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Conservation and tissue culture studies of a wild species of eggplant-*Solanum torvum*

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Abstract
The wild relatives of crop plants constitute a pool of genetic diversity which is invaluable for breeding programme. The existence of these wild plants are in danger due to induction of new cultivars and other environmental hazards. There is urgent need of for germplasm conservation and germplasm improvement of this plant beside mass propagation. Keeping these objectives into consideration, tissue culture studies of *Solanum torvum*, a wild relatives of eggplant were being undertaken to develop protocol for *in vitro* mass propagation and conservation.
Regeneration of shoots and callus was obtained using sterilized segments of node (8-10mm), internode (10-15mm) and shoot-tip (8-10mm) of *Solanum torvum* (about 2 years old). These explants were 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 and callus differentiation. Techniques were used for shoot regeneration directly from node and shoot-tip explants as well as from callus. Shoot regeneration was best achieved on 3mgl-1 Kn in nodal and shoot-tip cultures. Callus mediated shoot regeneration was promising in the culture and was obtained on 2mgl-1 NAA and 4mgl-1 Kn on sub-culture. 2,4-D alone or 2,4-D+Kn resulted in callus differentiation from explants. Callus in general was white/greenish- white, compact, hydrated and crystalline in appearance. Callus was maintained for about 2 years on 1mgl-1 NAA & 1 mgl-1 Kn on regular sub-culture after 25 days. Callus turned brown on higher concentration (10mgl-1) of auxin and Kn on sub-culture. Rooting of microshoots (about 5cm) was obtained on RM (½ MS Salts) containing 1mgl-1 NAA & 2 mgl-1 IBA. Plantlets were successfully transferred to soil and they survived well in nature. Explants taken during December to May were most regenerative. Plantlets obtained through *in vitro* were morphologically identical to parent plants. Nodal explants were superior to other explants (internode, Shoot-tip) with respect to shoot regeneration, whereas internodal explants was superior for callogenesis.

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

Introduction
Tissue culture is a method of *in vitro* culture of cell, tissue and organ in a sterile culture medium. 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 being applied to a wide range of biotechnology ventures and in particular to the clonal propagation and genetic up gradation of crop and medicinal plants (Jagannathan, 1988, Rao, 2008, & Singh 2011) [16, 33, 36].
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; Chaturuvedi *et al.*, 1994; Kannan & Ksrai 1996, Naseem and Jha, 1997; Bhojwani and Razdan, 2004 and Sharma *et al.*, 2008 Kumar *et al.*, 2010) [8, 9, 17, 28, 5, 40, 21].
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, Siva *et al.* 2002 & Rathore *et al.* 2005) [27, 41, 34].
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, Ganapathi *et al.* 1992) [10, 7, 14].

Fruits contain a number of potentially pharmacologically active chemicals including sapogenin, steroid, sterolin, chlorogenin and solasonine (Badola *et al.* 1993 & Srivastava *et al.* 1995) [3, 42] Tapia and others (1996) [43] 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) [29, 11], pimples, skin diseases and leprosy. This plant is also used medicinally for the treatment of epilepsy (Kumar *et al.*, 2012, 2014) [20].

Conservation of germplasm of this wild crop is highly needed for developing perennial brinjal variety, a common vegetable for millions of people 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, 1987; Sarthi and Annexavier, 2006; Mathew and Prasad, 2007 and Bahera *et al.*, 2008) [24, 39, 23, 4].

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 (D'Souza, 1988; Sreelatha *et al.*, 2008) [12, 35]. 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 regimes. Attempts were also made to suggest methods for germplasm conservation through tissue culture.

Material and Methods

The experimental plant, *S. torvum* was subjected to tissue culture experiments with a view to explore the possibilities of micropropagation protocol and genetic upgradation through the use of somaclonal variants among regenerated plants. 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. A wide range of culture media differing in their elemental composition has been described in the literature. In the present study, MS medium devised by Murashige and Skoog (1962) [25] and Nitsch's medium devised by Nitsch (1969) [31] were used as basal media. However, all works were carried out on Murashige and Skoog's medium (1962) [25] as this medium was suitable for regeneration and callus induction. The sequence of steps involved in preparing the medium was as follows:

1. Required quantities of agar (0.8% w/v) and sucrose (3% w/v) were weighted out.
2. Sucrose was dissolved in some amount of distilled water to give a concentrated solution and was filtered through the Whatman filter paper No. 1 (9.0cm) to remove the particulate impurities, if any
3. Appropriate quantities of various stock solutions and growth regulators were added.
4. 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.
5. The final volume of the medium was made upto 1 litre / required volume with distilled water.
6. 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.
7. About 20 ml of the medium was poured into the culture tube (25x100mm)
8. 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.
9. The culture vessels were transferred to appropriate baskets and autoclaved at 1210C (1.06 Kg/cm2) for 20 minutes.
10. Slants were prepared by keeping the tubes titled during cooling.

The pH of the medium was adjusted to 5.8 before autoclaving at 121 ^{o}C for 20 minute. Various growth regulators and adjuvants used as supplement of the basal medium were IBA, NAA, 2,4-D & Kn.

Stem segments (nodes and internodes) and leaf segments from youngest shoots and shoot-tip segments 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 sterilization of tissues or organ explants.

1. Washing the explants in running tap water
2. The explants were treated for 2 min. in 1% cetavlon (cetrimide I.P. 20% w/v isopropyl alcohol B.P. 10% v/v) solution followed by thorough washing in running tap water.
3. Washing and disinfecting the explants in 0.2% HgCl2 solution for 3 to 5 min. depending upon the nature of the explants.
4. Further, washing them three or four times thoroughly with sterile distilled water in an aseptic condition.
5. Finally using sterile forcep, tissue explants were transferred to sterile petri dishes 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 maintained at 25$\pm$2 ^{o}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±2 ºC 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 1/2 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±2 ºC, Light-2000 lux, continuous, cool, white and fluorescent).

Results and Discussion

In the present experimental system, nodal segment (8-10mm), internodal segment (8-10mm), shoot-tip (10-15mm) and leaf (5x5mm) of these sizes were taken for experimentation and these explants yielded 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. MS medium was proved equally well in many other plants too (Naseem, and Jha, 1997; Shahzad Rathore *et al*. 1999, Haque *et al.*, 2000, Siva Subramaniam *et al.*, 2002 & Rathore *et al*., 2005) [28, 37, 15, 41, 34].

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 (Ganapathi *et al.*, 1992; and Kumari and Shivanna, 2005, Verma *et al.*, 2008) [14, 18, 44]. Identical response of cytokinin was encountered in *S. torvum* cultures. Kn (2-3mgl-^{1}) induced direct development of shoots from nodal and shoot-tip segments in *S. torvum*, optimum response was obtained on 2mgl-^{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 3mgl-^{1} had adverse effect on shoot regeneration in nodal and shoot-tip cultures of *S. torvum*.

In the present investigation, the best shoot regeneration in nodal explant was obtained on 4mgl-1 Kn+2mgl-1 NAA and in shoot-tip explants on 3mgl-^{1} Kn+ 2mgl-^{1} NAA and 2mgl-^{1} 2,4-D + 2mgl-^{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 5mgl-^{1} Kn and 2mgl-^{1} NAA and 2mgl-^{1} 2,4-D + 4mgl-^{1} Kn. This is also in conformity of the above facts. Thus, a fine balance of exogenous auxin & cytokinin/cytokinin alone is necessary before successful regeneration can occur. This was also confirmed in many plants by Naseem and Jha (1997) [28] and Kumari and Shivanna (2005) [18] & 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 sub culture on 2mgl-^{1} NAA and 3-5mgl-^{1} Kn/2,4-D+Kn. The optimum response of callus mediated callogenesis was recorded on 5mgl-^{1} Kn + 2mgl-^{1} NAA and 2mgl-^{1} 2,4-D + 4mgl-^{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 1mgl-^{1} NAA + 1mgl-^{1} Kn and no regeneration was noted on maintenance medium (1mgl-1 NAA + 1 mgl-^{1} Kn). The rejuvenation in callus subculture was recorded on 2mgl-^{1} 2,4-D + 2mgl-^{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. Calli were maintained in culture till 1½ years for regeneration on 1mgl-^{1} NAA + ^{1} mgl-^{1} Kn and no regeneration was noted on maintenance medium (1mgl-^{1} NAA + ^{1} mgl-^{1} Kn). The rejuvenation in callus subculture was recorded on 2mgl-^{1} 2,4-D + 2mgl-^{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 2mgl-1 Kn in nodal and shoot-tip cultures of *S. torvum* where as NAA (2mgl-1) + Kn (2-4mgl-1) and 2,4-D (1-2mgl-1) + Kn (1-2mgl-1) were most responsive combinations for callus growth as well as shoot formation in the present system. In general, auxin and cytokinin above 5mgl-1 were inhibitory for differentiation and regeneration. Direct formation of shoots was frequent in primary cultures of nodal and shoot-tip segments in *S. torvum*. Differentiation of callus was obtained on 2,4-D and NAA/2,4-D + Kn combinations from nodal, internodal, leaf and shoot-tip cultures, callus mediated shoot regeneration was frequent in culture during the present investigation. Identical effects of auxin-cytokinin combination on shoot multiplication/callus induction were observed in many plants (Naseem, 1990; Srivastava *et al.*, 1995; Haque *et al.*, 2000; Nair and Seeni, 2002; Verma *et al.*, 2008) [27, 42, 15, 44].

The role of sugars in tissue culture experiments were extensively studied (Agrawal, 1996, Wagner *et al.* 1999 & Shahzad and Siddique, 2000, Dhawan 2009) [1, 45, 38, 13]. They provide energy source and maintain a minimum osmotic potential for the cultured tisued. Many carbohydrates have been used in tissue culture but among them sucrose is the most effective except in a few plants where glucose was found superior to sucrose (Boxus, 1974; Pierik and Steegmans, 1976) [7, 32]. Sucrose is generally used at the concentrations of 20-30 g/l and in the present investigation, 30g/l sucrose was found most effective.

Table 1: 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
BM	-	-	-	Single shoot
NAA	1-5	-	-	-
2,4-D	1	90.4	1±0.2	C+
	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

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+2mgl-1 Kn, MS+2mgl-1 NAA + 3-4mgl-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 3.8). 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) [45].

Table 2: 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	30	27	90, Fast growth
	ST	30	27	90, Fast growth
June-August	N	30	29	96, Fast growth
	ST	30	28	91, Fast growth
Sept.-Nov.	N	30	24	80, Moderate growth 76, " "
	ST	30	23	
Dec.-Feb	N	30	15	50, Slow growth
	ST	30	14	42, Slow growth

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

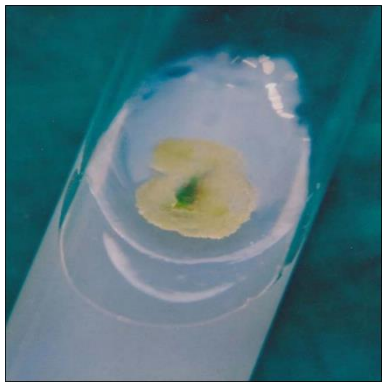

Fig 2: Explant showing development callus from entire surface on MS+2mgl-1 2,4-D+2mgl-1 Kn; 18days old culture

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

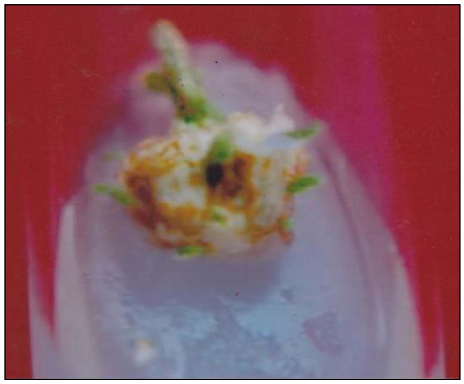

Fig 4: Culture showing development of callus mediated green shoots on MS+2mgl-^{1} NAA + 5mgl-^{1} Kn; 25 days old culture

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

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

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

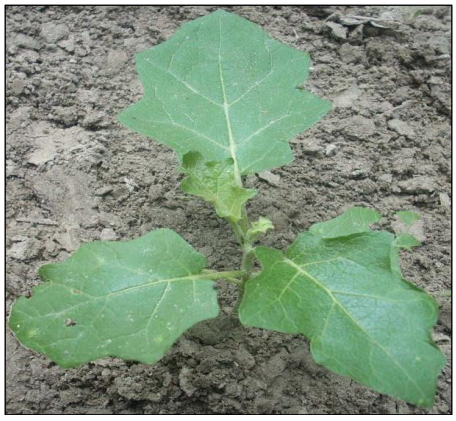

Fig 8: *In vitro* plant transferred in garden soil

Conclusion

In vitro tissue culture could be an important means of improving crop tolerance and yield through genetic transformation as well as by induced somaclonal variation. Therefore it is important to devise an efficient protocol of callus proliferation to broader opportunities for genetic manipulation of *S. torvum* through tissue culture, including trying various explants and media. Classical hormone based technologies of plant propagation or transformation are applicable only to limited species or accessions insights gained from basic callus research also have promising downstream application potentials.

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