

IN VITRO ORGANOGENESIS OF *TRICHOSANTHES DIOICA* ROXB. PLANTLETS THROUGH CALLUS CULTURE

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An efficient protocol for plantlet regeneration from shoot tip and axenic node derived callus was developed for *Trichosanthes dioica* Roxb. The optimum callus production was observed in MS + 2mg/l BAP + 4mg/l 2, 4-D, after 45 days of culture. Proliferation of multiple shoots (8) was obtained from the node derived callus cultures on MS + 1.5mg/l BAP + 0.3mg/l NAA, after 30 days of culture with an average shoot length of 4.88 ± 0.74 cm. The elongated shoots rooted within seven to eight days in half-strength MS medium supplemented with 1.0mg/l IBA. The *in vitro* raised plantlets were sequentially acclimatized by transferring them in an inorganic MS salt solution, then to sterile distilled water and tap water before transferring to potted soil. The plantlets were successfully established in earthen pots filled with soil, sand and farmyard manure in a ratio of 1:1:1, showing 70% survival rate.

Keywords: Callus formation, multiple shoot regeneration, organogenesis, *Trichosanthes dioica* Roxb.

Trichosanthes dioica Roxb. (pointed gourd) commonly known as parwal/patal in India, is one of the most important and expensive summer vegetable crops and holds a coveted position in the Indian market during the summer and rainy season (Kumar *et al.* 2003, Komal *et al.* 2010). It is said to be the native of South East Asia and probably the Northern and Eastern states of India, especially, of Bengal, Assam and Bihar (Nath and Subramanyam, 1972) and has high economic value with export potential. It is mainly cultivated along the riverine belts of Bihar. Sudden flood and water logging destroys the fruits and plant/vines leading to shortage of planting materials. Micropropagation may help overcome these problems to a great extent. Moreover the plant's dioecious nature and its vegetative mode of propagation make its reproduction and multiplication labour intensive. Genetic improvement of this crop has not been attempted adequately, hence, there is much work to be done from an agricultural point of view. Since, the crop is of immense economic importance, much work has to be done on its maintenance, conservation and *in vitro* propagation.

Though there are earlier reports on adventitious proliferation of shoots from the shoot tip and nodal explants of *Trichosanthes dioica* Roxb. (Malek *et al.* 2007, Komal *et al.*

2010, Komal, 2011) still plantlet regeneration from the callus cultures has to be achieved from genetic variation and agricultural point of view. The availability of an efficient protocol for *in vitro* regeneration is a prerequisite to harness any biotechnological approach for genetic improvement of crop plants. Development of an efficient and reproducible protocol for differentiation of multiple shoots from the callus has a great potential in obtaining soma clonal variants and *in vitro* mutation breeding (Tejavathi *et al.* 2010). Hence, the present study is an attempt to induce callus and regenerate plantlets from the callus cultures of shoot apices and axillary nodes of *Trichosanthes dioica* Roxb.

MATERIALS AND METHODS

The explants such as axenic nodes, shoot tips, tender leaves and roots were collected from the field grown plants. The explants were washed in running tap water in plastic pots for 10 to 15 min, followed by surface sterilization with detergent and 1-2 drops of Tween-20 (soap water) for 5 to 10 min. The explants were further transferred in an autoclaved plastic pot and treated with 0.1% (w/v) aqueous mercuric chloride solution and 2-3 drops of Tween-20 and shaken for 8 min under laminar air-flow cabinet. Then explants were shaken in ethyl

alcohol (70%) for 3 min. Further the explants were rinsed thrice with sterile double distilled water in an aseptic condition. Finally using sterile forceps, explants were transferred to sterile petri dishes and cut into small pieces (0.5-1.0 cm) with sterile scalpel.

MS medium supplemented with 100 mg/l meso-inositol, 3% sucrose and 0.8% agar-agar was used as basal medium (Murashige and Skoog, 1962). Different concentrations and combinations of BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l), Kn (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l), 2, 4-D (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l), NAA (0.1, 0.2, 0.3 and 0.5 mg/l), IAA (0.1, 0.2, 0.3 and 0.5 mg/l) and IBA (0.1, 0.2, 0.3 and 0.5 mg/l) were tested for callus induction and organogenesis. The pH of the medium was adjusted and maintained to 5.8 with the help of 0.1N NaOH or 0.1N HCl before adding agar (Hi-media, Mumbai). The cultures were incubated in the culture room maintained at $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a relative humidity of about 55-65% and 16 hour photoperiod, 2000-2500 lux light intensity from fluorescent tubular lamps. The medium was sterilized by autoclaving at 121°C for 20 min at 15-psi pressure. The cultures were maintained by sub culturing at four weeks intervals to fresh medium with the same composition. After successful callus induction and proliferation these calluses were transferred to a cytokinin rich medium for shoot initiation. The cultures for callus induction were kept in the dark for a week prior to exposing them to 16 hrs photoperiod. After successful callus induction and proliferation these calluses were transferred to a cytokinin rich media for shoot initiation. Each proliferated and adventitious shoot was cut from the basal end and individual shoot was placed in half - strength MS medium supplemented with NAA (0.1, 0.3, 0.5, 1.0 and 1.5 mg/l), IAA (0.1, 0.3, 0.5, 1.0 and 1.5 mg/l) and IBA (0.1, 0.3, 0.5, 1.0 and 1.5 mg/l) for rooting. Sub cultures were done after every 30 days. The rooted micro propagules were transferred to sterilized pots containing a mixture of sterilized soil, sand and farmyard manure in the ratio of 1:1:1. These were then

transferred to natural soil and environment.

RESULTS AND DISCUSSION

Morphogenetic response of different explants viz. axenic nodes, shoot tips, leaves and roots of *in vitro* regenerated pointed gourd plants were observed on MS medium fortified with different concentrations of BAP, Kn, 2,4-D and NAA alone and BAP + 2,4-D in different combinations. Average effect of different concentrations (2.0, 2.5, 3.0, 3.5, 4.0, 4.5mg/l) of growth regulators with MS medium on different explants of *Trichosanthes dioica* Roxb. on callus induction was observed (Table-1). This showed good callus growth in all the three explants except the root explants (Fig.1A, B, C). Almost 80-84% of shoot tip and nodal explants and about 58% of leaf explants showed callus induction in all media used (Fig.1 A, B, Table-2). The root explants showed some swelling but no callus formation was observed. Similar result related to the response of explants to growth regulators on callus induction was reported in *Stevia rebaudiana* through indirect organogenesis (Gunaseena *et al.* 2019).

In spite of the fact that BAP was found best for multiple shoot induction but higher concentration (3.0, 3.5, 4.0mg/l) produced a large amount of callus which suppressed shoot elongation (Table 3). These findings are similar to those of Joshkutty *et al.* (1993) in *Coccinia*

Table 1: Average effect of different concentrations (2.0, 2.5, 3.0, 3.5, 4.0, 4.5mg/l) of growth regulators with MS medium on different explants of *Trichosanthes dioica* Roxb. on callus induction.

Plant growth regulators (mg/l)	Callus induction (%)			
	Node	Shoot tip	Leaf	Root
BAP	84	80	58	-
Kn	80	76	50	-
2,4-D	78	80	60	-
NAA	70	75	58	-

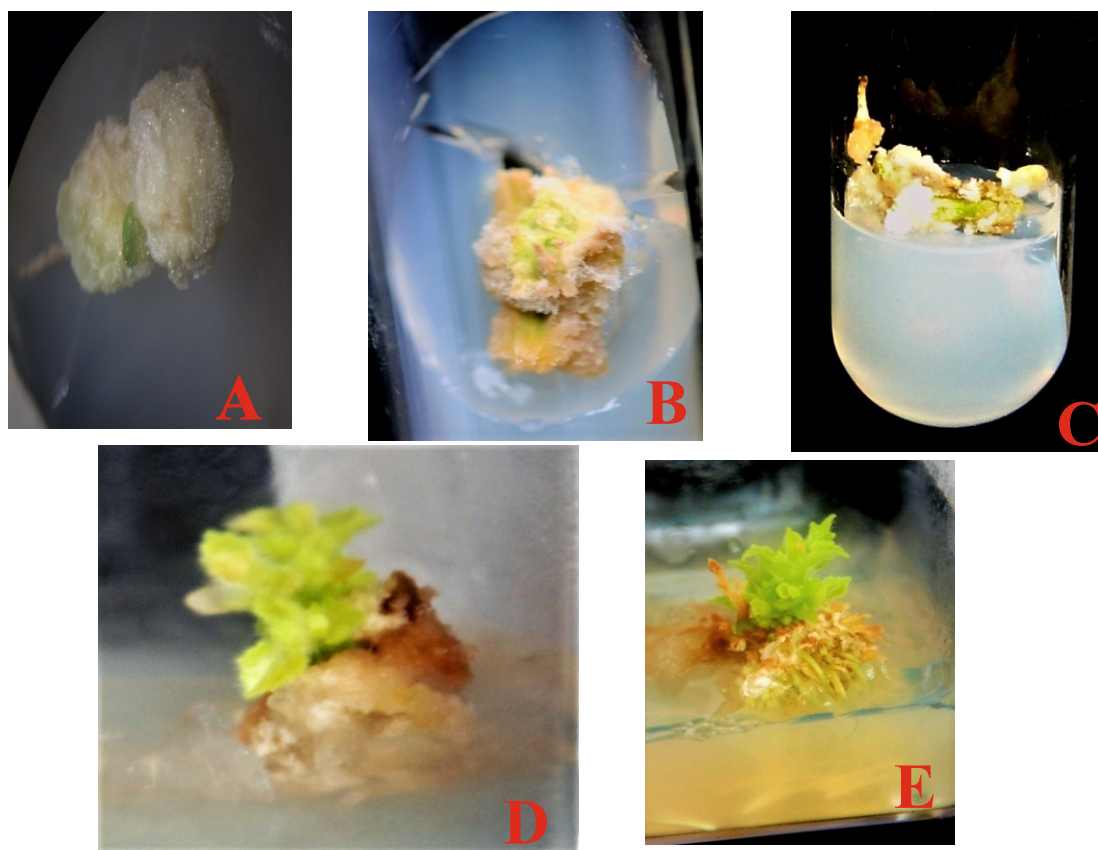


Figure 1 (A – E): *In vitro* plantlet regeneration through callus culture in *Trichosanthes dioica* Roxb. **A.** White crystalline friable callus after 45 days of inoculation in MS media supplemented with 2mg/l BAP+ 4mg/l 2,4-D. **B.** Off white to greenish white callus formed after 30 days of inoculation. **C.** Origin of organogenesis seen in pale yellow callus sub cultured on MS media supplemented with 1.5mg/l BAP + 0.3 mg/l NAA after 14 days of culture. **D.** Shoot elongation on MS media supplemented with 1.5 mg/l BAP + 0.3 mg/l NAA and 3% (w/v) sucrose after 30 days of subculture. **E.** Regeneration of multiple shoots with roots from node derived callus on MS media supplemented with 1.5 mg/l BAP + 0.3 mg/l NAA after 30 days of culture.

indica and Hoque *et al.* (1998) and Komal *et al.* 2010) in *Trichosanthes dioica* Roxb. Although visible callus induction started after 15 days of inoculation but the optimum callus formation was observed in MS + (2mg/l BAP + 4mg/l 2,4-D) media after 45 days of inoculation (Fig.1A, Table-2). Contrary to the result obtained in the present investigation (Saurabh *et. al.* 2017) reported that MS medium with lower concentration of BA (0.5mg/l) and 2,4-D (0.5mg/l) was the best medium for efficient callus induction in case of *Trichosanthes dioica* Roxb. The callus formation and regeneration varied both with the type of explants and kind of supplements used. The callus varied from shiny white, creamy white, pale yellow to greenish white in colour (Table-2, Fig.1A, B, C, D, E, Fig.2A). It was crystalline and friable

in nature and its friability retained even after several subcultures without any morphogenesis. The colour and nature of the callus also varied with the type of explants used and the concentration of different hormones (Table-2, Fig.1A - E). In some leaf explants, roots were also generated along with callus which turned brown after 30 days of inoculation (Fig.2C). Gunasena and Senarath, (2019) also reported that leaf was better than nodal segment as explants for callus induction in case of stevia. Saurabh *et al.*, (2017) observed that nodal explants were the best explants for callusing and direct organogenesis. Genotypic dependent response in callus formation was also reported by (Al-Hussaini *et al.* 2015).

Well-developed callus was sub cultured on

Table 2: Effect of different concentrations and combinations of growth regulator on nature, colour, callus induction and callus proliferation using shoot tip and nodal explants of *Trichosanthes dioica* Roxb. after 45 days of culture.

Growth regulators (mg/l)	Morphogenic response of callus induction from explants		Amount of callus produced	
	Nodal explants	Shoot tip explants	Nodal explants	Shoot tip explants
BAP				
1.5	Swelling of the explants	Swelling of the explants	-	-
2.0	White, Crystalline,	Creamy, Soft, Friable	++	++
2.5	White, Crystalline	Creamy, Soft, Friable	+++	+++
3.0	White, Crystalline	Creamy, Soft, Friable	++	+
3.5	White, Crystalline	Creamy, Soft, Friable	++	+
4.0	White, Crystalline	Swelling of the explants	-	-
4.5	-	-	-	-
Kn				
1.5	Swelling of the explants	Swelling of the explants	-	-
2.0	Swelling of the explants	Pale, Soft, Friable	-	++
2.5	Pale, Compact	Creamy, soft, Friable	+	++
3.0	White, Crystalline	Creamy, Compact	++	+
3.5	White, Compact	Creamy, Compact	++	+
4.0	White, Compact	Swelling of the explants	+	-
4.5	-	-	-	-
BAP+2,4-D				
2.0+ 2.0	Creamy, Soft,Ccompact	Creamy, Soft, Friable	+	+
2.0+2.5	Creamywhite, Compact	Creamy, Soft, Friable	+	+
2.0+3.0	White, Crystalline, Friable	White, Crystalline, Friable	++	++
2.0+3.5	White, Crystalline, Friable	White, Shiny Crystalline, Friable	+++	+++
2.0+4.0	White, Crystalline, Friable	White, Shiny Crystalline, Friable	++++	++++
2.0+4.5	Creamy, Soft	Creamy, Soft	++	+

- = No growth, + = Low growth, ++ = Normal growth, +++ = High growth, ++++ = Optimum growth

MS medium supplemented with different concentrations of BAP, Kn, 2,4-D and NAA alone and on MS medium supplemented with different combinations of BAP and NAA (Table-2). In earlier reports of Komal (2011) it is evident that a combination of BAP and NAA showed the highest percentage of shoot regeneration and maximum number of shoots per explant in *Trichosanthes dioica* Roxb. In the present study also similar data was observed. Sub cultures were done at every 45 days interval. To avoid browning of the medium, frequent transfer of explants to a fresh medium has been earlier reported by (Shutter *et al.* 1997 and Amjad and Srivastava, 2011) in regeneration of strawberry and the same was found to be effective in the present

investigation.

Efficient regeneration of multiple shoots was observed from shoot tip and nodal explants on MS medium fortified with 1.5mg/l BAP. An average of 02.50 ± 0.73 and 03.52 ± 0.72 shoot buds were regenerated having a length of ($0.2.42 \pm 0.59$ and 04.42 ± 0.35) cm from shoot tip and nodal explants respectively (Table-2). The similar effect of BAP for regeneration of multiple shoots were also observed in different plants like *Momordica charantia* Islam *et al.* (1994), *Trichosanthes cucumerina* L. var. *cucumerina* Devendra *et al.*, (2008) and in *Trichosanthes dioica* Roxb. (Lee and Thomas, 1985, Komal *et al.* 2010, Saurabh *et al.* 2017). BAP was found to be more potent in the induction of shoot buds as earlier observed by

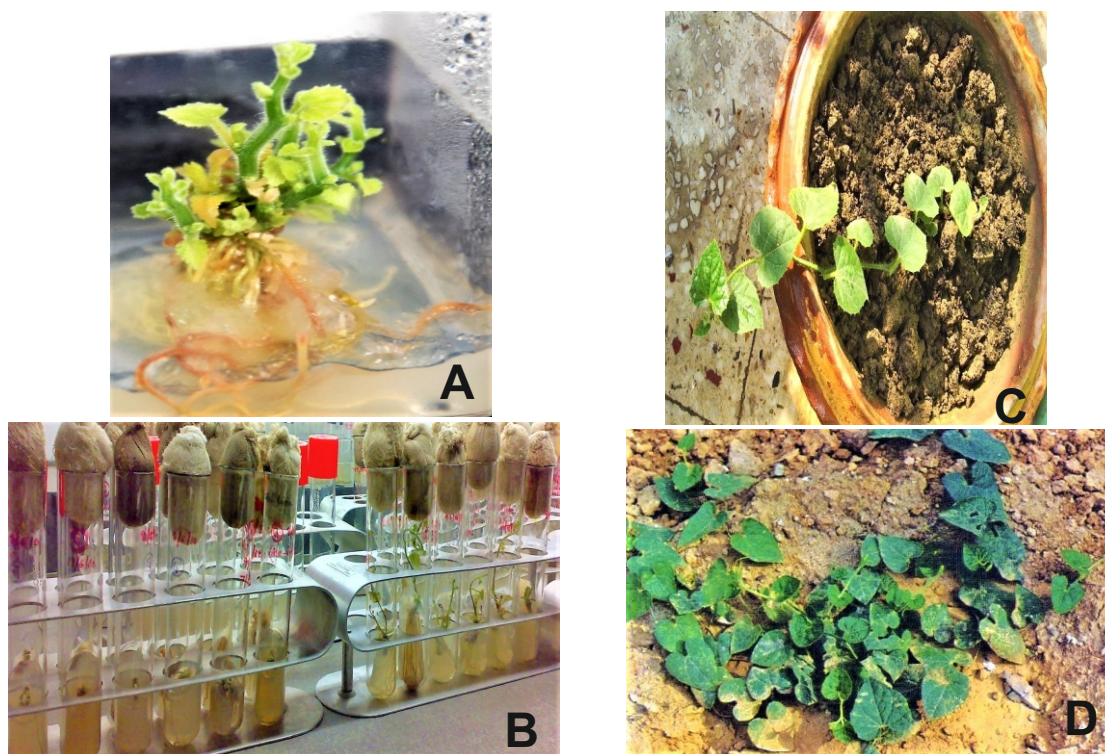


Figure 2 (A– D): *In vitro* plantlets regeneration and their successful field transfer through callus culture in *Trichosanthes dioica* Roxb. **A.** Professed rooting of *in vitro* obtained shoot on half strength MS media +1.0 mg/l IBA +3% (w/v) sucrose after four weeks of culture. **B.** Plants with professed roots after 35 days of inoculation in half strength MS media fortified with 1.0mg/l IBA. **C.** The plantlets successfully established in earthen pots containing a mixture of sterilized soil, sand and farmyard mixture in the ratio of 1:1:1. **D.** Plants after one month of transfer to natural soil and environment.

Table 3: Effect of different combinations of growth regulators on shoot multiplication from shoot tip and axenic node derived callus on MS medium after four weeks.

Plant growth (cm) regulators (mg/l)	Shoots regenerated (%)		No. of shoots per culture		Shoot length	
	Shoot tip derived callus, explants	Node derived callus, explants	Shoot tip derived callus	Node derived callus	Shoot tip derived callus	Node derived callus
BAP						
0.5	40.00	44.00	00.75±0.35	00.64±0.54	01.20±0.28	01.88±0.57
1.0	60.00	58.00	01.85±1.00	01.40±0.64	01.62±0.50	03.42±0.54
1.5	75.00	80.60	02.50±0.73	03.52±0.72	02.42±0.59	04.42±0.35
2.0	68.00	75.00	02.00±0.62	02.22±1.00	02.00±0.44	02.44±0.52
2.5	55.00	65.00	01.00±0.62	01.57±0.32	01.00±0.20	01.52±0.38
2,4-D						
1.0	-	-	-	-	-	-
1.5	-	-	-	-	-	-
2.0	20.00	33.00	02.00±0.54	02.44±0.34	02.50±0.79	03.00±0.42
2.5	10.00	22.00	01.00±0.54	01.34±0.56	02.00±0.88	02.70±0.39
BAP+NAA						
1.5+0.1	74.00	78.00	03.33±0.74	05.00±0.66	02.12±0.34	02.45±0.44
1.5+0.2	80.00	86.00	05.44±0.62	06.68±0.34	02.34±0.56	02.89±0.56
1.5+0.3	84.00	90.00	05.78±0.34	08.00±0.64	03.22±0.84	04.88±0.74
1.5+0.5	80.00	85.00	04.86±0.48	05.54±0.22	03.46±0.66	03.56±0.24
1.5+1.0	78.00	80.00	03.66±0.22	04.48±0.24	02.24±0.54	02.66±0.64
1.5+1.5	60.00	69.00	02.22±0.78	02.76±0.54	01.45±0.23	01.88±0.44

- = No response

Table 4. Effect of different growth regulators on rhizogenesis of regenerated shoots on half strength MS medium after four weeks.

Auxin (mg/l)		Rooting (%)	Rooting period (days)	No. of roots/shoot	Root length (cm)
IAA					
	0.1	-	-	-	-
	0.5	42.44	12-00	10 ± 1.14	3.78 ± 0.22
	1.0	60.62	10-00	22 ± 1.14	6.42 ± 0.44
	1.5	52.20	10-12	21 ± 0.88	4.46 ± 0.14
IBA					
	0.1	-	-	-	-
	0.5	46.60	08-10	06 ± 0.14	3.22 ± 0.24
	1.0	76.34	06-07	29 ± 0.36	8.48 ± 0.52
	1.5	58.24	10-00	18 ± 0.72	7.46 ± 0.24
NAA					
	0.1	-	-	-	-
	0.5	46.44	14-16	06 ± 0.24	2.16 ± 0.36
	1.0	71.22	14-00	19 ± 0.48	3.36 ± 0.44
	1.5	59.00	12-14	11 ± 0.16	2.00 ± 0.34

many workers (Shutter *et al.* 1997, Komal *et al.* 2010, Islam *et al.* 1994). Although shoot buds were formed in most of the combinations after 14 days of culture (Fig.1C, D) but proliferation of about 8 shoots was obtained from the callus cultures on MS medium fortified with (1.5mg/l BAP + 0.3mg/l NAA) after 30 days of culture with an average length of 4.88±0.74cm (Table-3, Fig.1E, Fig.2A). Results of the present investigation agrees with the investigations done in different plants like *Momordica dioica* Nabi *et al.* (1995), *Cucurbita moschata* Rahman *et al.* (1993), *Stevia rebaudiana* Gunasena and Senarath, (2019), *Trichosanthes dioica* Komal *et al.* (2010). BAP was more potent in induction of shoot buds as earlier observed by many workers (Shutter *et al.* 1997, Komal *et al.* 2010, Islam *et al.* 1994). BAP plays a significant role in the regeneration of multiple shoots also reported in different plants like *Trichosanthes cucumerina* L. var. *cucumerina* Devendra *et al.* (2008), *Trichosanthes dioica* Roxb. Komal *et al.* (2010) and *Momordica charantia* Hoque *et al.* (1998).

Each proliferated and adventitious shoot was cut and sub cultured on half - strength MS media supplemented with IBA, IAA and NAA (Table-4). Either IAA or IBA at various concentrations are commonly used auxins to

induce rooting from the explants both in culture and *in vivo* conditions. George *et al.* (1984) and Varisai *et al.* (1998) were successful in inducing roots of the shoot tip derived shoots on MS medium supplemented with IBA at various concentrations. In most of the cucurbits the root induction was achieved on either basal MS medium alone or with very low level of auxin as also observed in *T. dioica* (Mythili and Thomas 1999). In the present study also half-strength MS media supplemented with 1.0 mg/l IBA showed efficient rooting (29 ± 0.36 roots/shoot) and an average length of 8.48 ± 0.52 cm within seven to eight days (Fig.2A, B). With a higher concentration of IBA (2.0-3.5 mg/l) malformed and very thick roots were observed which were not suitable for field transfer. This finding is similar to the findings of Komal *et al.* (2010). In lower concentrations of IBA (0.3-0.1mg/l) direct rooting/ rooting with some callusing was observed in most of the cases. Number of roots per shoot and the percentage of shoots showing rhizogenesis varied with different concentrations of IAA, IBA and NAA (Table-4). Within 30-35 days the plantlets were ready with strong professed roots to be hardened (Fig.2A, B). So, it was found that half strength MS supplemented with IBA proved to be better than IAA and NAA for rooting of pointed gourd. IBA has proved to be an effective

rooting hormone and is continuously used by many workers (George and Sherrington, 1984, Pradhan *et al.* 1998, Ndoeye *et al.* 2003). Contrary to the findings of present investigation Saurabh *et al.* (2017), Rout *et al.* (2000) reported that 0.5 mg/L IAA was found more efficient for root development in pointed gourd.

The *in vitro* raised plantlets were sequentially acclimatized by transferring them in an inorganic MS salt solution, then to sterile distilled water and tap water before planting in potted soil. Within 30-35 days the plantlets were ready with strong professed roots to be hardened (Fig.2 B). The plantlets were successfully established in earthen pots containing a mixture of sterilized soil, sand and farmyard manure in the ratio of 1:1:1. (Fig.2 C) showing 60% survival rate. These were then transferred to natural soil and environment (Fig.2 D).

The present study reveals an efficient and reproducible protocol for multiple shoot regeneration from callus cultures in *Trichosanthes dioica* Roxb. an important medicinal and commercial vegetable plant. The plantlets obtained through callus cultures have a great potential in obtaining soma clonal variants and *in vitro* mutation breeding. There may be a difference in the ploidy level of these plants. These variations are still to be analysed both qualitatively and quantitatively.

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