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Germplasm conservation through plant tissue culture of a wild species of *Solanum torvum*.

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Abstract
A wild relatives of crop plants constitute a pool of genetic diversity which is invaluable for breeding programme. Due to environmental hazards and ruthless exploitation, there is a rapid depletion of wild flora. It is highly desirable to make systematic survey of the locality and suggested protocol for its conservation and propagation. Micropropagation technique has emerged as the best tool for mass propagation and germplasm conservation. Keeping these objectives into consideration, tissue culture studies of *S. torvum* were being undertaken to develop protocol for *in vitro* mass propagation and conservation. Sterilized segments of nodal (8-10 mm) & shoot-tip (8-10 mm) of *Solanum torvum* (about 2 years old) were used as explants and cultured on MS (Murashige & Skoog's,1962) medium containing 0.8% agar, 3% sucrose and different combination and concentration of NAA / 2,4-D and Kinetin (Kn) to obtain regenerants / plantlets. Techniques were standardized for shoot regeneration directly from node and shoot-tip explants as well as from callus. Callus was in general white /greenish-white, compact, hydrated and crystalline appearance. *In vitro* obtained plantlets were morphologically identical to parent plants. Nodal and shoot-tip explants were superior to other explants with respect to shoot regeneration, where as nodal explants was superior for callogenesis. Work are in progress to develop protocol for isolation of active constituents of medicinal importance of *S. torvum* plants growing as wild species of egg plants.

Keywords: Callus, crystalline, germplasm, in vitro, plant regeneration.

1. Introduction
Propagation of valuable economic plants through tissue culture is based on the principle of totipotency. During this principle, new plants may be raised in an artificial medium from very small parts of the plant called explant. The explant develops into a plant or grows into unorganized cells depending on the genetic potential of the tissue and the chemical as well as physical environments of the culture.

The application and development of this technique is opening the door to a second green revolution. The available protocols of plant tissue culture include mass micropropagation through organogenesis, somatic embryogenesis and shoot-tip culture, preservation and conservation of germplasm, anther and pollen culture, ovule and embryo culture, protoplast isolation and culture, somatic hybridization, mutagenesis at single cell level, genetic manipulation through transgenosis and production of polyploidy derivatives and have been appllied to a wide range of disciplines-genetics, physiology, cell biology, anatomy and biochemistry to solve various problems (Rotino GL 2016, Gatti E *et al*., 2017) [33, 13]. The potential use of *in vitro* techniques in tree improvement programe has been clearly established (Perez and Postigo, 1989) [29] and these methods have been successfully employed for large scale multiplication of a number of woody plants (Mascarenhas *et al*., 1988; Purohit and Dev, 1996, Rameshkumar R *et al*., 2017) [22, 30, 34], however, success in obtaining plantlets through organogenesis or embryogenesis in tree species is rather limited (Lakshmi Sita *et al*., 1986; Rai and Chandra, 1989, Devendra *et al*. 2011) [21, 31, 11]. Sub culture is a method of *in vitro* culture of cell, tissue and organ in a sterile culture medium.

Besides propagation of superior clones, *in vitro* clonal propagation serves as mean of germplasm conservation, isolation of secondary metabolites and maintenance of diseae free stocks (Ahuja, 1994 & Singha *et al*. 2018) [1, 39]. Tissue culture techniques have become in a big way to rescue endangered rare platns known for their medicinal, timber and ornamental value (Purohit and Dev, 1996) [30]. Success has been achieved in tissue culture of several

medicinal and oranmental plants (Chaturvedi *et al*., 1974; Naseem and Jha, 1997; Sharma *et al*., 2008) [6, 26, 38] and quite a large number of other medicinal and ornamental plants are being explored in different laboratories of world. Any attempt to improve the active constituents of medicinal importance and conserve germplasm through plant tissue culture technique must be preceded by finding out its morphogenic potentialities. In this background, tissue and organ cultures of the present experimental plant having desirable gene pool as well as known medicinal property would be beneficial in deciphering the pathway of differentiation and morphogenesis.

This technique can be referred to as "botanical laser" whose numerous uses are yet to be explored and fully understood. The tools of plant tissue culture are bieng applied to a wide range of biotechnology ventures and in particular to the clonal propagation and genetic upgradation of crop and medicinal platns (Jagannathan, 1988 & Singh 2011) [14, 35]. In recent years, tissue culture techniques have become useful tools in the hands of plant scientists of all disciplines because these techniques are more handy, less time consuming and less labour involving over the conventional methods of breeding and propagation (Chandra *et al*., 1985; Chaturvedi *et al*., 1974; Naseem and Jha, 1997; Bhojwani and Razdan, 2004, Sharma *et al.*, 2008 and Kumar *et al*., 2010) [7, 6, 26, 4, 38].

The primitive cultivars and wild relatives of crop plants constitute a pool of genetic diversity which is invaluable for future breeding programme. However, the existence of these plants are in danger due to induction of new cultivars and other environmental hazards. Germplasm includes plant parts which are used for maintenance, conservation and propagation of any biotype, it also acts as genetic pool.

Solanum torvum, commonly known as titbaigun and devil's fig is a bushy perennial wild plant measuring 150-300 cm in height and usually growing in sub-tropical areas throughout the world as a weed of disturbed areas. In Muzaffarpur, it is found growing in pastures, road sides and waste lands but not significantly in cultivated land. It prefers moist and fertile soil and also tolerates drought and saline soils (Naseem 1990, & Rathore *et al.* 2005) [25, 32].

Fruits are eaten as vegetable and used as ingredient of pickles, it is said to be good for enlargement of the spleen (Chopra *et al.*, 1956, Boxus 1974, Ganpati *et al.* 1992) [9, 5, 12]. Fruits contain a number of potentially pharmacologically active chemicals including sapogenin, steroid, sterolin, chlorogenin and solasonine (Badola *et al.* 1993) [3]. Tapia and others (1996) [40] reported that aqueous extracts of turkey berry *(S.torvum)* were lethal to mice or depressed the erythrocytes, leukocytes and platelets in their blood. Extracts of the plant are reported to be useful in the treatment of hyperactivity, colds and cough (Null, 2001; CPR Environmental Education Centre, 2001) [28, 10], pimples, skin diseases and leprosy. This plant is also used medicinally for the treatment of epilepsy (Kumar *et al.*, 2012,2014, Jaiswal BS 2012) [18, 15].

Conservation of germplasm of this wild crop is highly needed for developing perennial brinjal variety, a common vegetable for millions of poeple of the world and its medicinal uses are also required to be investigated in right perspectives. In this background, it is necessary to multiply this plant through *in vitro* methods. Calli and regenerants obtained through *in vitro* methods can be used for germplasm conservation as well as for biochemical analysis. For rapid multiplication of these wild plants, micropropagation is being increasingly applied to supplement conventional methods of propagation (Mascarenhas and Murlidharan, 1988) [22].

Our investigation is based using explants collected from mature *in vivo* grown plant (about 2 years old) and the cultures were maintained under continuous, cool and white fluorescent light (2000 lux) during the whole experiment. In my opinion, the present investigation would be the first thorough studies on organ cultures of this taxon. As the tissues of mature plant are as a rule recalcitrant, the tissue culture studies with explants taken from mature plant are of great significance. Hence, the present studies were aimed at *in vitro* regeneration of *S. torvum* through direct and callus mediated shoot regeneration using explants taken from *in vivo* grown plant (about 2 years old) under different hormonal concentration. Works were also made to protocol through different methods for germplasm conservation through *in vitro* propagation.

Material and Methods

The experimental plant, *Solanum torvum* swartz belonging to family Solanaceae is a bushy perennial wild plant. It is used horticulturally as a root stock for egg plant (*Solanum melongena*) and also used medicinally for the treatment of epilipsy (Wagner *et al*., 1999) [42] Grafted plants are very vigrous and tolerate diseases affecting the root system. Tissue culture studies on vegetative parts (nodal & shoot-tip) of this plant were carried out under normal *in vitro* conditions. This study was also aimed to develop protocol for *in vitro* conservation of germplasm. The methodology of tissue culture experiments includes the following steps:

a. Preparation of culture media
b. Preparation of Explants
c. Inoculation and Transfer
d. Maintenance of Cultures
e. Effect of Seasonal Variation on Regeneration
f. Rooting and Transfer of Plantlets

Nutritional requirements for optimal growth of a tissue *in vitro* may vary from species to species. Even tissues from different parts of the same plant may have some specific requirements for their satisfactory growth. In the present study, MS (Murashige and Skoog's 1962 & Nistch 1969) [23, 27] were used as basal media. However, all works were carried out on Murashige and Skoog's medium (1962) [23] as this medium was suitable for regeneration and callus induction. The sequence of steps invovled in preparing the medium was as follows:

i. Required quantities of agar (0.8% w/v) and sucrose (3% w/v) were weighted out.
ii. Sucrose was dissolved in some amount of distilled water to give a concentrated solution and was filtered through the Whatman filter paper No. 1 (9.0 cm) to remove the particulate impurities, if any
iii. Appropriate quantities of various stock solutions and growth regulators were added.
iv. Agar was dissolved in distilled water (in about ¼ of the final volume of the medium) by heating in a water bath. The dissolved agar solution & sucrose solution were mixed with stock solution.
v. The final volume of the medium was made upto 1 litre / required volume with distilled water.
vi. After proper mixing, the pH of the medium was adjusted to 5.8 using 0.1N NaoH or 0.1N HClwit the help of "Systronic" digital pH meter model no. 335.
vii. About 20 ml of the medium was poured into the culture tube (25 x 100 mm)

viii. The culture tubes were plugged with non-absorbent cotton wrapped in cheese cloth. The cotton plugs were wrapped with aluminium foils to prevent wetting during autoclaving.
ix. The culture vessels were transferred to appropriate baskets and autoclaved at 121^{0}C (1.06 Kg/cm2) for 20 minutes.
x. Slants were prepared by keeping the tubes titled during cooling.

The pH of the medium was adjusted to 5.8 before autoclaving at 121 ^{0}C for 20 minute. Various growth regulators and adjuvants used as supplement of the basal medium were IBA, NAA, 2,4-D & Kn. Stem segements (nodal & shoot-tip) segements collected from *in vivo* grown mature plant (about 2 years old) of *Solanum torvum* during March to November were used as explants and were surface sterilized. These adjuvants were used in a wide range of concentration (1-10 mgl^{-1}) either alone or in various combinations. The stocks of various growth regulators were prepared.

All the precautions were taken while sterilizing the tissues avoiding any damage to them. The following steps were undertaken for sterlization of tissues or organ explants.

i. Washing the explatns in running tap water
ii. The explants were treated for 2 min. in 1 % cetavelon (cetrimide I.P. 20 % w/v isopropyl alcohol B.P. 10 % v/v) solution followed by thorough washing in running tap water.
iii. Washing and dsinfecting the explants in 0.2 % HgCl2 solution for 3 to 5 min. depending upon the nature of the explants.
iv. Further, washing them three or four times thoroughly with sterile distilled water in an aseptic condition and
v. Finally using sterile forcep, tissue explants were transferred to sterile petridishes and were cut into required size with sterile scalpel or blade. Usually, node and internode of 8-10mm, leaf segments of 5x5 mm and shoot-tip 10-15 mm were trimmed out for explant preparation.

The cultures were incubated in culture room mainted at 25 $\pm$ 2 ^{0}C with a relative humidity of about 60% under continuous fluorescent light (2000 lux, cool and white). Calli obtained from different explants were taken out of the culture tubes aseptically and kept in a presterilized. The callus was cut into several pieces of almost equal sizes with the help of a sterilized blade. Pieces of calli from the growing portions were inoculated into the culture tubes containing MS medium with different combinations and concentrations of growth regulators. The calli were incubated at 25$\pm$ 2 ^{0}C for further growth and differentiation.

Microshoots (3-4 cm) obtained from shoot-tip, nodal segment and regenerative callus in *S. torvum* were cultured on MS and rooting media (RM ½ MS salts + full strength vitamins & amino acid) supplemented with IBA and NAA singly and in combination for rhizogenesis. Culture conditions were kept constant as in shoot regeneration (Temp. 25 $\pm$ 20 C, Light - 2000 lux, continuous, cool, white and fluorescent).

Results and Discussion

The explants of nodal segment (8-10 mm) and shoot-tip (10-15 mm) were taken for experiment and these explants were shown better results in culture. It was also remarkable in the present system that a proper amount of growing callus was must for inoculum to had better differentiation and regeneration, a small piece of callus having few cells could not survive in culture.

The composition of culture medium is the most important factor for the establishment of tissue culture. From screening of literature, it is confirmed that there is no fixed combination of the medium which is suitable for all the plants and even the different organs of the same plant. A particular combination of the nutrient medium is suitable for a certain group of plants but the same combination proves ineffective for other plants. So, the selection of proper culture medium is essential for the tissue culture experiment of any plant. The response of two basal media viz MS and Nitsch was tested in case of present experimental system and the results have been presented. MS medium was found most suitable for shoot regeneration and callus growth.

Normally, a high cytokinin to auxin ratio promotes shoot formation while a higher auxin to cytokinin ratio favours root differentiation. In a number of cases, cytokinin alone is sufficient for shoot regeneration and callus formation. Identical response of cytokinin was encountered in *S. torvum* cultures. Kn (2-3 mgl^{-1}) induced direct development of shoots from nodal and shoot-tip segments in *S. torvum*, optimum response was obtained on 2 mgl^{-1} Kn. The frequency of shoot regeneration was better in nodal culture of *S. torvum* than shoot-tip culture. No callus formation was obtained on Kn supported media in nodal and shoot-tip explants. Kn above 3 mgl-1 had adverse effect on shoot regeneration in nodal and shoot-tip cultures of *S. toruvm*.

In the present investigation, the best shoot regeneration in nodal explant was obtained on 4 mgl^{-1} Kn + 2 mgl^{-1} NAA and in shoot-tip explants on 3 mgl^{-1} Kn + 2 mgl^{-1} NAA and 2 mgl^{-1} 2,4-D + 2 mgl^{-1} Kn. Shoot regeneration through callus subculture was frequent in the present experimental system on NAA/ 2,4-D and Kn supplemented media, the optimum response with better shoot regeneration from callus was noted on 5 mgl^{-1} Kn and 2 mgl^{-1} NAA and 2 mgl^{-1} 2,4-D + 4 mgl^{-1} Kn. This is also in confirmity of the above facts. Thus, a fine balance of exogenous auxin & cytokinin / cytokinin alone is necessary before successful regeneration can occur (Table 1). This was also confirmed in many plants by Naseem and Jha (1997) [26] & Ansari (2011) [2].

Kn in combination with NAA / 2,4-D proved effective for shoot regeneration and callus growth in this experimental system. The callus in general was greenish-white/white, compact, hydrated and crystalline in appearance. However, in some hormonal combinations, the node derived callus was creamy, white, compact, hydrated and crystalline in appearance. Callus mediated regeneration was frequent in subculture on 2 mgl^{-1} NAA and 3-5 mgl^{-1} Kn / 2,4-D + Kn. The optimum response of callus mediated callogenesis was recorded on 5 mgl^{-1} Kn + 2 mgl^{-1} NAA and 2 mgl^{-1} 2,4-D + 4 mgl^{-1} Kn. In addition to direct shoot regeneration in nodal and shoot-tip explants, protocol for callus mediated shoot regeneration can also be adopted in the present experimental system as these shoots were morphologically identical to parent plants. Calli were maintained in culture till 1½ years for regeneration on 1 mgl^{-1} NAA + 1 mgl^{-1} Kn and no regeneration was noted on maintenance medium (1 mgl^{-1} NAA + 1 mgl^{-1} Kn). The rejuvination in callus subculture was recorded on 2 mgl^{-1} 2,4-D + 2 mgl^{-1} Kn, the callus gradually turned brown in the beginning and after a month profuse shining white callus grew from degenerated callus mass on the same combination of hormones. The best response for shoot regeneration was obtained on 2 mgl^{-1} Kn in nodal and shoot-tip cultures of *S. torvum* where as NAA (2 mgl^{-1}) + Kn

(2-4 mgl^{-1}) and 2,4-D (1-2 mgl^{-1}) + Kn (1-2 mgl^{-1}) were most responsive combinations for callus growth as well as shoot formation in the present system (Table-2). Work are in progress to develop protocol for isolation of active constituents of medicinal importance of *S. torvum* plants growing as wild species of egg plants.

Effect of seasonal variation on Regeneration

Influence of explant age and season of explant collection from in vivo grown *Solanum torvum* were studied on suitable combinations of hormones (MS + 2 mgl^{-1} Kn, MS + 2 mgl^{-1} NAA + 3-4 mgl^{-1} Kn) in case of nodal and shoot-tip explants. Age of the explant and seasonal variations (March-May, June-Aug, Sept.-Nov., Dec.-Feb.) greatly influenced shoot regeneration in culture (Table 3). The frequency of shoot regeneration was promising in explants collected during March to November however juvenile explants collected during March to August were most regenerative (Wagner *et al.* 1999) [42].

Table 1: Effect of different combinations of growth hormones on callus induction and shoot regeneration in nodal culture of *Solanum torvum**

Hormones (MS medium)	Hormonal Concentration (mgl-1)	% culture Showing response	No. of shoots/ culture	Other response
BM	-	-	-	-
NAA	1-3	-	-	Greening of explants
	5	-	-	Do (died)
2,4-D	1	80.5	-	Callus (1840±15.74
	2	86.4	-	Callus(2040 ± 30.6)
	5	95.6	-	excellent callus (2840± 48.6)
	10	48.8	-	Callus (1650±14.60) (Browning)
Kn	1	70.4	1.0±0.2	Bulging
	2	95.4	5.0±0.3	Bunchy shoots
	3	90.5	3.0±0.4	Hypertrophy, Enlargement
	5	92.2	3.0±0.2	Curling of shoots
	10	49.4	1.0±0.4	Hypertrophy (Browning)
NAA+Kn	2+1	91.4	1.0±0.2	Bulging,greening, callus at base
	2+3	92.6	1.0±0.2	Good callus (1550±25.64)
	2+4	96.4	3.0±0.2	Excellent callus (2650±48.6)
	5+5	46.8	-	Callus (Brown)
2,4-D+Kn	1+2	80.6	1.0±0.2	Callus, greening & bulging of explants
	2+2	89.6	-	Vigorous callus (4050±55.60)
	5+3	84.6	-	Excellent callus (3590±50.40)
	5+5	51.2	-	Callus (Browning)

*Growth Period : 21 days
No. of replicates : 20
Culture medium : MS with 3% sucrose & 0.8% agar
Figures in parentheses indicate fresh callus weight (mg)

Table 2: Callus induction and *in vitro* shoot regeneration in shoot-apex culture of *Solanum toruvm* on suitable combinations of growth hormones*

Hormones (MS medium)	Hormonal Concentration (mgl-1)	% culture Response	No. of shoots/ per culture	Other Response
	-	-	-	Single shoot
BM	1-5	-	-	-
NAA	1	90.4	1±0.2	C+
2,4-D	2	95.6	-	C++, deformed shoot
	5	65.4	-	C++, Brown
Kn	1	82.2	1±0.3	Green shoot
	2	92.4	3±0.2	Robust shoot
	3	90.4	2±0.3	Shoots
	5	72.6	1±0.2	Deformed shoot
NAA+Kn	1+1	90.2	2±0.3	C+, Shoot
	2+2	95.4	2±0.4	C+
	2+3	95.6	4±0.3	Shoots
	5+5	48.8	2±0.2	Deformed shoot (Browning)
2,4-D+Kn	1+1	82.5	2±0.2	Shoot
	2+2	92.5	2±0.2	C+
	3+2	95.4	-	C++
	5+5	62.4	-	C+ (deformed)

*Growth Period : 21 days
Culture replicate : 20
Culture medium : MS + 3% sucrose & 0.8% agar
- - No response
C+ = Poor Callus, C++ = Moderate Callus, C+++ = Excellent Callus

Table 3: Effect of seasonal variations on regeneration potential of nodal (N) and shoot-tip (ST) segments of *Solanum torvum**

Age of explants (Duration in month)	Explant	Total no. of explants treated	Number regenerating	% regeneration
March-May	N ST	30 30	27 27	90, Fast growth 90, Fast growth
June-August	N ST	30 30	29 28	96, Fast growth 91, Fast growth
Sept.-Nov.	N ST	30 30	24 23	80, Moderate growth 76, " "
Dec.-Feb	N ST	30 30	15 14	50, Slow growth 42, Slow growth

Fig 1: *Solanum tovum* Swartz plant growing in nature

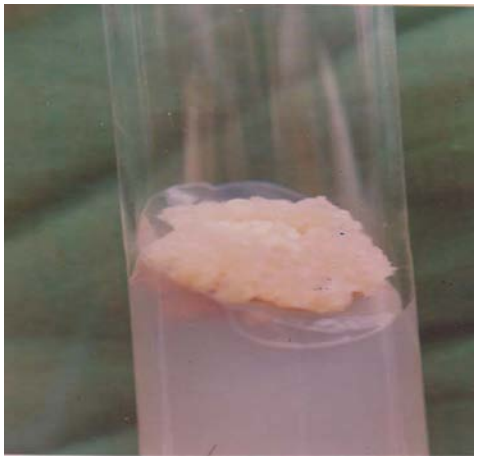

Fig 2: Explant showing excellent growth of callus on MS + 5mgl-1 2, 4-D; 25 days old culture, mark white and crystalline callus

Fig 3: 25 days old culture on MS+2mgl^{-1} NAA+3mgl^{-1}Kn

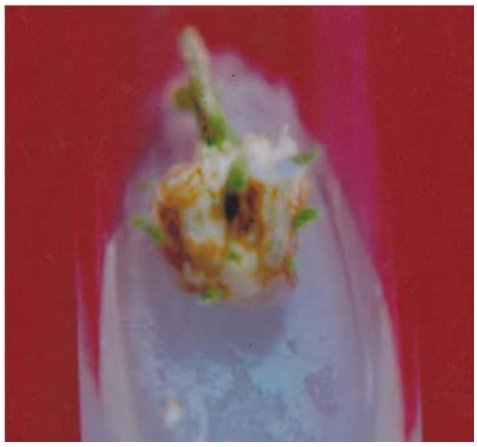

Fig 4: Culture showing development of callus mediated green shoots on MS+2mgl^{-1} NAA + 5mgl^{-1} Kn;

Fig 5: 25 days old culture showing of development of vigorous callus on MS+ 2mgl^{-1} 2-4 D+ 2mgl^{-1} Kn

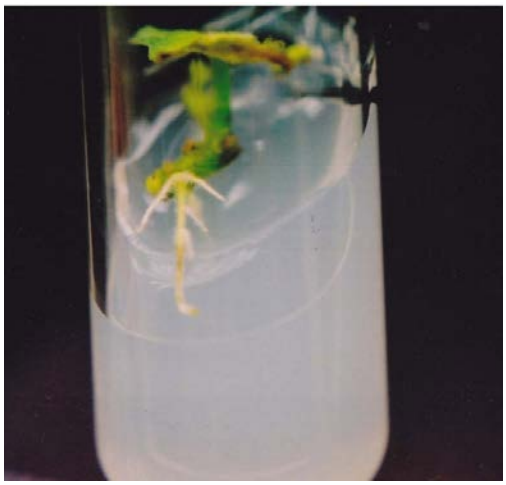

Fig 6: Culture showing development Profuse rooting; 25 days old culture

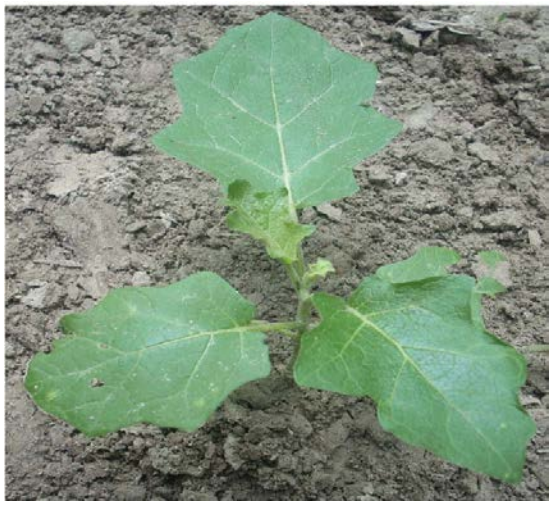

Fig 7: Culture showing development of Greening of stem and well developed rooting; 35 days old culture

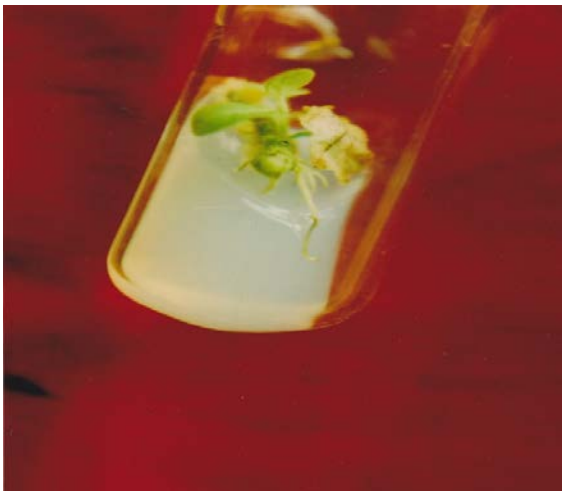

Fig 8: *In vitro* plant transferred in garden soil

Conclusion

It may be said that specific hormone or combination of hormones particularly auxin (NAA / 2,4-D) and cytokinin (Kn) are essential for shoot regeneration and callus growth. Organ formation is also governed by a number of factors such as environmental conditions, source of explants tissue, its genotype, its age and endogenous levels of growth hormones. The present investigations indicate that growth and proliferation of shoots in nodal and shoot-tip segments of *S. torvum* can be stimulated easily by using *in vitro* techniques. Protocols for shoot regeneration and callus mediated shoot regeneration as described in this manuscript may be used for clonal multiplication in this species which is otherwise, difficult to propagate by conventional methods. Further, on the basis of present findings, it can be inferred that calli obtained from different explant sources can be employed as an ideal system for preservation of germplasm which will be used as gene pool for the improvement of brinjal cultivars by biotechnological tools.

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