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IN VITRO MULTIPLE SHOOT INDUCTION IN *TRICHOSANTHES CUCUMERINA* L.

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SUMMARY

An efficient, rapid and large scale *in vitro* multiplication protocol was developed for the medicinally potent climber *Trichosanthes cucumerina* L. of family Cucurbitaceae. Various factors like source of explant and its age, nutrition and hormonal supplement have been found to affect morphogenesis of *T. cucumerina.* 8 days old cotyledonary node without cotyledon was found to be most efficient explant in regard to multiple shooting. The highest regeneration frequency of 61.1(20.3 shoots/explant) was found with White's media supplemented with 0.1mg/l kinetin and 2mg/l BAP. Although roots were induced on MS basal medium, but frequency was increased when supplemented with either IAA or IBA. Regenerated plants were successfully acclimatized and about 40% plantlets survived under *ex vitro* conditions. They flowered, fruited and were morphologically uniform and identical to donor plants.

***Key words*: *In vitro* multiplication, multiple shoot induction, regeneration, *Trichosanthes cucumerina*.**

INTRODUCTION

Trichosanthes cucumerina Linn. commonly known as 'Kadu padval' or 'Jangli padval' belonging to family Cucurbitaceae. Cucurbitaceae is well known for presence of bitter principle, which is generally found in almost all members of this family. The bitter principle present in *T. cucumerina* contains number of cucurbitacins of tetracyclic triterpenoid group. This group of compounds is of considerable interest because of its antitumor (Oh *et al.* 2002), anti-inflammatory and anti-leucodermal activities (Jayaprakasam *et al.* 2003). In addition to these, number of workers have reported various other activities of cucurbitacins and crude extracts of *T. cucumerina*. Roots and seeds are cooling, anthelmintic, purgative, vermifuge, syphilis, verminosis and are useful in vitiated conditions of Pitta (Kirtikar and Basu 1975). They are also reported to be useful against burning sensation, anorexia, dyspepsia, flatulence, constipation, helminthiasis, fever and general weakness (Longman 1997). In addition to this, it is traditionally used in conditions of fever, intestinal worms, boils and skin diseases in tropical countries like Thailand and India (Anonymous 1997).

The use of *T. cucumerina* has increased a lot in countries like India and China during last few years, which has affected the natural population of this plant (Anonymous 1997). The indiscriminate use of this plant may put it under endangered category. In addition to this, the plant is naturally available only during its season. Thus continuous supply of plant material is not available throughout the year. Another problem associated with *T. cucumerina* is that the seeds of this plant are very difficult for germination because of the hard seed coat. The seed germinates only when the coat is removed. Under natural condition the seeds germinate only in the regions, which contain high population of mycoflora, which degrades the seed coat (Kirkpatrick and Bazzaz 1979). One of the way out to overcome these problems is the micropropagation. Micropropagation ensures not only continuous supply of plant throughout the year but also prevent the destruction

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of the natural population of this plant (Datta 1993, Hamilton 2004). *In vitro* multiplication of various medicinally important plants have been reported earlier by Koroch *et al*. (2003) in *Echinaceae pallida*, Wawrosch *et al.* (1999) in S*wertia chirata* and Nguyen *et al.* (2005) in *Curcuma zedaria* etc. However, such attempts have not been reported for *T. cucumerina.* Hence, we attempted to study various factors affecting the *in vitro* morphogenesis of *T. cucumerina*.

MATERIALS AND METHODS

The seeds used in this investigation were collected in the month of August 2002-03 from naturally growing plants in the Deolapar village, 65 km away from Nagpur (MS) India. Before surface sterilization, the seed coat was removed by scalpel without damaging the embryo because the hard seed coat restricts the germination. The seeds were surface sterilized by sequential washing with 5% sodium hypochlorite, 0.1% mercuric chloride and 70% ethanol for 5 min. each. In between each sterilizing agent 3 rinses of sterile distilled water for 2 min. each. Seeds were finally thoroughly washed with distilled water and then inoculated over wet filter paper under aseptic condition for germination.

Four different media viz. MS (Murashige and Skoog 1962), B5 (Gamborg *et al.* 1968), N6 (Chu 1978) and White's (White 1963) were used for the study of effect of nutritional requirements. The media were augmented with various concentrations of kinetin and BAP and autoclaved at 121^0C for 15 min. The cultures were maintained at 25^0C $\pm 2^0$C under 16/8 h photoperiod from cool white fluorescent lamps with 3000 lux light.

The explants like hypocotyl, cotyledonary node with or without cotyledon, cotyledonary leaf, true leaf and epicotyl of 8 days old seedling were harvested and inoculated over media containing MS + 0.1 mg/l kinetin + 2 mg/l BAP (benzyl amino purine). Then best responded explant i.e cotyledonary node without cotyledon of 2-12 days old seedlings tested on the media containing MS + 0.1 mg/l kinetin + 2 mg/l BAP. After the selection of explant and age of explant, four different media viz. MS, B5, White's and N6 were tested and finally hormones standardized by using different concentrations of kinetin and BAP either singly or in combination.

The regenerated shoots of about 5 cm in length were excised aseptically with the help of sterile scalpel under laminar air flow hood. The shoots were then inoculated over MS medium containing different concentrations of IAA (indole 3- acetic acid), IBA (indole 3-butyric Acid) and NAA (naphthaleneacetic acid) for root induction.

Well rooted plantlets were gently removed from the culture vessel, without damaging the roots, and then plantlets were placed in a thermacol cup containing sterile sand-soil mixture (3:1). Then the plant alongwith cup was wrapped with plastic bag having two or three holes for aeration. The plastic bag was opened for 1 h after every 2 days. After two weeks, the plastic bag was completely taken out and the plants were allowed to adjust to the ambient condition. Well-acclimatized plants were transferred to non-sterile soil for one week and then transferred to field.

Percentage response of explants was recorded on daily basis for 4 weeks. In all experiments, each hormone concentration consisted of 40 replicates and each experiment was repeated thrice. Standard error of the mean was calculated by Software Graphpad Prism.

RESULTS AND DISCUSSION

Organogenesis is dependent on the factors like explant type, physiological state of donor plant or organ and endogenous level of phytohormones (Thanh and Trinh 1990). In explant standardization, cotyledonary node without cotyledon of seedling showed the best response for shoot regeneration (68.33%) after 12 days of culture. This was followed by cotyledonary node with cotyledon and hypocotyls, which showed the frequency of multiple shoot induction of 27.17% and 15.67%, respectively on 13^{th} day of culture. Rest of the explants i.e. epicotyl and true leaf did not respond at all (Fig. 1). The best responsive explant is totally depends upon plant, different explants responses variedly in different plants. But in this plant cotyledonary node without cotyledon was found to be best. The similar type of response was observed by Debnath *et al.* (2000) in *Trichosanthes dioica*. The reason behind this may be the competent cells for adventitious shoot formation in cucurbits seem to be restricted to specific cotyledon regions (Ananthakrishman *et al.* 2003). This differential

response of various explants towards multiple shoot induction might be due to different levels of endogenous plant growth regulators (Ghosh and Sen 1994).

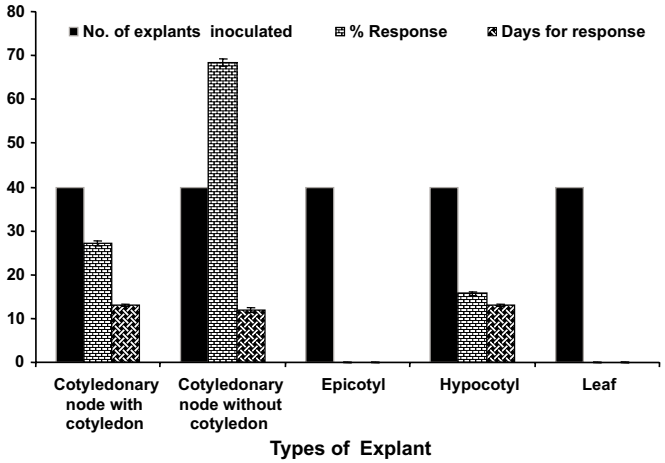

Fig. 1. Frequency of induction of multiple shoots using various explants (MS + 0.1 mg/l Kin + 2 mg/l BAP). Vertical bars indicate SEm±

In seedling age selection, cotyledonary node without cotyledon from 2, 4, 6, 8, 10 and 12 day old seedlings were inoculated over MS medium containing 0.1 mg/l kin and 2 mg/l BAP. The explants from 2 and 4 day old seedling showed no response, while the explants of 6-12 days old seedlings showed induction of multiple shoots around 12 days. The frequency of shoot induction increased up to 8 days old seedling where it was maximum 67.17% and later on it gradually declined (Fig. 2). Ten days old seedlings also showed good frequency (50%) of shoot induction, while 32% in 12 days seedlings. In 8 and 10 days old seedlings, shoot induction initiated 12^{th} day while it was on 13^{th} day in 6^{th} and 12^{th} day seedlings.

Seedling age is reported to be an important factor for obtaining high frequency regeneration in watermelon (Choi *et al.* 1994). The endogenous hormone level is developmentally regulated. Hence, age of explant plays a crucial role in response of explant towards the media (Mandal *et al.* 2001). There are many reports on explant age and regeneration response of plants. All these results showed that the physiological maturity of that seedling play an important role in regeneration response (Ozyigit *et al.* 2007).

Four different media viz. MS, White's, B5 and N6 supplemented with 0.1 mg/l kin and 2 mg/l BAP were tested for medium selection. Out of these media MS and White's supported the shoot induction most. The regeneration frequency was found to be 57.23% in MS, followed by White's medium with regeneration frequency (53.8%) and B5 medium with regeneration frequency of 6.2% (Fig. 3). N6 medium did not support shooting at all. Generally, it was found out that White's medium respond less as compare to that of MS (Nikam and Shitole 1997), but in this investigation both these media responded almost equally. One of the reasons behind this type of result might be the concentration of vitamins.

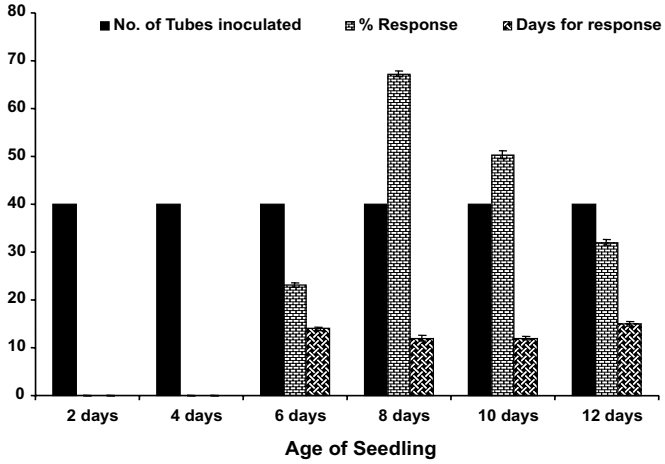

Fig. 2. Effects of seedling age on the frequency of multiple shoot induction (MS + 0.1 mg/l Kin + 2 mg/l BAP). Vertical bars indicate SEm±

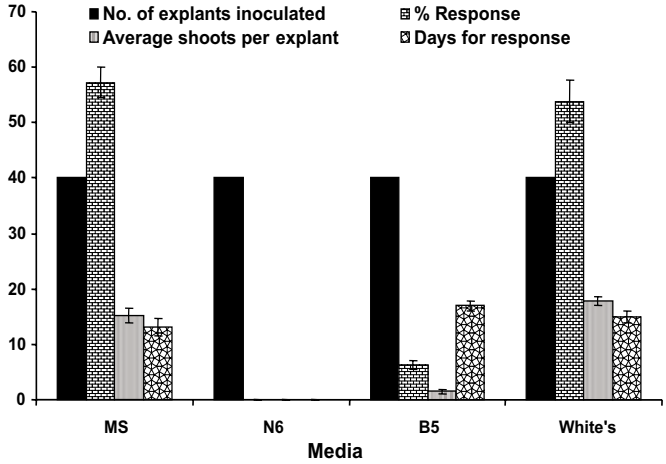

Fig. 3. Effects of media on multiple shoot induction using cotyledonary node without cotyledon as an explant. Vertical bars indicate SEm±.

In both these media vitamins are present in lesser amount as compared to that other two media used (Narayanswamy 1994).

It has been reported by various workers that the balance of auxin to cytokinin is a determining factor (Zheng *et al.* 2001). However, in this experiment, media containing combination of kin and BAP showed higher response of multiple shoot induction than either of the individual hormones. Kinetin alone was not much effective. It induced only 3.7 -5.5% of regeneration response with 2 to 3 shoots per explants over MS medium, similarly over White's medium response was only 4.5 – 6.2% with 2-3 shoots per explant (Table 1).

The media supplemented with BAP alone triggered shoot induction with appreciable frequency. BAP at moderate concentration (2-3 mg/l) was most effective in both the media. The response on MS and White's was found to be 28.2-33.3% and 25.7-34.2% respectively. However, the increase in concentration of BAP reduced the frequency of shoot induction (Table 1).The average number of shoots per explant also followed the same trend. BAP alone at the concentration of 2.5 mg/l produced maximum shoot per explant. The numbers of shoots per explant were 10 and 9.6 shoots over White's and MS media, respectively.

When BAP was used in combination with kinetin, the frequency of shoot induction was further increased. The 0.1 mg/l and 0.2 mg/l kin in combination with different concentrations of BAP produced the maximum response in MS and White's media. The kinetin (0.1 or 0.2 mg/l) with BAP 2 mg/l was found to be the best combination for multiple shooting. The numbers of shoots per explant were also maximum at 2 mg/l BAP over MS medium (15.3) and White's (17.8). (Table 1) The cytokinin combinations involving low concentrations of BAP (0.1 or 0.2 mg/l) and various higher concentrations of kinetin were less effective and the frequency of multiple shooting considerably dropped to less than 10% (Table 1) (Fig. 4A).

Srivastava *et al.* (1989) found that BAP to be highly effective in cucurbit organogenesis. Moreover, Chaturvedi and Bhatnagar (2001) found synergism between 2-isopentyladenine or kinetin and BAP, thus improving organogenesis in watermelon. Debnath *et al.* (2000) reported that BAP in combination with NAA was found to be the most responsive amongst all hormones used in *Trichosanthes dioica.* When shoot formation occurs on a medium containing a cytokinin alone, it suggests explants must contain sufficient endogenous auxin or be capable of its *de novo* synthesis (Julliard *et al*. 1992).

The induction of roots was observed on basal MS media containing either IAA or IBA. However, NAA completely inhibited the root induction. The frequency of root induction increased with concentration of IAA and IBA from 1 mg/l to 4 mg/l. However, at the concentration higher than this the frequency of root induction decreased. In case of IAA the roots were induced at the frequency between 0.0 to 24.1%, while in case of IBA the frequency was higher and it varied between 18.7 to 40.8% (Table 2) (Fig. 4B). In MS basal (full strength) media root induced with frequency of 14.4 on regenerated shoots which is comparatively less than IAA and IBA supplemented media.

It is well known that root induction depends on the auxin concentration in tissue (Narayanswamy 1994). Similar results were obtained in the study of Rout (2006) on *Camellia sinensis* where IBA showed more positive response as compared to IAA and NAA. Rout (2006) explained the reason behind such results that total phenolics content and peroxidase activity were higher in IBA treated cutting and phenolics plays key role for induction of advantaceous root.

From the overall regenerated shoots, 62% plantlets rooted properly. These regenerated and well rooted complete plants then transferred for hardening into autoclaved sand : soil mixture (1:1) (Fig. 4C) where the plants showed 100% survival rate. All of these plants were transferred for acclimatization to field, where 58% plants died and only 42% plants survived. These 42% plants adjust very well in natural environment and out of which 53.84% plants flowered 42.85 plants set fruits and in 66.66% plants seeds were formed (Table 3).

Table 1. Effect of different hormone combinations on multiple shoot induction using cotyledonary node without cotyledon as a explant.

Hormone		MS Medium			White's Medium		
Kin (mg/l)	BAP (mg/l)	No. of explants inoculated	Shoot regeneration response (%)	Avg. no. of shoots/ explant	No. of explants inoculated	Shoot regeneration response (%)	Avg. no. of shoots/ explant
0.2	-	40	0	-	40	0	-
0.4	-	40	0	-	40	0	-
0.6	-	40	0	-	40	0	-
0.8	-	40	0	-	40	0	-
1.0	-	40	3.7	2	40	4.7	2
1.5	-	40	5.5	2.5	40	5.7	2.5
2.0	-	40	3.8	3	40	6.2	2.5
2.5	-	40	0	-	40	4.5	3
3.0	-	40	0	-	40	0	-
3.5	-	40	0	-	40	0	-
4.0	-	40	0	-	40	0	-
4.5	-	40	0	-	40	0	-
5.0	-	40	0	-	40	0	-
-	0.2	40	6.6	2.5	40	6.5	3
-	0.4	40	7.8	3.3	40	6.7	3
-	0.6	40	9.3	3.3	40	8.8	4
-	0.8	40	8.1	3.6	40	9.3	4
-	1.0	40	13.5	5.4	40	12.8	4
-	1.5	40	19.4	7.2	40	21	4
-	2.0	40	31.5	7.1	40	30.7	8
-	2.5	40	33.3	9.6	40	34.2	10
-	3.0	40	28.2	8.3	40	25.7	9
-	3.5	40	23.6	4.2	40	20.5	9
-	4.0	40	18.9	4.4	40	21	6
-	4.5	40	12.1	3.6	40	14.2	4
-	5.0	40	9.3	3.3	40	10.2	3
0.1	1.0	40	20.5	6.2	40	18.4	7.3
0.1	2.0	40	57.23	15.3	40	53.8	17.8
0.1	3.0	40	42.1	12.1	40	48.6	14.6
0.1	4.0	40	27.7	9.4	40	25	13.4
0.1	5.0	40	19.4	6.3	40	23.1	10.4
0.2	1.0	40	23.6	8.2	40	19.6	6.1
0.2	2.0	40	57.8	18.2	40	61.1	20.3
0.2	3.0	40	50	15.4	40	48.4	14.5
0.2	4.0	40	30.7	11.3	40	28.6	12.2
0.2	5.0	40	21	6.4	40	19.1	9.3
1.0	0.1	40	4.7	2	40	3.8	2.5
2.0	0.1	40	5.1	2.5	40	4.9	2.5
3.0	0.1	40	5.4	2.5	40	7.6	3
4.0	0.1	40	5.8	3.5	40	10.9	3.3
5.0	0.1	40	6.2	3.5	40	12.7	3.3
1.0	0.2	40	6.2	2.5	40	4.9	3.6
2.0	0.2	40	6.6	3.5	40	5.7	3.3
3.0	0.2	40	6.4	3.5	40	5.1	3.3
4.0	0.2	40	7.1	3.5	40	9.4	3.6
5.0	0.2	40	6.8	4	40	12.4	3.6

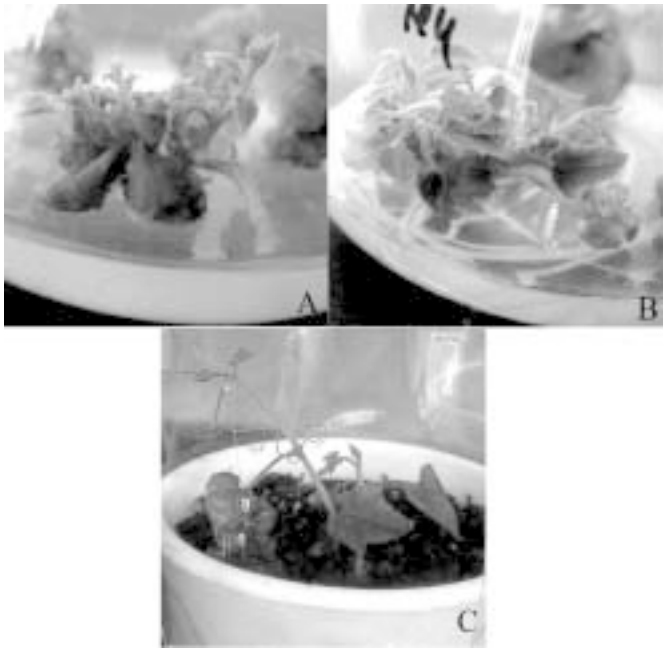

Fig. 4. *In vitro* multiplication of *Trichosanthes cucumerina*, (A) Induction of multiple shoots (MS + 0.1mg/l kin + 2mg/l BAP), (B) Induction of roots in regenerated shoots (MS + 4mg/l IBA), (C) Hardening of regenerated plant in a sand : soil mixture (1 : 1)

Table 2. Effect of different hormones and strength of basal medium on root induction of *in vitro* shoots. Values are mean of three replicate.

Medium	Root induction response (%)
MS basal+ IAA (mg/l)	
1	0.0
2	9.9 ±0.82
3	17.5 ±0.7
4	24.1 ±1.3
5	5.7 ±0.5
MS basal + IBA (mg/l)	
1	18.7 ±0.38
2	21.1 ±1.5
3	35.4 ±1.4
4	40.8 ±0.9
5	24.4 ±0.6
MS basal + NAA (mg/l)	
1	0.0
2	0.0
3	0.0
4	0.0
5	0.0
MS basal (Full strength)	14.4 ±1.3

Table 3. Data on survival of transferred regenerated plants.

	No. of plants	Plants survived	% Response
Transferred for rooting	50	31	62
Transferred for hardening (to sand : soil mixture)	31	31	100
Transferred for acclimatization (to field)	31	13	42
Set flowers	13	7	54
Set fruits	7	3	43
Set seeds	3	2	67

In this investigation, during the process of transfer to field the survival percentage was found to be decreased. There are various reasons for low survival of regenerated plants after transfer to natural soil. Gangopadhyay *et al.* (2002) reported that *in vitro* derived roots are often delicate and easily damaged during transplantation and often show limited physiological functioning when in contact with the soil.

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REFERENCES

Ananthakrishnan, G., Xia, X., Elman, C., Singer, S., Paris, H.S., Galon, A. and Gaba, V. (2003). Shoot production in squash (*Cucurbita pepo*) by *in vitro* organogenesis. *Plant Cell Rep.* **21:** 739-746.

Anonymous. (1997). World bank report. In: J. Labert, J. Srivastava and N. Vietmeyer (eds.), Medicinal Plants-Rescuing a Global Heritage. Technical Paper No. 355, pp. 61, World Bank.

Chaturvedi, R. and Bhatnagar, S.P. (2001). High frequency shoot regeneration from cotyledon explants of watermelon cv. Sugarbaby. *In Vitro Cell Dev. Biol. (Plant).* **37:** 255-258.

Choi, P.S., Soh, W.Y., Kim, Y.S., Yoo, O.J. and Liu, J.R. (1994). Genetic transformation and plant regeneration of watermelon using *Agrobacterium tumifaciens*. *Plant Cell Rep.* **13:** 344-348.

Chu, C.C. (1978). The N6 medium and its applications to anther culture of cereal crops. In: Proc. Symposium on Plant Tissue Culture, pp. 45-50. Science Press, Beijing, China.

Datta, P.C. (1993). Biotechnology and Tissue culture of some medicinal plants. In: J.N. Govil, V.K. Sing and S. Hshmi (eds.), Glimpses in Plant Research, Medicinal plants: New Vistas of Research part II, Vol XI, pp. 337-342.

Debnath, R.K., Roy, S.K., Ahmedi, G. and Hossaini, M. (2000). Micropropagation of Patal (*Trichosanthes dioica* Roxb) from nodal segment and shoot tip. *Plant Tiss. Cult.* **10:** 125-130.

Gamborg, O.L., Miller, R.A. and Ojima, V. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50:** 151-158.

Gangopadhyay, G., Das, S., Mitra, S.K., Poddar, R., Modak, B.K. and Mukherjee, K.K. (2002). Enhanced rate of multiplication and rooting through the use of coir in aseptic liquid culture media. *Plant Cell Tiss. Org. Cult.* **68:** 301-310.

Ghosh, B. and Sen, S. (1994). Micropropagation of *Asparagus cooperi* Baker as affected by growth regulators. *Plant Sci.* **82:** 119-124.

Hamilton, A.C. (2004). Medicinal Plants, Conservation and livelihoods. *Biodiversity Conser.* **13:** 1477-1517.

Jayaprakasam, B., Seeram, N.P. and Nair, M.G. (2003). Anticancer and anti-inflammatory activities of cucurbitacins from *Cucurbita andreana*. *Cancer Lett.* **189:** 11-16.

Julliard, J., Sosountzov, K., Habricot, Y. and Pelletier, G. (1992). Hormonal requirement and tissue competency for shoot organogenesis in two cultivars of *Brassica napus*. *Physiol. Plant.* **84:** 512-530.

Kirkpatrick, B.L. and Bazzaz, F.A. (1979). Influence of certain fungi on seed germination and seedling survival of four colonizing annuals. *J. Appl. Ecol.* **16:** 515-527.

Kirtikar, K.R. and Basu, B.D. (1975). Indian Medicinal Plants Vol: I-IV. (Second Edn.) M/s Bishen Singh Mahendrapal Singh, New Delhi.

Koroch, A.R., Kapteyn, J., Juliani, H.R. and Simon, J.E. (2003). *In vitro* regeneration of *Echinacea pallida* from leaf explants. *In Vitro Cell Dev. Biol. (Plant).* **39:** 415-418.

Longman, O. (1997). Indian Medicinal Plants: a compendium of 500 species. Orient Longman Limited, Chennai.

Mandal, A.K.A., Gupta, S.D. and Chatterji, A.K. (2001). *In vitro* plant regeneration in safflower. In: I.R. Khan and A. Khanum, (eds.), Role of Biotechnology in Medicinal and Aromatic Plants, Vol IV, pp. 159-167.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with Tobacco tissue cultures. *Physiol. Plant*. **15:** 473-497.

Narayanswamy, S. (1994). Plant Cell and Tissue Culture. Tata McGraw Hill Publishing Company Limited, New Delhi.

Nguyen, L., Doan, D., Tae, K. and Moon, Y. (2005). Micropropagation of Zedoary (*Curcuma zedoaria* Roscoe)- a valuable medicinal plant. *Plant Cell Tiss. Org. Cult.* **81:** 119-122.

Nikam, T.D. and Shitole, M.G. (1997). *In vitro* plant regeneration from callus of niger (*Guizotia abyssinica* Cass.) cv Sahyadri. *Plant Cell Rep.* **17:** 155-158.

Oh, H., Mun, Y.J., Im, S.J., Lee, S.Y., Song, H.J., Lee, H.S. and Woo, W.H. (2002). Cucurbitacins from *Trichosanthes kirilowii* as the inhibitory components on tyrosinase activity and melanin synthesis of B16/ F10 melanoma cells. *Plant Med.* **68:** 832-833.

Ozyigit, I.I., Kahramn, M.V. and Ozgene, (2007). Relation between explant age, total phenol and regeneration response in tissue cultured cotton (*Gossypium hirsutum* L). *African J. Biotech.* **6:** 003-008.

Rout, G. (2006). Effect of auxins on adventitious root development from single node cuttings of *Camellia sinensis* (L.) Kuntze and associated biochemical changes. *Plant Growth Regul.* **48:** 111-117.

Srivastava, D.R., Andrianov, V.M. and Piruzian, E.S. (1989). Tissue culture and Plant regeneration of water melon (*Citrullus vulgaris* Schrad cv. Melitopolski). *Plant Cell Rep.* **8:** 300-302.

Thanh, K.T. and Trinh, T.H. (1990). Organogenic differentiation. In: S.S. Bhowani (ed.), Plant Tissue Culture application and limitations, pp. 34-53. Elsevier Amsterdam.

White, P.R. (1963). The Cultivation of Animal and Plant Cells. Ronald Press, New York.

Wawrosch, C., Maskay, N. and Kopp, B. (1999). Micropropagation of the threatened Nepalese medicinal plant *Swertia chirata* Buch. Ham ex Wall. *Plant Cell Rep.* **18:** 997-1001.

Zheng, S.S., Yuan, H.Y., Wang, L.J., An, C.C. and Chen, Z.L. (2001). The tissue culture of medicinal plant *Trichosanthes kirilowii* and its protein analysis. *Sheng wu Gong cheng Xue Bao.* **17:** 420-422.