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# Micropropagation of *Trichosanthes anguina L.* via cotyledonary node

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Micropropagation of *Trichosanthes anguina L.* (Snake Gourd) was achieved using cotyledonary node explants derived from 7day old *in vitro* grown seedlings in a two step regeneration process. In the first step, the explants were cultured for four weeks for shoot bud induction and emergence of shoots in MS medium containing BA (0.2-1.4 mg/l) or Kn (0.2-1.2 mg/l) along with NAA (0.2 mg/l). At the end of the first step, higher number of shoots (14 shoots/explant) was produced in MS medium fortified with BA (1.2 mg/l), Kn (1.0 mg/l) along with NAA (0.2 mg/l). Individual effect of BA (1.2 mg/l) and Kn (1.0 mg/l) resulted in lower number of shoots induction with 12 and 7 shoots/explant respectively. In the second step, the explants with emerging shoots were subcultured in MS medium amended with BA (1.2 mg/l), Kn (1.0 mg/l) and L-glutamine (20 mg/l), which resulted in the maximum proliferation of shoots (18 shoots/explant). Elongation of shoots was obtained in MS medium containing $GA_3$ (0.5 mg/l). Highest percentage (94.6) of root induction was achieved in MS medium supplemented with IBA (0.4 mg/l). Well-rooted plants were transferred to paper cups containing sand, soil and farmyard manure (1:1:1; w/v) and then to larger earthen pots. 90% of acclimatized plants survived in field.

*Trichosanthes anguina*/micropropagation

*Trichosanthes anguina* L. (Cucurbitaceae) is extensively cultivated for its long snake-like fruits which are used as one of the most nutritive cucurbit vegetables. The vegetable is constituted with proteins, carbohydrates, fibre, fat, vitamins A and E and minerals such as potassium, phosphorous, sodium, zinc and magnesium in trace. In addition, the plant contains a series of secondary metabolites such as flavonoids, carotenoids, phenolic acids etc., (Sandhya et al. 2010) which make the plant pharmacologically and therapeutically important. The plant has been used as medicine and it is known for its antidiabetic, hepatoprotective, cytotoxic, anti inflammatory and larvicidal properties (Longman, 2002).

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Recently, many species of *Trichosanthes* have been reported to possess anti-viral compounds like Trichobetacin and Trichoanguin, which are used for the treatment of skin diseases, intestinal disorders, cough and tumours. Most of the crop plants are propagated vegetatively and grown as clones, but in *Trichosanthes*, the propagation is usually done by seeds. The yield of *T. anguina*, like any other cucurbits, has been hampered by many abiotic and biotic factors. Hence, the present day cultivars of this species need to be improved. In vitro culture techniques would serve as alternative

techniques to conventional plant breeding for the genetic improvement programme of *T. anguina*. In Cucurbitaceae, there have been several reports on plantlet formation through *in vitro* micropropagation using different explants such as node of cucumber (Naseem et al. 2005); shoot tip of cucumber (Vasudevan et al. 2006; Mohammadi et al. 2007); shoot tip of pumpkin (Mahzabin et al. 2008), shoot tip, cotyledons and node of water melon (Gnamien *et al.*, 2010); Khantun et al.2010) and cotyledonary node of melon (Zhang et al.2011). Micropropagation has been done *T. cucumerina* L. *var. cucumerina* (Devendra et al. 2008; Geetha et al. 2008; Malek et al. 2010; Komal, 2011).

Micropropagation is an alternative way to get quality seedlings in the crop. *In vitro* multiplication of elite clones will be an attractive approach in order to meet the requirement of quality propagules at large scale for commercial cultivation (Kumar et al. 2003). The most outstanding merits of micropropagation are in a short time and space, large number of plants can be produced from single explants. Micropropagated plants are generally free from fungal and bacterial diseases. Virus eradication and virus-free state are readily achieved in tissue culture (Debnath et al. 2006). Hence, the present work has been designed to develop an efficient protocol for *in vitro* plant micropropagation of snake gourd using cotyledonary node as explant for the production of elite clones.

## Materials and Methods

### Plant material

Mature seeds of snake gourd (cv. UK B200561) procured from Mahyco (Maharastra Hybrid Seed Company, Mumbai, Maharastra, India) were used for the present study.

### Explant source and preparation

After removal of the seed coat, the de- coated seeds were surface sterilized by washing in 3-5 drops of Teepol (commercial bleach solution) and 2% (w/v) Bavistin each for 5 min, then rinsed with distilled water for 3-4 times. The rinsed seeds were surface sterilized in 0.1% mercuric chloride for 3-min followed by washing in sterile water 3-4 times and left to air dry. Seeds were then placed on MS basal medium (Murashige and Skoog,1962) with 30g/l sucrose and 8g/l agar to germinate at dark chamber for 3-days and then in light (coal-white fluorescent light at 50 µmol $m^{-2}s^{-1}$) for 4-days. Cotyledonary nodes (1cm long) were excised from 7-day old *in vitro* grown seedlings and used as explants.

### Shoot initiation and proliferation

The cotyledonary node explants were aseptically inoculated vertically with the cut end touching the medium containing various individual concentrations and combinations of BA (0.2- 1.4 mg/l), Kn (0.2-1.2 mg/l) along with NAA (0.2mg/l) [Shoot Induction Medium (SIM)]. The pH of the medium was adjusted to 5.8 prior to autoclaving for 15 min. with a pressure of 1.05 kg $cm^2$ at 121°C. The cultures were maintained at 25 ± 2°C under 16 hr photo period with light intensity of 50 µmol $m^{-2}s^{-2}$ provided by cool white tubes. After four weeks of initial culture, numerous shoot bud protuberances originated from the axillary region of cotyledonary node were subjected to two subcultures at an interval of four weeks in MS medium supplemented with the optimal concentration of BA (1.2 mg/l) and Kn (1.0mg/l) fortified with L-Glutamine (20 mg/l) [Shoot Proliferation Medium (SPM)]. The subcultures were maintained as stated above. The explants with emerging shoots were transferred to fresh medium with same composition at 7 days interval during the two sub-culture period. At the end of each subculture, multiplication rate of shoots per explant was noted.

### Shoot elongation, rooting and hardening

The proliferated multiple shoots with an average height of 2-3 cm were excised and transferred to Shoot Elongation Medium (SEM) containing $GA_3$ (0.2-1.0 mg/l). After 4 weeks, the elongated shoots (8-8.5 cm) were transferred to Root Induction Medium (RIM) containing NAA (0.2-1.0 mg/l) or IBA (0.2-1.0 mg/l). The cultures were maintained as described earlier. After 3 weeks, the rooted plants were transferred to paper cups containing sterile soil, sand, vermiculite (1:1:1; v/v/v) and irrigated with sterile water daily. After 3-4 weeks, the plants were transferred to earthen pots and ultimately to garden where 90% of acclimatized plants survived.

### Statistical analysis

In all experiments, each single treatment consisted of 20 replicates and all experiments were repeated 3 times. Data on multiple shoot

regeneration, elongation and rooting were statistically analyzed using one way analysis of variance (SPSS, Version 10) and comparison of means was performed at the 5% level of significance using Duncan's Multiple Range Test (DMRT).

## Results and discussion

The present study established an effective protocol for in vitro micropropagation of snake gourd using cotyledonary node explants derived from 7 day-old *in vitro* seedlings.

### Shoot initiation

In this study, the greenish shoot buds initiated and emerged from the axillary portion of cotyledonary node explants (Fig.1c, a), cultured in MS medium supplemented with individual treatment of BA or Kn along with NAA (0.2 mg/l). About 78.63% of explants produced an average of 12 shoots per explant in medium containing BA (1.2 mg/l) whereas 86.56% of explants produced 7 shoots per explant in medium with Kn (1.0 mg/l) only. Development and number of shoots produced per explant were less when compared to combined effect of optimal concentration of BA (1.2 mg/l) and Kn (1.0 mg/l) in which 92.53 % of explants responded and produced 14 shoots/explants during the initial culture of 4 weeks. Previous reports on several cucurbits established the individual effect of either BA or Kn on the induction of multiple shoots. Devendra et al. (2010) observed that BA was superior to Kn in inducing high frequency shoot regeneration in the nodal explants of *T. cucumerina var. cuccumerina*. BA was also found better in shoot induction from cotyledon explants of *T. dioica* (Malek et al. 2010). Although BA (1.2 mg/l) induced more number of shoots from cotyledonary node explant in the present study, the higher percentage of explants (88.5%) response occurred in Kn (1.0 mg/l) treatment, which however resulted in lower shoot production.

On the other hand, combined effect of BA, Kn with auxins on multiple shoot induction in cucurbits has also been reported. In *T. dioica*, nodal explants produced maximum number of shoots in BA, Kn and NAA combination (Komal et al. 2011) and in the shoot tip explants of *C. maxima* (Mahzabin et al.2008). Zhang et al. (2011) reported the effective combination of BA and IAA in the induction of shoots from cotyledonary node of melon. In the present investigation, we achieved higher shoot multiplication (14 shoots/explant) in the combination of BA (1.2 mg/l), Kn (1.0 mg/l) with NAA (0.2 mg/l) at the end of four weeks of initial culture.

Table -1: Effect of PGRs and L-glutamine (20.0 mg/L) on induction and proliferation of multiple shoots from cotyledonary node explants derived from *in vitro* 7 day-old seedlings of *Trichosanthes anguina* on MS medium.

| PGRs (mg/l) | | Initial culture [MS medium + NAA (0.2 mg/l)] | | Sub- culture on MS medium + BA (1.2 mg/l) + Kn (1.0 mg/l) + L-Glutamine (20 mg/l) | |
|---|---|---|---|---|---|
| BA | | Explants developing shoots (%) | Number of shoots per explants | I Sub- culture | II Sub- culture |
| 0.2 | | 32.93±0.15$^{e}$ | 2.49± 0.15$^{i}$ | - | - |
| 0.4 | | 46.26±0.19$^{d}$ | 3.27±0.13$^{h}$ | - | - |
| 0.8 | | 62.99±0.20$^{c}$ | 5.75±0.12$^{f}$ | - | - |
| 1.0 | | 73.06±0.14$^{b}$ | 8.14±0.15$^{c}$ | - | - |
| 1.2 | | 78.63±0.19$^{b}$ | 12.52±0.16$^{b}$ | - | - |
| 1.4 | | 58.35±0.10$^{d}$ | 6.74±0.13$^{e}$ | - | - |
| Kn | | - | - | | - |
| 0.2 | | 38.20±0.18$^{e}$ | 1.80± 0.18$^{j}$ | - | - |
| 0.4 | | 62.99±0.32$^{c}$ | 2.47±0.17$^{i}$ | - | - |
| 0.8 | | 82.92±0.16$^{b}$ | 4.66±0.12$^{g}$ | - | - |
| 1.0 | | 88.56±0.28$^{a}$ | 7.42±0.15$^{d}$ | - | - |
| 1.2 | | 68.49±0.18$^{c}$ | 5.11±0.16$^{f}$ | - | - |
| BA | Kn | - | - | - | - |
| 1.2 | 1.0 | 92.53±0.09$^{a}$ | 14.62±0.19$^{a}$ | 15.44±0.15$^{a}$ | 18.60±0.12$^{a}$ |

CN- Cotyledonary Node

Each value represents means ± SE of three replicates with 20 explants. Values with the same letter within columns are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level

**Shoot proliferation**

The multiple shoots obtained in the combination of BA, KN along with NAA in the initial culture with an average height of 2-3 cm were transferred to MS medium containing optimal concentrations of BA (1.2 mg/l), and Kn (1.0 mg/l) along with L-glutamine (20 mg/l). At the end of the first sub culture, 15 shoots/explant were produced. In the present experiment, it was observed that more number of shoots (16 shoots / explant) was produced at the end of second sub culture (Table -1, Fig.1d and e). Sub-culture induced shoot multiplication in many cucurbits (Singh et al.1996; Vasudevan et al. 2006). In the present study, the enhancement of shoot production during the second step process was due to repeated transfer of explants to fresh medium at an interval of 7 days. Repeated transfer of explants in media resulted in rejuvenation of explant tissues. This promoted activation and conditioning of meristems (Shekhawat et al. 1993). Addition of L-glutamine, is a relatively non-toxic compound during the sub culture (Gamborg et al.1968), provided a sustainable nitrogen source to the explants to maintain a high growth rate to produce shoots for a long period of culture *in vitro*. The role of L-glutamine on shoot multiplication in cucumber was already established (Selvaraj et al. 2007).

Table- 2: Effect of $GA_3$ along with BA (1.0 mg/l) on elongation of shoots regenerated from cotyledonary node explants of *Trichosanthes anguina* on MS medium

| Concentration of $GA_3$ (mg/l) | Culture showing response (%) | Mean shoot length (cm) |
|---|---|---|
| 0.2 | $65.47\pm0.08^{e}$ | $4.05\pm0.09^{e}$ |
| 0.4 | $86.47\pm0.06^{b}$ | $5.18\pm0.10^{c}$ |
| 0.5 | $96.45\pm0.05^{a}$ | $8.50\pm0.14^{a}$ |
| 0.6 | $84.51\pm0.07^{c}$ | $6.79\pm0.21^{b}$ |
| 0.8 | $64.40\pm0.04^{f}$ | $4.22\pm0.10^{d}$ |
| 1.0 | $52.56\pm0.07^{g}$ | $3.43\pm0.13^{f}$ |

Each value represents means ± SE of three replicates with 20 explants. Values with the same letter within columns are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Table- 3: Effect of auxins [NAA and IBA] on *in vitro* rooting of shoots regenerated from cotyledonary node explants of *Trichosanthes anguina* on MS medium.

| Auxins (mg/l) | | Rooting response (%) | Mean number of roots per shoot | Mean root length (cm) |
|---|---|---|---|---|
| **NAA** | 0.2 | $44.48\pm0.13^{d}$ | $8.48\pm0.13^{d}$ | $1.49\pm0.08^{da}$ |
| | 0.4 | $62.42\pm0.12^{b}$ | $12.57\pm0.13^{b}$ | $3.62\pm0.05^{b}$ |
| | **0.6** | $\mathbf{75.52\pm0.10^{a}}$ | $\mathbf{16.31\pm0.10^{a}}$ | $\mathbf{4.49\pm0.06^{a}}$ |
| | 0.8 | $52.39\pm0.09^{c}$ | $9.36\pm0.08^{c}$ | $2.59\pm0.06^{c}$ |
| | 1.0 | $38.12\pm0.17^{e}$ | $7.50\pm0.16^{e}$ | $4.40\pm0.09^{db}$ |
| **IBA** | 0.2 | $72.87\pm0.34^{c}$ | $15.54\pm0.14^{c}$ | $4.53\pm0.53^{b}$ |
| | **0.4** | $\mathbf{94.60\pm0.12^{a}}$ | $\mathbf{26.29\pm0.13^{a}}$ | $\mathbf{5.50\pm0.06^{a}}$ |
| | 0.6 | $82.50\pm0.12^{b}$ | $18.49\pm0.13^{b}$ | $3.51\pm0.06^{c}$ |
| | 0.8 | $68.30\pm0.18^{d}$ | $11.51\pm0.12^{d}$ | $1.48\pm0.0^{cb}$ |
| | 1.0 | $48.19\pm0.15^{e}$ | $8.39\pm0.10^{e}$ | $1.45\pm0.08^{d}$ |

Each value represents means ± SE of three replicates with 20 explants. Values with the same letter within columns are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

**Shoot elongation, rooting and acclimatization**

Shoots regenerated from *in vitro* derived cotyledonary node explants remained stunted.

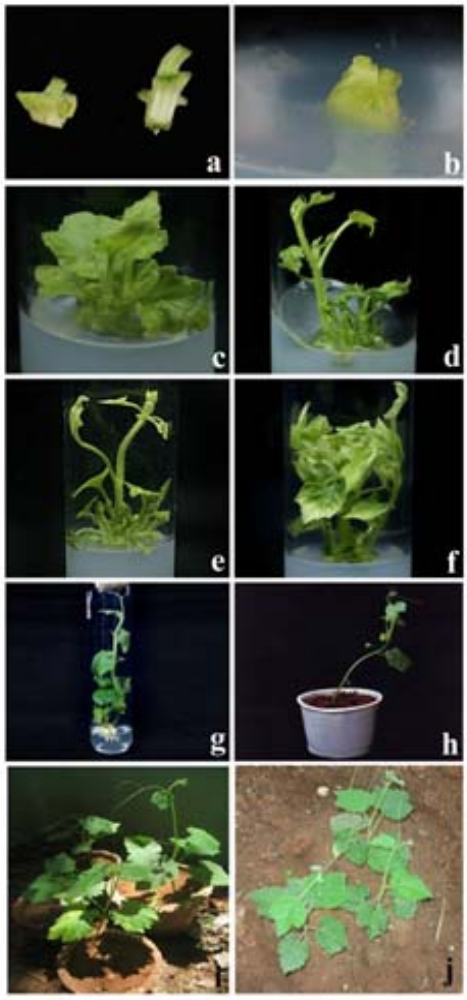


Fig.1: Micropropagation using cotyledonary node explants derived from 7 day-old in vitro seedlings of *T. anguina* on MS. a. Cotyledonary node explant, b. Cotyledonary node explant inoculated in MS medium, c. Shoot initiation and proliferation on MS medium containing BA (1.2 mg/l), Kn (1.0 mg/l) and NAA (0.2 mg/l), d. Shoot multiplication on MS medium fortified with BA (1.2 mg/l), Kn (1.0 mg/l) and L-glutamine (20 mg/l), e and f. Shoot elongation (MS medium containing 0.5 mg/l $GA_3$), g. Rooted shoot (Ms medium fortified with IBA 0.4 mg/l), h and i. Hardened plants, j. Acclimatized plants.

Therefore, the shoots (2-3cm length) were transferred to shoot elongation medium (SEM). After 4-weeks, an optimum shoot elongation (8.5cm) was achieved (Fig.1 and Table- 2) in MS medium containing $GA_3$ (0.5 mg/l). Stipp et al. (1991) induced shoot elongation in medium containing only BA (0.2 mg/l). Selvaraj et al. (2002) reported that $GA_3$ to be effective in shoot elongation in cucumber. About 94.6% of *in vitro* rooting was achieved with the production of 26.6 roots/shoot from MS medium fortified with IBA (0.4 mg/l) (Table-3). The present investigation revealed that IBA had better effect than NAA on root induction in snake gourd. The elongated (8.5cm length) rooted plants were successfully hardened and after 4 weeks of hardening (Fig.1h & i), the plants were transferred to field (Fig.1j). We have observed no phenotypic variations in the regenerated acclimatized plants and they behaved normally. This protocol would help in the clonal propagation and production of virus-free plants of *Trichosanthes anguina* for commercial exploitation.

## Conclusion

The present study established repeatable micropropagation protocol for *T. angunia* using cotyledonay node explants derived form 7 day-old in vitro grown seedlings. A combination of BA (1.2 mg/l), Kn (1.0 mg/l) with NAA (0.2 mg/l) induced maximum number of shoots in a culture duration of 4 weeks. High frequency shoot proliferation (16 shoots/explant) occurred in MS medium fortified with BA (1.2 mg/l), Kn (1.0 mg/l) along with L-glutamine (20 mg/l). Proliferated shoots were elongated, rooted and acclimatized successfully. The two step process involving shoot induction and shoots proliferation is the first report on micropropagation of *T. angunia*. This protocol would serve in clonal propagation of *T. angunia* at commercial scale.

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