred in explants placed on MS, SH and WPM with kinetin. However, the cultures on SH and WPM began to dry during first subculture (data not given) suggesting specific nutritional requirement at maintenance stage. The requisite for auxin or cytokinin is essential and their level in medium is vital to differentiation. Datta et al. (2003) used MS medium augmented with NAA, BA, aspergine and glutamine to produce hypocotyl callus and organogenesis in *Heliotropium indicum*. Manjkhola et al. (2005) reported simultaneous organogenesis and somatic embryogenesis in leaf-derived callus of *Arnebia euchroma* on MS medium containing different levels of IBA and BA. However, the embryo-derived callus of *T. indicum* differentiated only into shoot buds. The nodal segments of *Cordea verbenacea* proliferated shoots on MS medium containing kinetin and NAA (Lameira and Pinto, 2006), and that of *Rotula aquatica* on MS medium with BA and IBA (Martin, 2003); and *Tournefortia* cf *paniculata* on WPM fortified with BA (Bertoluci et al., 2001).

The micro shoots of different species of family-Boraginaceae showed variation in nutritional and hormonal requirements during rooting. Martin (2003) reported *ex vitro* rooting in *R. aquatica,* whereas *in vitro* rooting was found successful on hormone-free MS medium in *C. verbenacea* (Lameira and Pinto, 2006,) and on hormone-free WPM in *T. paniculata* (Bertoluci et al., 2001). Addition of activated charcoal has been reported to establish dark environment (Pan and Staden, 1998) suitable for root initiation, but the micro shoots of *T. indicum* formed callus at the base and subsequently dried when placed on MS medium containing IBA and activated charcoal (data not given). The micro shoots of *T. indicum* best rooted on half-MS medium supplemented with IBA for 72 h.

The regenerated plants looked normal without any visible variability. Thus, it is possible to produce clones of *T. indicum* for medicinal purpose.

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