# RESEARCH ARTICLE

## PROTEIN POLYPEPTIDES CHANGES DURING DIRECT ORGANOGENESIS CULTURE STAGES OF *SOLANUM TRILOBATUM* L.

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**ABSTRACT**

The present investigation was aimed to detect the specific protein polypeptide(s) appeared during the organogenesis stages of differentiation in *Solanum trilobatum* L. Among different explants, only internode showed good results for micropropagation. To analyze and compare their variation of protein profiles were assessed from callus, organogenic calls, multiple shoots, root, and regenerated plants. The highest response of callus induction (92.8%) was observed in 2.5 mg/l of 2,4-D (2, 4-dichlorophenoxyacetic acid) supplemented in Murashige and Skoog's (MS) medium. Organaogenic callus induction was maximum (34.6%) in medium supplemented with 2,4-D 3.0 mg/l. α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) and 2,4-D with BAP various combinations were showed variable redifferentiation response along with callus formation and multiple shoot initiation. The medium supplemented with 3.0 mg/l BAP with 3.0 mg /l of 2,4-D showed better shoot multiplication (87.6%) and elongation. High rate of root induction (68.3%) was observed on medium supplemented with NAA (1.0 mg/l). Simultaneous induction of shoot and root (58.4%) was observed in MS medium supplemented with BAP 2.0 mg/l with NAA 1.0 mg/l. For best plantlet regeneration was achieved in (86.2%) medium supplemented with BAP 2.0 mg/l with NAA 1.0 mg/l. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed a unique protein bands in tissues. Significantly different pattern and concentration of total soluble proteins were seen in differentiation of callus to plantlet regeneration stages. Polypeptide banding pattern of regenerated plants were different from naturally growing plants. When comparing the protein bands in cultures of each stage they were different to each other. The molecular weights of detected bands for all samples ranged from 62.66 to 86.51 KDa. Three similar culture specific callus, Organogenic callus and root polypeptides (MW 86.65, 83.45 and 80.10 kDa) and four shoot specific polypeptides (MW 86.51, 83.25, 80.18, and 62.66 kDa) and were synthesized during the differentiation. Similarly, one shoot specific (MW 62.66 kDa) and two regenerated plants specific (MW 77.52 and 75.32 kDa) polypeptide band were appeared during differentiation in *S. trilobatum*. *In vitro* regenerated plant cultures showed totally five (MW 86.34, 83.78, 80.44, 77.52 and 75.32 kDa) plant specific polypeptides.

## INTRODUCTION

Medicinal plants provide the most exclusive source of life saving drugs for the majority of the world's population. World Health Organization (WHO) has enlisted over 21,000 plants, which have medicinal value. More than 2000 plant species are used in the traditional medicines as evident from the *Charak Samhita* and *Sushruta Samhita* and 159 pharmaceutical companies and 3.5 billion people rely on these traditional medicines (Chandel *et al.*, 1996). Clonal propagation through tissue culture, popularly called, micropropagation, can be

achieved in a short time and space. Thus, it is possible to produce plants in large numbers from a single individual. Endangered species are being proliferated and taken off the 60 endangered list even though they are no longer found in their native habitat. Use of plant tissue culture for micropropagation was initiated by Morel (1960) who found that this is the only commercially viable approach for orchid propagation. Micropropagation technology owns unique distinction as the quick and easy method of deriving plants with identical genetic constitution (Hussy, 1986). Plant tissue culture techniques have been successfully used for a rapid clonal multiplication of high yielding genotypes or for the production of specific virus-free plants. Although remarkable progress has been made in the area of gene transfer technology, little is known as to how plant cells differentiate in the cultures or about molecular

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mechanism of *in vitro* differentiation (Goyal *et al*., 2009). This unique property also offers an opportunity to investigate the cellular and molecular basis of differentiation. Little is known about the intervening biochemical events occurring in the cultured cells undergoing organogenesis (plant regeneration), therefore elucidation of biochemical changes accompanying the differentiation could decipher the underlying mechanism (Singh *et al*., 2006). During the process of differentiation, protein profile studies would allow the identification of embryogenic potential or serve as an indication of the loss of regeneration capacity with the culture age. Detection of specific protein(s) synthesized during the sequential stages of differentiation would give an insight into the biochemical changes occurring during the differentiation (Mhatre *et al*., 1991). *Solanum trilobatum* L. belongs to the family of solanaceae, it is a popular medicinal plant used in Indian alternative system of medicines like siddha, ayurveda, herbal medicines, folk and home remedy for curing many diseases. It is a thorny creeper that bears bluish purple flowers and small sized fruits native to India especially it is found every were in Tamil Nadu. This herbal plant is used as medicine for asthma, vomiting of blood and bilious matter, phlegmatic rheumatism, several kinds of leprosy. It is also antibacterial, antifungal, antimitotic and antitumourous (Ramakrishna *et al*., 2011; Gandhiappan and Rengasamy, 2012). The leaves and stem of *S. trilobatum* are reported to possess antimitotic, anti-inflammatory and anti-ulcerogenic properties. The leaf extracts are used to increase male fertility and to cure snake poison (Kumar *et al*., 2011). It is used with ghee in siddha for treating tuberculosis, as decoction in case of acute and chronic bronchitis, root and berries for treating cough (Swathy *et al*., 2010).

The major alkaloids identified in the alcoholic extract from leaves and stem part of *S. trilobatum* has been shown to possess antimitotic and antimicrobial activity against bacteria and fungi. Biological screening of the alkaloid mixture of this plant revealed anticancer activity against certain type of cancer and its effectiveness as an adjuvant in cancer chemotherapy (Kumar *et al*., 2011). Increased demand due to medicinal properties and depletion of natural sources has initiated the development of plants through micropropagation, Manisha Sharan *et al*. (2010). Only limited success has been reported for *in vitro* micropropagation and organogenesis of *S. trilobatum*, Arulmozhi and Ramanujam, (1997). Pawar *et al*. (2002), reported cytokinins to be mainly responsible for shoot initiation Plant regeneration through leaf-derived calli and organogenesis from stem explants has been achieved in *S. xanthocarpus* Schrad. &Wendl. (Baburaj and Thamizhchelvan, 1991). The presence of solasodine, a glycoalkaloid, which can be used for steroidal drug biosynthesis, has been reported in this species (Krishnamurthy and Reddy 1996). The natural production of solasodine is restricted. Hence, the pharmaceutical industry is exploring alternative methods such as *in vitro* production of natural compounds (Xu *et al*., 2008). *In vitro* regeneration potential was high in *S. trilobatum* (Rejitha *et al*., 2002, Alagumanian *et al*., 2004). Thus, the aim of the present study was to evaluate the differential behavior in different stages of cultures of *Solanum trilobatum* L. with responsible for specific polypeptides.

## MATERIALS AND METHODS

### Plant Material and Explants source

Healthy and disease free *Solanum trilobatum* L. plants were collected from the departmental nursery in December (2015), PG and Research Department of Botany, Government Arts College, Dharmapuri, Tamil Nadu, India. The well spread leaves, nodes, internodes and shoot tips were used as explants to establish cultures. Then the explants were surface sterilized in 10% commercial phenolic cleaner (Domex) for 5 minutes followed by 5% sodium hypochlorite for 15 minutes and 0.5% mercuric chloride 2-5 minutes then rinsed three times in sterile distilled water in the laminar air flow cabinet. The surface sterilized explants were used for induction of different cultures *in vitro* condition with MS medium supplemented with various hormones.

### Culture establishment

The internode explants were excised from the wild plants. For callus induction, multiple shoot induction and root induction were established on Murashige and Skoog (MS) (1962) medium containing 3% sucrose and solidified with 0.8% agar, supplemented with different concentration of 6-benzylaminopurine (BAP) (0.5-3.0 mg/l), α-naphthalene acetic acid (NAA) (0.5-1.0 mg/l) and 2,4 dichlorophenoxy acetic acid (2,4-D) (0.5-3.0 mg/l). Medium pH was adjusted to 5.8 with 1 N NaOH or HCl before autoclaving. After 25 days, the aseptic cultures were transferred to fresh medium of the same composition to study the response of explants. All cultures were placed in a culture room at $25\pm2$°C temperature and a photoperiod of 16/8 light/dark under fluorescent light.

### Callus, Organogenic callus, and Multiple shoot culture

The internode explants were placed aseptically in 100 ml culture vials containing 20-25 ml of MS medium containing 3% sucrose and 0.8% agar plus 1.0 mg/l of 2,4-D or 1.0 mg/l of BAP was subjected to different serial transfer periods. The percentage of explants initiating callus, organogenic callus, multiple shoots, roots and basal callus percentage were recorded after 4 weeks of culture. The callus with the internode explants were transferred into the fresh medium containing 1.0 - 2.0 mg/L of BAP alone as well as 0.5-1.0 mg/L of NAA combinations for callus, shoot, multiple shoos and root induction. The micropropagation cycle consisted of a 28 days subculture of leaf segments. Data were collected five times at a two month interval and subjected to Mean ± Standard Deviation.

### Rooting and plantlet regeneration

Well developed shoots with roots on second subcultures to promote shoots and root by transferring on to the rooting and plantlet regeneration medium supplemented with different concentrations of NAA for their rooting. The rooting medium consisted of MS medium supplemented with 3% sucrose and solidified with 0.8% of agar. Hormone NAA was tested either alone (at 0.5, 1.0 mg/l) or in combination with BAP (0.5-1.0 mg/l). The aseptic shoots were cut into single nodes with their respective 2 leaves and placed, randomly distributed, into the different proliferation media. In general, from each shoot 4-5 nodes were obtained. The cultures were initially maintained for 6 days under dark and then exposed to light and temperature as mentioned above. Multiple shoots were rooted by following the procedure of Akram and Aftab, (2007). All experiments were repeated five times. The percentage of callus, multiple shoots, rooted shoots, and regenerated plants evaluated after 4 weeks of culture on the rooting medium. The regenerated young plants were removed from the culture vials and washed

thoroughly with tap water. They were acclimatized and then planted in plastic bags in culture room a mixture of vermiculite: sand: soil (1:1:1 v/v) mixture enriched with half strength of MS solution and placed in a glass house (33±2ºC, RH 85%). Two-month-old plants were planted in the field and their survival is being observed.

### Hardening

Initially plantlets were covered with a polyethylene film, which was gradually eliminated in two weeks time. Plants were transferred to pots and placed in a greenhouse (28±2ºC, RH 90%) for completing their acclimatization. The hardened plants were transferred to a greenhouse before transferring them in to the field. Photographs were taken using a Nikon DSLR 5500 camera with a macro lens with or without a bellows attachment.

### Extraction and determination of soluble proteins

One gram of each cultures tissues were homogenized separately with the help of pre-chilled pestle and mortar in 2.5 mL of chilled 0.1M Tris-buffer containing 0.1% PVP, pH 8.0. The homogenate was centrifuged at 10,000 g at 4°C for 15 min. The supernatant containing the soluble proteins was taken in chilled test tube. The amount of protein in the extract was determined following Bradford (1976).

### Protein profile resolution (SDS-PAGE)

A 25 µL of crude protein extract, containing 50 µg of protein extract was transferred to an equal volume of Laemmli's 2X sample buffer (0.5 M Tris-HCl, pH 6.8) containing 20% glycerol, 4% SDS, 0.5% bromophenol blue (w/v) and 10% β-mercaptoethanol and heated at 100°C for 3 min and cooled. Electrophoresis was carried out by the method of Laemmli (1970). The cooled samples were then loaded on to a SDS discontinuous gel system with a 0.1 mm thick stacking gel of 4% polyacrylamide in Tris-HCl buffer (pH 6.8) and a resolving gel of 10% polyacrylamide in Tris-HCl buffer (pH 8.8). The gels were run at 15 mA in the stacking gel and 25 mA in the resolving gel. After electrophoresis, gels were fixed and stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol with 7% glacial acetic acid (v/v) and then destained in 10% methanol (v/v) with 7.5% glacial acetic acid (v/v). After destaining, the gels were stored at 7% glacial acetic acid (v/v).

### Data analysis

A completely randomized design was used for all experiments. Callus formation, multiple shoot initiation, root induction and plantlet regeneration were carefully calculated based on the number of explants used. Data of all experiments were statistically analyzed and expressed as Mean ± Standard Deviation (Snedecor and Cochran, 1994).

# RESULTS

To initiate the present study, the healthy and disease free intermodal explants were collected from well established field grown plants of *Solanum trilobatum*. The surface sterilization of explants was washed with tap water followed by standard disinfectants and sodium hypochlorite for 10 minutes. The surface sterilization of explants showed initially large percent

found to be contaminated with microbes either bacteria or with fungi. Large number of explants (>70%) were found to be fungal contaminated. However, inclusion of phenolic cleaner (Domax) and increase of sodium hypochlorite concentration upto 0.1-0.5% as well as increase the time (15 minutes) and 0.5% of mercuric chloride treatment for 2-5 minutes then washed with sterile distilled water three times in 5 minutes interval. Surface sterilization method gave a very satisfactory result (clean culture 95-98%). From intermodal explants, callus, organogenic callus, multiple shoots, roots and plantlet regeneration were established on MS medium supplemented with various concentrations auxins (2,4-D and NAA) and cytokinin (BAP), alone or in combination.

### Callus and Organogenic callus culture

For callus induction from internode explants of *S. trilobatum*, were cultured on MS medium supplemented with 3% sucrose, 0.8% agar as well as different hormones at different concentrations. The hormone treatments were at the concentration of 0.5-3.0 mg/l of 2, 4-D and BAP in alone and in combinations. The internode explants were respond different frequencies in all hormone treatments. Callus induction 2,4-D was respond well than BAP. Callus initiation and proliferation was noticed in all explants at the proximal cut end when place horizontally on MS medium (Figure 1A). When compare to BAP treatments, 2,4-D (0.5 to 3.0 mg/l) showed high percentage of callus induction. Increasing the concentration of hormones, the response of callus induction also increases upto 3.0 mg/l, and above (3.0 mg/l) of concentration the internode explants were respond abnormally. Maximum percentage of callus induction (92.8%) was observed on MS medium supplemented with 2,4-D (2.5 mg/l) followed by 85.3% in 3.0 mg/l, 70.6% (2.0 mg/l), 48.5% (1.5 mg/l), 37.5% (1.0 mg/l) and 25.5% (0.5 mg/l), respectively. Very minimum amount of callus induction (15.3%) was observed in medium supplemented with BAP 3.0 mg/l (Table 1). None of the explants were failed to produce callus in medium supplemented with NAA (0.5-1.0 mg/l). Formation of the morphogenetic varied characteristic callus cultures (like green, pale yellow green and bright white callus) was obtained within two weeks of inoculation in the medium containing hormones at the concentration of 2.5 mg/l of 2,4-D. In combination of BAP (1.0-3.0 mg/l) with 2,4-D (1.0-3.0 mg/l) and BAP (1.0 - 2.0 mg/l) with NAA (1.0 mg/l) were also tested for callus proliferation from internode explants. The hormone BAP+2,4-D combination developed better callus mass then BAP and NAA at lower concentrations. Whereas other combinations of BAP and NAA yielded slightly friable and brownish callus. The results were observed for 4-8 weeks of time period without altering the culture conditions under light. The hormone BAP + 2,4-D combination developed better callus mass (23.3%) in BAP (3.0 mg/l)+2,4-D (2.5 mg/l) then BAP (2.0 mg/l) and NAA (1.0 mg/l) at lower (22.1%) level. The rate of growth of callus increased from the second week of culture initiation until the eighth week after the rate of callusing declined. Pale yellow green callus developed from the medium was repeatedly subcultured after every 4 weeks interval for shoot initiation (Table 1).

For callus mass propagation, MS medium supplemented with (BAP, 2,4-D, NAA) alone or in different combinations at the concentration of 1.0-3.0 mg/l. Internode explants were respond different frequencies in all hormone treatments. For organogenic callus induction was higher (37.4%) in medium

supplemented with BAP (2.0 mg/l) + NAA (1.0 mg/l) than (26.5%) BAP (3.0 mg/l) + 2,4-D (3.0 mg/l) 2,4-D (Table 1, Figure 1B). Five to eight days after inoculation on MS medium, the internode explants were started to respond first to formation of callus. Percentage of callus initiation was calculated based on number of explants inoculated. Well-developed callus cultures were sub-cultured on medium supplemented with same concentration of hormone. After three to five passage, the calli were developed into as organogenic calli. From these organogenic calli were further developed into multiple shoots in subsequent subcultures.

developed from cut surface of internodal explants as well as callus cultures. The multiple shoot induction and multiplication was observed in medium supplemented with BAP (0.5-3.0 mg/l) in combination of 2,4-D (1.0-3.0 mg/l) or NAA (0.5-1.0 mg/l). Maximum percentage of shoots (23.6%) was induced on MS medium supplemented with BAP 3.0 mg/l alone and minimum percentage of multiple shoot induction (10.6%) in BAP (2.0 mg/l). Similarly maximum multiple shoots induction (87.6%) was also achieved in combination of BAP (3.0 mg/l) + 2,4-D (2.5 mg/l) and 67.6% in BAP (2.0 mg/l) + NAA (1.0 mg/l), respectively. Followed by (80.1% and 56.2%)
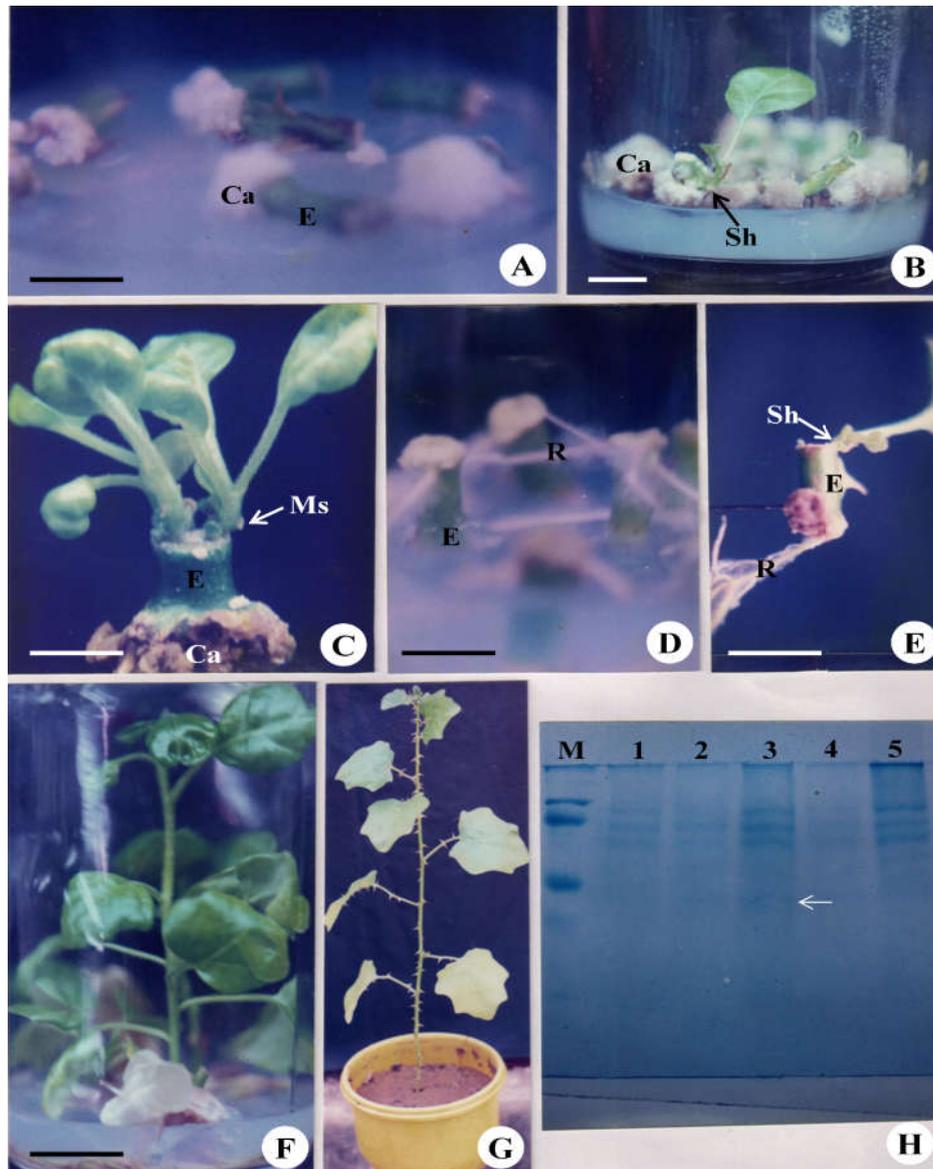


**Figure 1. Direct organogenesis and protein profile changes in *Solanum trilobatum* L. A. Young internode explants showed callus induction on the proximal cut end. B. Organogenic callus showed multiple shoot induction. C. Explants showed direct multiple shoots in proximal cut end, callus induction at distal cut end. D. Explants showed direct root induction at distal cut ends. E. Explants showed shoot and root development. F & G. Plantlet regeneration and hardening of micropropagated plants. H. SDS-PAGE showed the protein profiles of different stages during organogenesis. (Bar = 1 cm)**

**E - Internode, Ca - Callus, Ms - Multiple shoot, Sh - Shoot, R - Root, M - Protein marker,**
**1 - Callus, 2 - Organogenic callus, 3 - Multiple shoot, 4 - Root,  5 - Regenerated plants**

## Shoot and Multiple shoot Culture

Shoot initiation and establishment from internodal explants were cultured on MS medium supplemented with various concentrations of BAP alone or in combination with NAA and 2,4-D is described in Table 1. Initially multiple shoots were

BAP 3.0 mg/l + 2,4-D 3.0 mg/l and BAP 2.0 mg/l + NAA 0.5 mg/l, (76.2% and 45.8%) BAP 3.0 mg/l + 2,4-D 2.0 mg/l and BAP 1.5 mg/l + NAA 1.0 mg/l, (52.1% and 38.3%) BAP 2.0 mg/l + 2,4-D 2.5 mg/l and BAP 1.0 mg/l + NAA 1.0 mg/l, (48.3% and 27.3) BAP 2.0 mg/l + 2,4-D 2.0 mg/l and BAP 1.0 mg/l + NAA 0.5 mg/l, respectively.

**Table 1. Response of internode explants of *Solanum trilobatum* Linn. on MS medium supplemented with different hormones**

| Hormones (mg/l) | Percentage of Induction (%) | | | | | |
|---|---|---|---|---|---|---|
| | Callus | Org. callus | Mul. Sh. | Root | Sh & Rt | Plt. reg. |
| Control | 2.7 ± 0.1 | - | - | 2.2 ± 0.1 | - | 6.2 ± 0.4 |
| 2,4-D 0.5 | 25.5 ± 1.2 | 4.5 ± 0.3 | - | - | - | - |
| 2,4-D 1.0 | 37.5 ± 3.2 | 9.5 ± 1.0 | - | - | - | - |
| 2,4-D 1.5 | 48.5 ± 3.7 | 14.5 ± 1.1 | - | - | - | - |
| 2,4-D 2.0 | 70.6 ± 6.5 | 20.6 ± 1.5 | - | - | - | - |
| 2,4-D 2.5 | 92.8 ± 8.2 | 28.8 ± 2.2 | - | - | - | - |
| 2,4-D 3.0 | 85.3 ± 7.8 | 34.6 ± 2.8 | - | - | - | - |
| BAP 0.5 | - | 2.2 ± 0.1 | - | - | - | - |
| BAP 1.0 | - | 6.1 ± 0.4 | - | - | 2.0 ± 0.2 | 7.2 ± 0.6 |
| BAP 1.5 | - | 10.5 ± 1.0 | - | - | 6.2 ± 0.4 | 11.4 ± 1.0 |
| BAP 2.0 | 5.4 ± 0.3 | 20.6 ± 1.5 | - | - | 10.6 ± 0.8 | 15.7 ± 1.3 |
| BAP 2.5 | 12.1 ± 2.2 | 24.2 ± 2.1 | 8.1 ± 0.5 | - | 18.7 ± 1.5 | 20.3 ± 1.8 |
| BAP 3.0 | 15.3 ± 2.8 | 30.6 ± 2.9 | 14.6 ± 1.2 | - | 23.6 ± 2.0 | 24.6 ± 2.1 |
| NAA 0.5 | - | - | - | 50.1 ± 4.5 | 37.5 ± 3.2 | 28.8 ± 2.2 |
| NAA 1.0 | - | - | - | 68.3 ± 6.1 | 59.5 ± 5.2 | 34.6 ± 2.8 |
| BAP 1.0 + 2,4-D 1.0 | 6.3 ± 0.5 | 7.3 ± 0.5 | 15.4 ± 1.2 | - | - | - |
| BAP 1.0 + 2,4-D 1.5 | 10.1 ± 0.4 | 10.1 ± 0.8 | 20.5 ± 1.8 | - | - | - |
| BAP 1.0 + 2,4-D 2.0 | 12.6 ± 1.5 | 14.6 ± 1.1 | 32.3 ± 2.8 | - | 5.6 ± 0.3 | - |
| BAP 2.0 + 2,4-D 2.0 | 10.2 ± 0.5 | 20.2 ± 1.5 | 48.3 ± 4.1 | - | 9.7 ± 0.8 | - |
| BAP 2.0 + 2,4-D 2.5 | 13.4 ± 0.9 | 23.4 ± 2.0 | 52.1 ± 4.7 | - | 14.6 ± 1.1 | - |
| BAP 2.0 + 2,4-D 3.0 | 16.2 ± 1.0 | 24.2 ± 2.1 | 65.8 ± 5.6 | - | 16.1 ± 1.2 | - |
| BAP 3.0 + 2,4-D 2.0 | 11.0 ± 1.2 | 21.0 ± 1.6 | 76.2 ± 7.0 | - | 24.9 ± 2.5 | - |
| BAP 3.0 + 2,4-D 2.5 | 23.3 ± 2.1 | 23.3 ± 2.0 | 87.6 ± 8.1 | - | 30.1 ± 2.8 | 16.5 ± 1.2 |
| BAP 3.0 + 2,4-D 3.0 | 17.2 ± 1.3 | 26.5 ± 2.3 | 80.1 ± 7.6 | - | 32.0 ± 2.9 | 18.2 ± 1.5 |
| BAP 1.0 + NAA 0.5 | 3.2 ± 0.2 | 6.4 ± 0.5 | 27.3 ± 2.4 | 42.1 ± 4.0 | 26.6 ± 2.1 | 56.2 ± 5.2 |
| BAP 1.0 + NAA 1.0 | 8.1 ± 0.6 | 9.5 ± 0.7 | 38.3 ± 3.5 | 48.4 ± 4.1 | 28.5 ± 2.6 | 61.5 ± 5.6 |
| BAP 1.5 + NAA 0.5 | 10.1 ± 1.0 | 12.4 ± 1.2 | 42.1 ± 4.0 | 44.7 ± 4.3 | 40.4 ± 3.8 | 67.2 ± 5.5 |
| BAP 1.5 + NAA 1.0 | 16.6 ± 1.5 | 18.8 ± 1.5 | 45.8 ± 4.2 | 51.5 ± 5.0 | 44.7 ± 4.1 | 72.4 ± 6.6 |
| BAP 2.0 + NAA 0.5 | 20.8 ± 2.2 | 23.7 ± 2.0 | 56.2 ± 5.1 | 50.1 ± 4.9 | 53.7 ± 5.0 | 82.3 ± 6.0 |
| BAP 2.0 + NAA 1.0 | 22.1 ± 2.3 | 27.4 ± 2.4 | 67.6 ± 6.2 | 54.9 ± 5.2 | 58.4 ± 5.1 | 86.2 ± 6.3 |

Note: Org.callus - Organogenic callus; Mul.sh - Multiple shoots; Sh = Shoot; Rt = Root; Plt.Reg. = Plantlet regeneration, Mean ± S.E for five experiments

Very minimum multiple shoots induction (15.4%) was observed on medium supplemented with BAP (1.0 mg/l) + 2,4-D (1.0 mg/l) (Table 1, Figure 1C). When compare to BAP alone supplemented, BAP and 2,4-D or BAP and NAA supplemented with MS medium showed higher multiple shoots induction.

**Root culture**

When intermodal explants were inoculated upside down, the distal cut ends facilitate to produce numerous roots on MS medium supplemented with NAA alone. After four to five weeks of culture on NAA at the level of 0.5-1.0 mg/l. Rooting was significantly affected with the increasing concentration of (1.0 mg/l) of NAA. The percentage of root induction, number of roots and mean of root length were calculated after 28 days. Maximum root induction 68.3% was observed in MS medium supplemented with NAA (1.0 mg/l). Minimum root induction was observed in MS medium supplemented with NAA (0.5 mg/l) at 50.1% (Table 1, Figure 1D). Similarly maximum root induction (54.9%) was also achieved in combination of BAP (2.0 mg/l) + NAA (1.0 mg/l), followed by 51.5% in BAP 1.5 mg/l + NAA 1.0 mg/l, 50.1% in BAP 2.0 mg/l + NAA 0.5 mg/l, 48.4% in BAP 1.0 mg/l + NAA 1.0 mg/l and 44.7% in BAP 1.5 mg/l + NAA 0.5 mg/l, respectively. Very minimum root induction (42.1%) was observed on medium supplemented with BAP (1.0 mg/l) + NAA (0.5 mg/l). When compare to NAA alone supplemented, BAP with NAA supplemented with MS medium showed less root induction.

**Shoot and Root culture**

Well grown mature healthy internode explants were cultured on MS medium supplemented with NAA (0.5-1.0 mg/l) alone as well as in combination of BAP (1.0-3.0 mg/l) + 2,4-D (1.0-3.0 mg/l) and BAP (1.0-2.0 mg/l) + NAA (0.5-1.0 mg/L). Simultaneous induction of shoot and root (58.4%) was observed in MS medium supplemented with BAP 2.0 mg/l with NAA 1.0 mg/l (Table 1, Figure 1E).

**Plantlets regeneration and Acclimatization**

*In vitro* developed shoots (4.0-7.0 cm) were removed from culture tubes and sub cultured on MS medium supplemented with BAP alone and combination of different concentration 2,4-D or NAA for plantlet regeneration. Except 2,4-D alone or combination of BAP treatments all other treatments were facilitate plantlet regeneration. Among all concentrations tested MS medium with BAP (2.0 mg/l) and NAA (1.0 mg/l) showed maximum plantlets regeneration (86.2%) followed by 82.3% in BAP (2.0 mg/l) + NAA (0.5 mg/l), 72.4% in BAP (1.5 mg/l) + NAA (1.0 mg/l), 67.2% in BAP (1.5 mg/l) + NAA (0.5 mg/l), 61.5% in BAP (1.0 mg/l) + NAA (1.0 mg/l), respectively. The minimum plantlet regeneration 56.2% was observed in BAP (1.0 mg/l) + NAA (0.5 mg/l) (Table 1, Figure 1F). After root initiation, the regenerated plantlets were removed from the culture vials and washed with sterile distilled water to remove agar sticking in the roots. Plantlets with fully expanded leaves and well-grown roots (25 d) were transferred to polythene bags containing vermiculite:soil:sand (1:1:1 w/v). *In vitro* raised plantlets were regenerated on liquid MS medium supplemented with 0.5 mg/l of NAA and acclimatized on vermiculate before transferring them to field. This method has been resulted in 85% survival of the plantlets. For acclimatization of micropropagated plants were kept under 16/8 photoperiod at 26±2°C and regularly poured half strength MS solution for one month. Relative humidity (80%) was

maintained by covering them with polythene bags. All the transferred plant were then hardened and later established in the field successfully (Figure 1G).

## Detection of polypeptide variation using SDSPAGE

The protein profile system revealed the biochemical variation and evolutionary relationship among *in vivo* plants, calli and regenerated plantlets of *S. trilobatum* was demonstrated in Figure 1H. The molecular weights of detected bands for all samples ranged from 62.66 to 86.51 KDa. There were five bands detected at molecular weight 62.66, 75.32, 77.52, 80.44, 83.45, and 86.65 KDa in *in vivo* plants, in addition to presence of one band detected at molecular weight 62.66 KDa in shoot specific polypeptide in multiple shoot cultures. Only three polypeptide bands of molecular weight 86.65, 83.45 and 80.44 KDa have been detected in callus, organogenic callus and root and absence of other specific polypeptide bands. On the other hand, bands with molecular weight 62.66 KDa was absent in the regenerated plantlets instead of that 77.52 and 75.32 KDa bands were appeared *in vitro* regenerated plants.

## DISCUSSION

In the present study, organogenesis of *Solanum trilobatum* was achieved from intermodal explants on MS medium supplemented various hormones at different concentrations and different combinations. Callus initiation and proliferation was achieved in all intermodal explants at the proximal cut end when place horizontally on MS medium. When compare to BAP treatments, 2,4-D showed high percentage of callus induction. No callus induction was observed on medium supplemented with NAA. Morphogenetic varied characteristic callus cultures was developed on medium with 2,4-D. In combination of BAP with 2,4-D or BAP with NAA also showed callus proliferation from internode explants. The hormone BAP+2,4-D combination developed better callus mass then BAP and NAA at lower concentrations. Callus induction usually requires the presence of auxins or cytokinins or both in the nutrient media. Various types of explants such as leaf segments, leaf segments with petiole, and internodal segments were used for callus initiation (Chatterjee *et al.*, 1992). These were inoculated in the media having different combination and concentration of BAP, NAA, Kinetin, and 2,4-D, the explants enlarged (swell) within 10-12 days of inoculation; however callus formation started after 20- 25 days at the cut ends of explants. The differential response of explants to callusing may be attributed due to varying concentration of endogenous levels of auxin and cytokinins which relatively influence the genes to trigger differentiation of cells (Rout and Das, 1997). The auxin commonly used for callus induction is 2,4- D, but NAA and IAA are also used (Chawla, 2002). Reliable callus induction and regeneration of viable plants considered as a limiting steps to the successful use of modern techniques in genetic improvement of the major crop (Murphy, 2003). Many researchers observed 2,4-D is the best hormone for callus induction in monocot and dicot plants (Evans *et al.*, 1981). The results of present study showed 2,4-D and combination of 2,4-D+NAA yielded high degree of callus mass. The auxin 2,4-D, by itself or in combination with cytokinins, has been widely used to enhance callus induction and maintenance (Castillo *et al.*, 1998). Moreover, depending on 2,4-D concentration there was a range of variations in callus initiation, percentage of explants developed from callus, callus texture, callus colour and degree of callus formation was

noticed. Callus initiation on cut ends of *in vitro* cultured explants could be observed in all 2, 4-D and 2,4-D+NAA levels after 7-17 days. Similar findings were reported by Yasmin *et al.* (2003).

The rooting response from the shoots cultured in MS medium supplemented with auxins NAA (at the concentration of about 0.5 - 1.0 mg/l) produced maximum roots. Similarly (Mutasim *et al.*, 2010; Fiegert *et al.*, 2000; Jayasree *et al.*, 2001) were also noticed similar results in developing allogenesis and somatic embryogenesis of *Solanum tuberosum*. Similar kind of results organogenic green callus obtained from *Eclipta alba* (Devendra *et al.*, 2011). Similarly direct regeneration of the same species of plant was reported by Jawahar *et al.* (2004) using different concentration of BAP and KIN for shoot regeneration and root formation. The present work concluded the indirect organogenesis protocol for the rapid *in vitro* proliferation of *Solanum trilobatum* L. an important medicinal plant of India using leaf explants. The induction of callogenesis was strongly dependent on the auxin and cytokinin concentrations used during the subcultures. This study will support improvement of the genetic characteristics of the species and further continuation for large and commercial scale production of the plant and this study helps to avoid further loss of species from natural environment. The efficiency of BAP in shoot induction may be due to the ability of plant tissue to metabolize natural hormones more readily than artificial growth regulators or due to the ability of BAP to induce production of natural hormones namely, zeatin within the tissue and thus, working through natural hormone system (Sharma and Wakhlu, 2003). The higher concentration of BAP and NAA was previously found to promote callus formation from the leaf explant in *C. asiatica* (Senthilkumar, 2017). It is well known that cytokinins suppress the growth of apical meristems and instead induce excess formation of lateral meristems, resulting in multiple shoots. The cytokinin-auxin combination has also been used widely for shoot regeneration in various protocols (Joshi and Dhar, 2003; Dhar and Joshi, 2005; Alagumanian *et al.*, 2003). SDS-PAGE is the most economical, simple and extensively used biochemical technique for analysis of genetic structure of germplasm and expression of gene by specific polypeptides. The expression of some proteins and enzymes is affected by growth stages (Fuentes *et al.*, 2000). The results of the present study also confirm the proteomic variations in the cultured plantlets. The specific protein profile could be used as a biochemical marker for the future plant breeding or genetic improvement programme to identify the mother plant and somaclonal variants of *S. trilobatum*.

Protein profile of *in vitro* calli, organogenic calli and root cultures were showed 100% of similarity between multiple shoots and regenerated plantlets of *S. trilobatum*. A bit lower similarity (95%) was observed between *in vitro* multiple shoots and regenerated plants. These results indicated that the genetic material is more stable at protein level than at DNA level under normal conditions. On the other hand, a moderate variation in protein profile was observed between calli and *in vitro* plants (90%). These results were in agreement with Barpete *et al.* (2014) in that similarity in protein profile of grasspea regenerated plantlets ranged between 37% and 85%. Also, our results coincide with Farid *et al.* (2014), where tissue culture-raised plants via shoot regeneration of *Arum palaestinum* revealed differences in protein profiles in the examined samples, suggests that a real genetic change might

have occurred. However our results were in contrary with the study of Rady *et al.* (2014) in the point of that no or little differences in protein profile were obtained between *in vivo* plants and *in vitro* regenerated plantlets of *Silybum marianum*. Great similarity in protein profile between *in vivo* plants and regenerated plantlets of sugar beet (Bekheet *et al.*, 2007), broccoli (El-Kazzaz and Taha, 2002) and garlic (Bekheet, 2004) has been reported.

Kairong *et al.* (2002) reported that addition of hydrogen peroxide in the media induced the synthesis of new protein in the embryogenic callus of *Lycium barbarum*. Ishizaki *et al.* (2002) found a new synthesized protein in calli grown from segments of spinach (*Spinacia oleracea* L.) root in the presence of gibberellic acid (GA3) plus auxin compared to absence of GA3 and presence of auxin. Elavumoottil *et al.* (2003) obtained new proteins in callus cultures of *Brassica oleracea* L. var. *botrytis* grown in media supplemented with different concentrations of sodium chloride. Silva *et al.* (2005) investigated that the protein content in the callus of *Glycine wightii* depends on the incubation period. Furthermore, Mohamed *et al.* (2011) concluded that changes in the protein pattern appear to correlate with colocynth (*Citrullus colocynthis*) callus percentage and different combinations of plant growth regulators. In conclusion, we think that leaf explants culturing increase the rate of polymorphism, which is also influenced by the cucumber cultivar genotype.

### Conclusion

In the present study, SDSPAGE analysis reported the presence of polypeptide variations in culture stages of organogenesis and *in vivo* regenerated plantlets generated from internodeal explants of *Solanum trilobatum*. In general, an efficient protocol was established for multiple shoot and plant regeneration from internode explants of *Solanum trilobatum* has lower level somaclonal variation. Our results revealed that cultured stages appeared lower level of polypeptide variation compared to other stages of organogenesis. The increasing demand for herbal medicines in the recent years due to fewer side effects in comparison to synthetic drugs and antibiotics, has high lightened the need for the conservation and propagation of medicinal plants and there *in vitro* conservation of germplasm.

### Acknowledgments

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