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In vitro Clonal Propagtion of *Trichosanthes cucumerina* L. var. *cucumerina*

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Abstract

An efficient protocol was established for *in vitro* shoot multiplication from shoot tip explant of *Trichosanthes cucumerina* L. var. *cucumerina* on semisolid MS basal medium supplemented with BA. NAA in the culture medium along with BA promoted higher number of shoot multiplication than BA alone. The rate of shoot multiplication was maximum 12.00 ± 0.70 after four weeks of culture on MS basal medium supplemented with BA 1.0 mg/l + NAA 0.1 mg/l. The elongated shoots rooted within seven - eight days in half strength of MS basal salts supplemented 1.0 mg/l IBA and 3% (w/v) sucrose. About 90% of the rooted plantlets were acclimatized and transferred to the greenhouse and successfully transferred to the field with 80% survival rate. The histological study shows that the organogenesis occurs directly, without callus formation on epidermal and sub epidermal layer of the explants. Adventitious shoots were characterized by the development of shoots apical meristem and leaf primordial.

Introduction

Trichosanthes cucumerina L. var. *cucumerina* belongs to Cucurbitaceae and is distributed in throughout India, Bangladesh, Sri Lanka, Burma, Malaysia, Australia (Chakravarty 1982). It is a perennial climber with an attractive white flower. It is highly bitter in taste which may be supposed to contain medicinal properties (Choudhary 1967) hence being used in various treatments as a cordiotonic, antipyretic, antiperiodic, useful for intestinal worms and leaf juice rubbed over the liver in remittent fever (Kirtikar and Basu 2000). Skin disease (Chopra et al. 1969). Appetizer, laxative, aphrodisiac and blood purifier (Shivarajan and Indira 1994) root is used to cure bronchitis, headache and boils. Leaves, for biliousness, emetic, externally applied over bald patches of alopecia to reduce congestion on congestive cardiac failure (Pullaih 2006). The seed posses anthelmintic and antifibrile properties the seeds are haemoagglutinating (Chakravarty 1982) seed is a good source of nutrients (Oloyede and Adebroye

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2005). Isolation and characterization of galactose specific lactin from the seed. The aqueous extract of root exhibited significant anti-inflammatory (Kolte et al. 1996). It is used as one of the important ingredients in 16 commercially available herbal products in India.

The species belong to genus *Trichosanthes* are considered as the future plants of Cucurbitaceae (http://www.pfaf.org/index.html 1996.) Trichosanthin is an antiviral protein purified from the root of *T. kirilowii* Maxim. It is an active component of Chinese medicine and is still being used in midterm abortion and to treat carcinoma. Trichosanthin shows inhibition of human immunodeficiency virus (HIV) because of its ribosome inactivating activity (Jian-Hua Wang et al. 2003). Karasurin is another new abortificient protein isolated from root of *T. kirilowii* (Shunsuke et al. 1991).

Due to large scale destruction of plant habitats and unrestricted over exploitation of this natural resource, coupled with limited cultivation and insufficient attempts for its replenishments, the propagation through seed is unreliable due to poor germination and death of young seedlings under natural conditions the wild stock of this species has been markedly depleted. The consequence is possible extinction of the species and this provides justification from conservation and propagation of this valuable germplasm. *In vitro* culture technique is an alternative method for conservation and propagation of this species. There is no report on *in vitro* studies on *Trichosanthes cucumerina* var. *cucumerina*. The present investigation describes an efficient protocol for micropropagation of *Trichosanthes cucumerina* var. *cucumerina* by using shoot tip and nodal explants.

Materials and Methods

Elongated shoots (10 -15 cm) were collected from plants grown at the Botany Department, Gulbarga University, Gulbarga, India with their cut ends placed in distilled water, leaves were removed from the stem and the stem is washed under running tap water for at least 10-15 min, followed by soaking in 5% (v/v) detergent solution (Teepol Qualigen, India) for 5 min. After thorough washing in sterilized distilled water, the explants were surface sterilized with freshly prepared 0.1% (w/v) aqueous mercuric chloride solution for 3 min. Followed by repeated washing with sterile distilled water, the stems were cut transversely in to 0.5 - 1 cm segments the explants were inoculated onto culture media.

Shoot tip and nodal explants were placed on semisolid MS supplemented with sucrose 3% (w/v), polyvinylpyrrolidone (PVP) 0.1% (w/v) and mesoinositol were used in all the experiments. Different concentrations of BA (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) or Kn (0.5, 1.0, 1.5, 2.0, and 2.5 mg/l), NAA (0.1, 0.2 and 0.5 mg/l), IAA (0.1, 0.2 and 0.5 mg/l) and IBA (0.1, 0.2 and 0.5 mg/l) were tested for

shoot multiplication. The pH of the medium was adjusted to 5.7 using 0.1N NaOH or 0.1N HCl prior to adding 0.8% agar (Hi-media, Mumbai). Medium was dispensed in 20 ml aliquots in to culture tubes (25 × 150 mm) covered with an aluminum foil. Media were steam sterilized at 121°C for 20 min. The cultures were incubated under a 16 hrs photoperiod in cool white florescent light (55 µ mol/m/s) (Phillips, India) and maintained at a constant temperature of 25 ± 2°C. The cultures were maintained by subculturing at four weeks intervals to fresh medium with the same composition.

The elongated shoots (4 - 5 cm) were excised from the eight-week-old culture grown on MS supplemented with 2.0 mg/l BA + 0.1 mg/l NAA. The excised shoots were transferred to half strength of MS basal semisolid medium supplemented with different concentrations of IAA (0.5, 1.0 and 1.5 mg/l), IBA (0.5, 1.0 and 1.5 mg/l) and NAA (0.5, 1.0 and 1.5 mg/l) with 3% (w/v) sucrose tested individually for root initiation. One excised shoot was cultured in each tube (25 × 150 mm) containing 15 ml of culture medium. Temperature and photoperiod were same as for shoot multiplication. Rooted micropropagules were thoroughly washed to remove the adhering gel and planted in earthen pots containing a mixture of soil, sand and farmyard mixture in the ratio of 1:1:1 and grown in the green house for acclimatization. Watering was made at two-day intervals. Percentage of survival was recorded one month after transfer.

All the experiments were repeated three times with ten replicates per treatment, observations were recorded every week on the bud proliferation state, percentage of regeneration, number of shoots per explants and shoot length. The comparison of means was analyzed using procedure of SPSS package version X.

To study the ontogeny of adventitious shoot bud differentiation in culture from shoot tip explant on shoot regeneration medium (MS + 3% sucrose, 1.0 mg/l BA, and 0.8 agar) were fixed after 2, 3, 5, 7, 10 and 12 days and fixed in glacial acetic acid and alcohol (1 : 3) dehydrated through a graded ethnol-xylene series, fallowed by and embedding histowax. Serial section 20 - 25 µm thick was cut with microtomy. The sections were affixed to slides, dewaxed, stained safranin - fast green combination, dehydrated and mounted in Canada balsam (Johansen 1940). Photomicrographs were taken with a Nikon Elipse E200 microscope, attached to Nikon coolpix 8400 camera.

Results and Discussion

Best regeneration of multiple shoots was observed from shoot tip than nodal segments on MS fortified with BA (1.0 mg/l). An average of 6.75 ± 0.52 shoot buds were regenerated having the length of 2.06 ± 0.50 cm from the shoot tip explant (Fig. 1A) with 70% response. Whereas 5.00 ± 0.53 shoot buds were regenerated from node explants with 66.6% response (Table 1). The similar

effects of BA for regeneration of multiple shoot were also observed in different plants like *Trichosanthes dioica* (Sanjeevkumar et al. 2003). *Momordica charantea* (Islam et al. 1994).

Table 1. Effect of growth medium (MS + different concentrations of BA and Kn + 3% (w/v) sucrose) on shoot multiplication from shoot tip and nodal explants after four weeks culture.

Growth regulators (mg/l)	Explant regenerated (%)		No. of shoots per culture		Average length of shoots per culture in (cm)	
	Shoot tip	Nodal explant	Shoot tip	Nodal explant	Shoot tip	Nodal explant
BA						
0.5	56.6	46.6	2.82 ± 1.00	1.42 ± 0.36	1.18 ± 0.28	1.28 ± 0.34
1.0	70.0	76.6	6.75 ± 0.52	5.00 ± 0.53	2.06 ± 0.50	2.85 ± 0.28
1.5	66.6	60.0	3.25 ± 0.55	2.77 ± 0.56	1.18 ± 0.29	1.85 ± 0.35
2.0	56.6	46.6	1.75 ± 0.41	1.42 ± 0.42	1.00 ± 0.28	0.92 ± 0.27
2.5	50.0	43.3	1.12 ± 0.39	1.00 ± 0.37	0.75 ± 0.50	0.42 ± 0.20
Kn						
0.5	-	26.6	-	0.60 ± 0.24	-	0.60 ± 0.24
1.0	20.0	43.3	1.00 ± 0.57	1.40 ± 0.24	1.16 ± 0.60	1.30 ± 0.20
1.5	16.6	36.6	1.33 ± 0.66	0.80 ± 0.20	1.33 ± 0.66	0.70 ± 0.18
2.0	26.6	20.0	3.00 ± 0.57	0.40 ± 0.24	2.50 ± 0.28	0.60 ± 0.24
2.5	10.0	-	0.33 ± 0.33	-	0.50 ± 0.50	-

- = Indicates no response.

Even though BA was found best for multiple shoot induction the higher concentration (3.0 and 3.5 mg/l) produced large amount of callus, which suppressed shoot elongation in *Coccinia indica* (Josekutty 1993). Pointed gourd (Hoque et al. 1998). There are reports that BA and Kn were required in optimal quantity for shoot proliferation in *Mentha arvensis* (Shasany et al. 1998), *Plubago zylenica* (Rout et al. 2000). Maximum number of multiple shoots produced from the shoot tip explant on medium containing BA 1.0 mg/l + NAA 0.1 mg/l an average number of 12.00 ± 0.70 shoots per culture with 3.62 ± 0.15 cm length were recorded from Four-week-old culture (Table 1, Fig. 1B). Results of the present investigation agrees in different plants like *Momordica dioica* (Nabi et al. 1995), *Cucurbita muschata* (Rahman et al. 1993), *Trichosanthes dioica* (Hossain et al. 1997), *Cucurbita fructidissima* (Lee and Thomas 1985). Incorporation of IAA or IBA with BA did not show better response than BA 1.0 + NAA 0.1 mg/l. Kn was found to produce less number of shoots either alone or in combination with NAA, IAA or IBA it was produced an average of 3.00 ± 0.57 per culture along with callusing and occasional rhizogenesis.

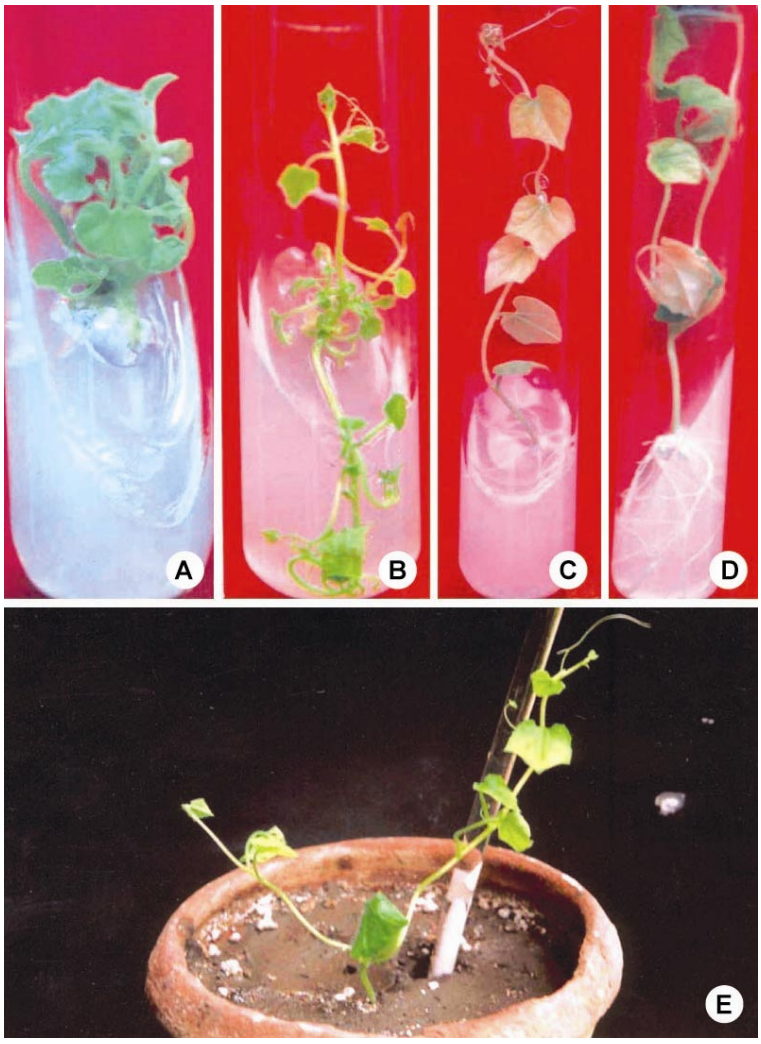

Fig. 1A - E : *In vitro* clonal propagation of *Trichosanthes cucumerina* var. *cucumerina* L. A. Induction of multiple shoot from shoot tip explants on MS supplemented with 1.0 mg/l BA after 30 days of culture. B. Shoot multiplication and elongation on MS supplemented with 1.0 mg/l BA + 0.1 mg/l NAA, and 3% (w/v) sucrose after four weeks of culture. C. Rooting of *in vitro* derived shoot on half strength MS + 1.0 mg/l IBA + 3% (w/v) sucrose after two weeks of culture. D. Rooting of *in vitro* derived shoot on half strength MS + 1.0 mg/l IBA + 3% (w/v) sucrose after four weeks of culture. E. *In vitro* raised planted grown in a pot.

In this experiment browning of explant was not seen in shoot tip explant but it was common in nodal explant when cultured on a medium containing BA. Similar results were reported in *Pistachia vera* cv. Kirmig (Ozden et al. 2005). Ethylene enhanced the activities of peroxidases and bond polyphenol oxidase, associated with the metabolism of phenolic products and tissue browning was reported in *Havea brasiliensis* (Housti et al. 1992).

Table 2. Effect of growth medium (MS + different concentrations of BA and Naa, IAA, IBA + 3% (w/v) sucrose) on shoot multiplication from shoot tip and nodal explants after four weeks of culture.

Growth regulators (mg/l)	Explant regenerated (%)		No. of shoots per culture		Average length of shoots per culture in (cm)	
	Shoot tip	Nodal explant	Shoot tip	Nodal explant	Shoot tip	Nodal explant
BA+ NAA						
1.0 + 0.1	76.6	73.3	12.00± 0.70	7.50 ± 0.42	3.62 ± 0.15	3.31 ± 0.16
1.0 + 0.2	50.0	56.6	5.62 ± 1.26	2.87 ± 0.78	2.25 ± 0.15	1.81 ± 0.43
1.0 + 0.5	36.6	50.0	2.87 ± 1.12	1.25 ± 0.36	1.00 ± 0.38	0.90 ± 0.28
BA + IAA						
1.0 + 0.1	56.6	53.3	3.30 ± 0.33	2.00 ± 0.00	1.10 ± 0.16	0.96 ± 0.27
1.0 + 0.2	66.6	60.0	4.00 ± 0.00	3.33± 0.33	1.56 ± 0.37	1.40 ± 0.10
1.0 + 0.5	60.0	36.6	1.66 ± 0.33	1.33 ± 0.33	1.25 ± 0.25	0.93 ± 0.16
BA+IBA						
1.0 + 0.1	60.0	53.3	2.33 ± 0.33	1.66 ± 0.33	1.20 ± 0.17	1.16 ± 0.16
1.0 + 0.2	66.6	56.6	2.66 ± 0.33	2.00 ± 0.00	1.76 ± 0.14	1.63 ± 0.26
1.0 + 0.5	46.6	43.3	1.66 ± 0.33	1.33 ± 0.33	1.63 ± 0.16	1.46 ± 0.24

Table 3. Effect of culture media (half strength MS + different concentrations of IAA, IBA and NAA + 3% (w/v) sucrose) on rooting response after four weeks of culture.

Type of auxins	Concentrations of auxins (mg/l)	Percentage of shoots rooted	Days to rooting	No. of roots per shoot	Average length of root in (cm)
IAA	0.5	43.3	13	4.00 ±1.04	3.14 ± 0.88
	1.0	66.6	9-10	13.71 ± 0.83	7.42 ± 0.57
	1.5	56.6	12-13	3.57 ± 0.71	3.28 ± 0.60
IBA	0.5	46.6	10	4.87 ± 1.45	3.12 ± 0.95
	1.0	73.3	7-8	20.87 ± 0.97	8.75 ± 0.45
	1.5	63.3	9-10	7.50 ± 1.65	5.75 ± 1.29
NAA	0.5	63.3	16-17	3.00 ± 0.75	2.25 ± 0.53
	1.0	70.0	12-13	7.62 ± 0.46	4.25 ± 0.53
	1.5	66.6	11-12	4.37 ± 0.80	2.75 ± 0.42

Although a high number of multiple shoot buds apparently formed on the explants, a very few buds were observed when histological study of culture was done (Fig. 2A-D) in a similar way to the one reported for other cucurbit species (Gaba et al. 1999, Stipp et al. 2001 and Maria et al. 2005). Adventitious shoots developed directly from the explant epidermal and sub epidermal layers. By day 2 on shoot regeneration medium random cell division activity had commenced in cells. By day 3 - 4 (Fig. 2B) rapid cell divisions were restricted to the peripheral area, which resulted in the formation of multiple meristematic nodules (Fig. 2A). These nodules gave rise to shoot bud meristems by day 5, which in turn formed leaf primordial by day 7 (Fig. 2C), on 10-11th day xylem tissue was developed, by day 12 xylem tissue was established the connection to the main vascular

bundle (phloem was not found) of explant (Fig. 2D). Shoot bud becomes macroscopic and fully differentiated shoots were obtained by 21 day (Fig. 1A).

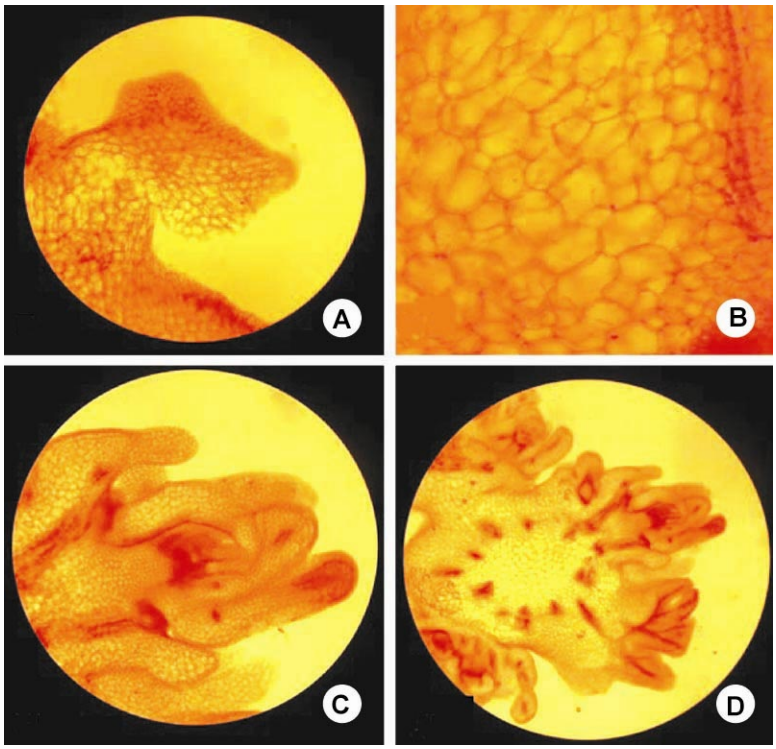

Fig. 2A - D : Ontogeny of *T. cucumerina* var. *cucumerina*. A. Transverse section of shoot tip cut end showing meristematic dome (day 2). B. Transverse section of shoot tip cut end at day 3-4 the sub epidermal cells are under going random cell divisions. C. Transverse section of shoot tip cut end at day 7 showing development of a shoot meristem along with leaf primordia. D. Shoot bud showing vascular connection at day 12 to the main explant.

Elongated shoots (3.5 - 4.5 cm) were excised from 8-wk old cultures, and transferred to half strength MS medium supplemented with various concentrations of IAA, IBA and NAA. Optimal rooting (73.3%) with no intervening callus was observed within seven - eight days of transfer to medium containing 1.0 mg/l IBA with 3% sucrose (Table 2, Fig. 1D). The percentage of shoots that formed root and the number of roots per shoot varied significantly with different concentrations of IAA, IBA and NAA. In most of the cucurbits the root induction was achieved on either basal MS medium alone or with very low level of auxin *T. dioica* (Mythili and Thomas 1999). In the present study rooting on medium containing even a low level of NAA 0.5 mg/l induced thick hairy and malformed roots, which were not suitable for transfer to pots. Similar results were observed in *Trichosanthes dioica* (Sanjeevkumar et al. 2003). The rooted plantlets (8-10 cm) tall were transferred to soil, sand and farmyard mixture. (1 : 1

: 1). About 90% survived after transfer to the filed. The acclimatized plants exhibited normal growth (Fig. 1E).

In conclusion, the present studies describe an effective protocol for *in vitro* regeneration of *Trichosanthes cucumerina* var. *cucumerina* an important medicinal plant. This may help in the conservation and propagation of the species and possibly lead to the synthesis and extraction of active compounds from plant sources.

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