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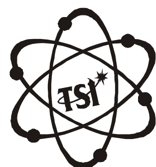

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In vitro clonal propagtion of *Trichosanthes cucumerina L.* var. *Cucumerina* L. - An important medicinal plant

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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* L. on semisolid Murashige and skoog's[18] basal medium supplemented with 6-benzylaminopurine (BA). Inclusion of 1- naphthaleneacetic acid (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 4 wk of culture on MS basal medium supplemented with BA 1.0 mg^{-1} + NAA 0.1 mg^{-1}. The elongated shoots rooted within 7-8 days in half strength MS basal salts supplemented 1.0 mg^{-1} IBA and 3% (w/v) sucrose. About 90% of the rooted plantlets were acclimatized and transferred to the green house 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.

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KEYWORDS

Trichosanthes cucumerina L. var. *cucumerina L.*;
Clonal propagation;
Shoot multiplication;
In vitro : ontogeny.

INTRODUCTION

Trichosanthes cucumerina L.var. *cucumerina* Linn. belongs to the family Cucurbitaceae and is distributed in throughout India, Bangladesh, Sri Lanka, Burma, Malaysia, Australia[2]. It is perennial climber with an attractive white flower. It is highly bitter in taste the bitter taste may suppose to contain medicinal properties[4] 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[14]. Skin disease[3] Appetizer, laxative, aphrodisiac and blood purifier[13]. Root is used to cure bronchitis, headache and boils. Leaves, for biliousness, emetic, externally applied over bald patches of alopecia[1]. To reduce congestion on congestive cardiac failure[25]. The seed posses anthelmintic and antifibrile properties the seeds are haemo-agglutinating[2] seed is a good source of nutrients[22]. Isolation and characterization of galactose specific lactin from the seed[5]. The aqueous extract of root exhibited significant anti-inflammatory[15]. It is used as one of the important ingredient in 16 commercially available herbal products in India.

The species belongs to *Trichosanthes* are considered as the future plants of Cucurbitaceae[23]. 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[6]. Trichosanthin shows inhibition of human immunodeficiency virus (HIV) because of its ribosome inactivating activity[11]. Karasurin is another new abortificient protein isolated from root of *T.kirilowii*[31].

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* L. var. *cucumerina*. The present investigation describes an efficient protocol for micropropagation of *Trichosanthes cucumerina* L. var. *cucumerina* by using shoot tip and nodal explants.

MATERIALS AND METHODS

Plant material and explant sources

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.

Culture media and culture conditions

Shoot tip and nodal explants were placed on semisolid MS medium[18] supplemented with Sucrose 3% (w/v), Polyvinylpyrrolidone (PVP) 0.1% (w/v) and mesoinositol were used in all the experiments. Different concentrations of cytokinins like 6- benzylaminopurine (BA: 0.5, 1.0, 1.5, 2.0, and 2.5 mg^{-l}) or Kinetin (0.5, 1.0, 1.5, 2.0, and 2.5 mg^{-l}) and auxins like 1-naphthalinacetic acid (NAA; 0.1, 0.2 and 0.5 mg^{-l}), Indole acetic acid (IAA 0.1, 0.2 and 0.5 mg^{-l}) and Indole butyric acid (IBA 0.1, 0.2 and 0.5 mg^{-l}) and were tested for shoot multiplication. The pH of the media 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 tube (25×150 mm), which were covered with an aluminum foil. Media were steam sterilized at 121^0C and 1.05 kg cm^{-2} s^{-1-} for 20 min. The cultures were incubated under a 16 h photoperiod in cool white florescent light (55μ mol m^{-2} s^{-1}) (Phillips, India) and maintained at a constant temperature of 25 ± 2^0C. The cultures were maintained by sub culturing at 4-wk intervals to fresh medium with the same composition.

Induction of rooting and acclimatization

The elongated shoots (4-5 cm) were excised from the 8-wk old culture grown on MS medium supplemented with 2.0 mg^{-l} BA + 0.1 mg^{-l} NAA. The excised shoots were transferred to half strength 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 2-day intervals. Percentage of survival was recorded 1 month after transfer.

Statistical analysis

All the experiments were repeated three times with 10 replicates per treatments, 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.

TABLE 1: Effect of growth medium (ms + different concentrations of cytokinin + 3% (w/v) sucrose) on shoot multiplication from shoot tip and nodal explants of *T.cucumerina* var. *Cucumerina*, after 4 wk of culture

Growth regulators (mg^{-1})	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

Histological studies

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^{-1} 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) de hydrated through a graded ethnol-xylene series, fallowed by and embedding histowax. Serial section 20-25μm 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

Effect of growth regulators on shoot multiplication

Different concentrations and combinations of cytokinins and auxin were used singly or in combinations on MS medium for optimizing multiple shoot regeneration from shoot tip and nodal segment of *Trichosanthes cucumerina* L.var. *cucumerina*. Among the various hormonal supplements used, best regeneration of multiple shoots were observed from shoot tip than nodal segments on MS medium fortified with BA (1.0 mg^{-1}) 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 (Figure 1A) with 70% response. Where as 5.00 ± 0.53 shoot buds were regenerated from node explant 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*[28]. *Momordica charantea*[10].

Even though BA was found best for multiple shoot induction the higher concentration (3.0 and 3.5 mg^{-1}) produced large amount of callus, which suppressed shoot elongation in *Coccinia indica*[13]. Pointed gourd[6]. Many authors have reported that cytokinins were required in optimal quantity for shoot proliferation in *Mentha arvensis*[29] *Plubago zylenica*[27]. Maximum number of multiple shoots produced from the shoot tip explant on medium containing BA 1.0 mg^{-1} + NAA 0.1 mg^{-1} an average number of 12.00 ± 0.70 shoots per culture with 3.62 ± 0.15 cm length were recorded from 4 wk old culture (TABLE 1, Figure 1B) results of the present investigation agrees in different plants like *Momordica dioica*[20], *Cucurbita muschata*[26], *Trichosanthes dioica*[8], *Cucurbita fructidissima*[16]. Incorporation of IAA or IBA with BA did not show better response than BA 1.0 + NAA 0.1 mg^{-1}. Kinetin was found to produce less number of shoots either alone or in combination with any of the auxins, it was produced an average of 3.00 ± 0.57 per culture along with callusing and occasional rhizogenesis.

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 as a cytokinins. Similar results were reported in *Pistachia vera* cv. Kirmig[24]. Ethylene enhanced the activities of per-

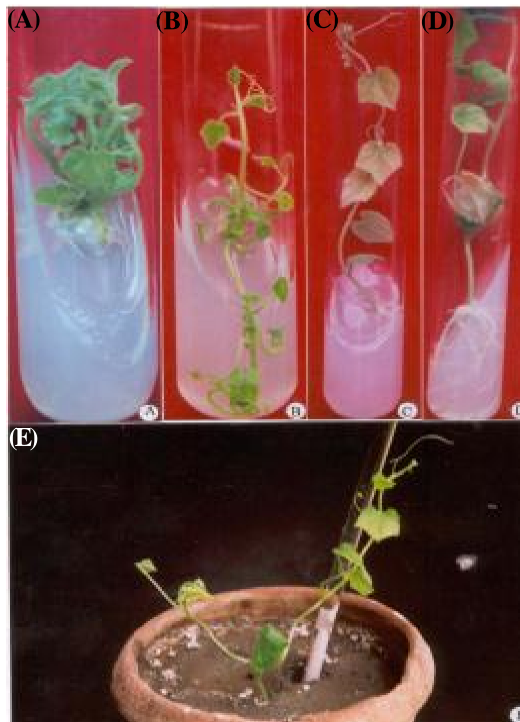

Figure 1(A-E): *In vitro* clonal propagation of *Trichosanthes cucumerina* L. var. *cucumerina* L.: A- Induction of multiple shoot from shoot tip explant of *T.cucumerina* var. *cucumerina* on MS medium supplemented with 1.0 mg^{-1} BA after 30 day of culture, B-Shoot multiplication and elongation of *T.cucumerina* var. *cucumerina* on MS supplemented with 1.0 mg^{-1} BA + 0.1 mg^{-1} NAA, and 3% (w/v) sucrose after 4 wk of culture, C - Rooting of *in vitro* derived shoot of *T.cucumerina* var. *cucumerina* on half strength MS + 1.0 mg^{-1} IBA + 3% (w/v) sucrose after 2 wk of culture, D- Rooting of in vitro derived shoot of *T.cucumerina* var. *cucumerina* on half strength MS + 1.0 mg^{-1} IBA + 3% (w/v) sucrose after 4 wk of culture, E- In vitro raised planted grown in a pot

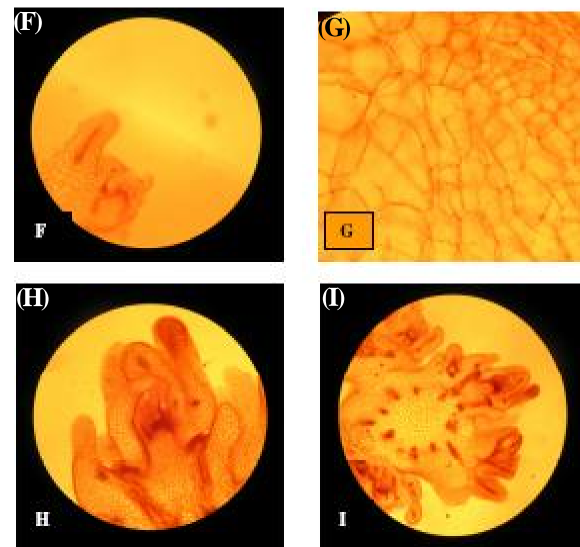

Figure 2(F-I) : Ontogeny of *T. cucumerina* var. *cucumerina:* F- Transverse section of shoot tip cut end showing meristematic dome (day-2), G- Transverse section of shoot tip cut end at day 3-4 the sub epidermal cells are under going random cell divisions, H-Transverse section of shoot tip cut end at day 7 showing development of a shoot meristem along with leaf primordial, I- Shoot bud showing vascular connection at day 12 to the main explant

oxidases and bond polyphenol oxidase, associated with the metabolism of phenolic products and tissue browning was reported in *Havea brasiliensis*[9].

Histogenesis of shoots primordial

Although a high number of multiple shoot buds apparently formed on the explants, very few buds were found when a histological study of culture was done (Figures 2(F-I)) in a similar way to the one reported for other cucurbit species[5,17,32]. 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, (Figure 2G) rapid cell divisions were restricted to the peripheral area, which resulted in the formation of multiple meristematic nodules (Figure 2F). These nodules gave rise to shoot bud meristems by day 5, which in turn formed leaf primordial by day 7 (Figure 2H), 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 (Figure 2I). Shoot bud becomes macroscopic and fully differentiated shoots were obtained by 21 day (Figure 1A).

Rooting of micro shoots

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 7–8 days of transfer to medium containing 1.0 mg^{-1} IBA with 3% sucrose (TABLE 2, Figure 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.dioic*[19]. In

TABLE 2 : Effect of growth medium (ms + different concentrations of cytokinin and auxins + 3% (w/v) sucrose) on shoot multiplication from shoot tip and nodal explants of *T.cucumerina* var. *Cucumerina,* after 4 wk of culture

Growth regulators (mg^{-1})	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 auxins + 3% (w/v) sucrose) on rooting response of *T.cucumerina* var. *Cucumerina*, after 4 wk of culture

Type of auxins	Concentrations of auxins (mg^{-1})	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

the present study rooting on medium containing even a low level of NAA 0.5 mg^{-1} induced thick hairy and malformed roots, which were not suitable for pot transfer similer results were observed in *Trichosanthes dioica*[28]. The rooted plantlets (8-10cm) height was transferred to soil, sand and farmyard mixture. (1:1:1) about 90% have survived after field transfer. The acclimatized plant exhibited normal growth (Figure 1E).

In conclusion, the present studies describe an effective protocol for in vitro regeneration of *Trichosanthes cucumerina* L. 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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