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Influence of hormonal and histological studies related to *in vitro* organogenesis of *Solanum trilobatum* L.

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Abstract
The present study was aimed to regenerate plants from internode explants and analyze the origin and developmental pattern of cells during different developmental stages of *Solanum trilobatum* L. To analyze and compare the developmental stages were assessed from callus, organogenic callus, multiple shoots, and root. The best response of callus and organogenic callus induction (78.4% and 65.2%) was obtained on 3.0 mg/l of 2,4-D (2,4-dichlorophenoxyacetic acid) supplemented in Murashige and Skoog's (MS) medium. Organaogenic callus induction was maximum (81.2%) in medium supplemented with 3.0 mg/l of BAP (6-benzylaminopurine) + 1.0 mg/l of NAA (α-naphthaleneacetic acid) in different combinations. A higher rate of multiple shoot induction (80.1%) was observed on medium supplemented with 3.0 mg/l BAP with 1.0 mg /l of NAA. For best root initiation (63.5%) was induced on medium containing 1.0 mg/l of IBA (indolebutyric acid). *In vitro* plantlets regeneration was successfully achieved in maximum (80.8%) medium supplemented with BAP 2.0 mg/l with NAA 1.0 mg/l and acclimatized in vermisulite:sand:soil (1:1:1) mixture and carefully transferred to field condition they showed 90% of survival. Histological analysis revealed that the shoots were originated directly on the proximal cut surface and root induction was distal cut end of internode.

Keywords: *Solanum trilobatum*, internode, multiple shoot, organogenes is, histology

1. Introduction
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 also antibacterial, antifungal, antimitotic and antitumourous [1, 2]. 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 [3]. 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 [4]. Increased demand due to medicinal properties and depletion of natural sources has initiated the development of plants through micropropagation [5]. Only limited success has been reported for *in vitro* micropropagation and organogenesis of *S. trilobatum* [6], Pawar *et al*. [7], reported cytokinins to be mainly responsible for shoot initiation Plant regeneration through leaf-derived calli and organogenesis from stem explants has been achieved [8]. The presence of solasodine, a glycoalkaloid, which can be used for steroidal drug biosynthesis, has been reported in this species [9].
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 mechanism of *in vitro* differentiation [10]. Micropropagation technology owns unique distinction as the quick and easy method of deriving plants with identical genetic constitution [11]. In addition, there are no precise histological studies on the different developmental stages during the initiation of direct multiple shoots and roots were reported in the literature cited, particularly from internode explants of *S. trilobatum*. The aim of the present work was to investigate the possible induction of direct multiple shoot by culturing internode explants on MS medium supplemented with different growth regulators. Furthermore, the morphological and histological changes during the ontogeny of the direct multiple shoot and roots from the initial cells of their development into plantlets were investigated.

2. Materials and Methods
2.1 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 grown internodes 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.

2.2 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) [12] medium containing 3% sucrose and solidified with 0.8% agar, supplemented with different concentration of 6-benzylaminopurine (BAP) (1.0-4.0 mg/l), α-naphthalene acetic acid (NAA) (0.5-1.0 mg/l) and 2,4 dichlorophenoxy acetic acid (2,4-D) (1.0-4.0 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±2ºC temperature and a photoperiod of 16/8 light/dark under fluorescent light.

2.3 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 1.0-4.0 mg/l of 2, 4-D or 1.0-4.0 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 1.0-4.0 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.

2.4 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 IBA for their rooting. The rooting medium consisted of MS medium supplemented with 3% sucrose and solidified with 0.8% of agar. Hormone IBA, IAA and NAA was tested either alone (1.0 mg/l) or in combination with BAP (1.0-3.0 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, [13].

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.

2.5 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.

2.6 Histology

Samples of plant material at different culture stages of cell differentiation were collected at various points during callus, shoot induction and root induction. The free-hand sections were taken and used to study the morphology, anatomy and histology of *in vitro* grown culture samples. Sections were immediately stained and observed. The samples were fixed in 50% formaldehyde/acetic acid/alcohol (FAA) (v/v) for 48h in accordance with the protocol. Histological and histochemical staining was carried out according to standard procedure [14]. Next, they were transferred into 70% ethanol and dehydrated in an ethanol series. The samples were embedded in paraffin wax (60ºC) following the procedure described by Feder and O'Brien [15]. Longitudinal sections were cut using a rotary microtome with a steel razor (type C) to a section thickness of 5 mm. The cuttings were placed onto slides and stained with 0.05% (v/v) toluidine blue for 10 min [16]. The permanent slides were mounted in DPX. Microphotographs were taken with a Nikon DSLR 5500 camera, microscope system having bright-field, dark-field, phase-contrast, Nomarski-DIC, polarized light and fluorescence modes. Macrophotographs were taken using a Nikon DSLR 5500 camera, Olympus stereozoom microscope.

2.7 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 [17].

3. Results

The present study revealed that the healthy and disease free intermodal explants of *Solanum trilobatum* were collected from field grown plants. The surface sterilization of explants was washed with tap water followed by standard disinfectants and sodium hypochlorite for 10 minutes. Initial cultures, the explants were largely contaminated with bacteria and fungi. 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 good result free of contaminations. 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, IAA, IBA and NAA) and cytokinin (BAP), alone or in combination.

3.1 Callus induction

Internode explants of *S. trilobatum* were showed best response than other explants 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 1.0-3.0 mg/l of 2, 4-D and BAP in alone and in combinations of NAA, IAA and IBA (1.0 mg/l). Callus induction frequencies were various based on hormone concentration. Callus induction 2, 4-D was respond well than IAA, NAA, IBA and BAP. Callus initiation and proliferation was noticed in all explants all over the surface ("Figure 1A, B") at the proximal cut end (Figure 1C) when place horizontally on MS medium. When compare to all other treatments, 2, 4-D (1.0 to 3.0 mg/l) showed high percentage of callus induction. Upto 3.0 mg/l concentration of 2, 4-D was improve the increased level of callus induction and above the concentration the callus induction was moderately decreased. Best callus induction (78.5%) percentage was observed in MS medium supplemented with 2, 4-D (3.0 mg/l) followed by 66.1% in 2.0 mg/l and 55.2% (4.0 mg/l), respectively. Very low level of callus induction (36.7%) was observed in medium supplemented with 2, 4-D 1.0 mg/l ("Table 1"). No other hormone treatments were respond remarkably or explants were failed to produce callus.

In combination of BAP (1.0-3.0 mg/l) with NAA (0.5-1.0 mg/l), BAP (1.0-3.0 mg/l) with IAA (0.5-1.0 mg/l) and BAP (1.0-3.0 mg/l) with IBA (0.5-1.0 mg/l) were also tested for callus proliferation from internode explants. The hormone BAP with NAA combination developed better callus mass then other combinations. The results were observed for 4-8 weeks of time period without altering the culture conditions under light. The hormone BAP (3.0 mg/l) with NAA (1.0 mg/l) combination developed better callus mass (20.1%) followed by 18.2% in BAP (3.0 mg/l) with NAA (0.5 mg/l), 15.6% in BAP (2.0 mg/l) with NAA (1.0 mg/l), 12.1% in BAP (2.0 mg/l) with NAA (0.5 mg/l), respectively. The lower level of callus induction (9.6% and 6.5%) was observed in BAP (1.0 mg/l) with NAA (1.0 mg/l), and BAP (1.0 mg/l) with NAA (0.5 mg/l), respectively. The callus growth rate was calculated upto eighth weeks of culture after that the rate of callusing declined. Green compact callus was repeatedly subcultured at every 4 weeks interval for shoot initiation ("Table 1").

Table 1: Effect of intermodal explants of *Solanum trilobatum* L. on MS medium supplemented with different hormones.

Hormones (mg/l)	Percentage of Induction (%)				
	Callus	**Org. callus**	**Mul. Shoot**	**Root**	**Plant reg.**
Control	2.2 ± 0.1	-	-	3.7 ± 0.2	15.3 ± 1.1
BAP 1.0	-	-	8.2 ± 0.6	-	-
BAP 2.0	-	-	12.8 ± 1.1	-	-
BAP 3.0	-	-	20.1 ± 1.6	-	-
BAP 4.0	-	-	16.3 ± 1.2	-	-
2,4-D 1.0	36.7 ± 3.2	44.6 ± 4.1	-	-	-
2,4-D 2.0	66.1 ± 6.2	52.5 ± 5.1	-	-	-
2,4-D 3.0	78.5 ± 6.7	65.2 ± 5.6	-	-	-
2,4-D 4.0	55.2 ± 5.1	40.5 ± 3.8	-	-	-
IBA 1.0	-	-	-	25.8 ± 2.2	-
IAA 1.0	5.2 ± 0.3	-	-	32.3 ± 3.1	-
NAA 1.0	3.4 ± 0.2	-	-	63.5 ± 6.5	-
BAP 1.0 + NAA 0.5	6.5 ± 0.4	22.6 ± 2.1	18.6 ± 2.9	5.2 ± 0.3	39.4 ± 3.7
BAP 1.0 + NAA 1.0	9.6 ± 0.8	37.3 ± 3.5	31.4 ± 2.9	13.2 ± 1.0	47.5 ± 4.5
BAP 2.0 + NAA 0.5	12.1 ± 1.1	46.7 ± 4.1	40.2 ± 3.5	20.8 ± 1.8	55.9 ± 5.3
BAP 2.0 + NAA 1.0	15.6 ± 1.5	58.1 ± 5.5	52.1 ± 4.8	28.4 ± 2.5	60.1 ± 5.9
BAP 3.0 + NAA 0.5	18.2 ± 1.6	62.4 ± 6.2	72.4 ± 7.0	31.0 ± 2.8	67.4 ± 6.1
BAP 3.0 + NAA 1.0	20.1 ± 1.9	81.2 ± 7.5	80.1 ± 7.2	39.4 ± 3.5	80.8 ± 6.1
BAP 1.0 + IAA 0.5	-	14.1 ± 1.2	5.8 ± 0.4	3.1 ± 0.2	11.0 ± 1.0
BAP 1.0 + IAA 1.0	-	21.0 ± 1.9	10.6 ± 0.9	6.1 ± 0.5	18.4 ± 1.6
BAP 2.0 + IAA 0.5	-	31.3 ± 2.8	18.4 ± 1.7	8.1 ± 0.7	26.5 ± 2.4
BAP 2.0 + IAA 1.0	-	42.2 ± 4.0	22.1 ± 2.0	11.4 ± 1.1	35.7 ± 3.2
BAP 3.0 + IAA 0.5	3.0 ± 0.2	56.1 ± 5.1	27.3 ± 2.4	15.1 ± 1.3	48.2 ± 4.4
BAP 3.0 + IAA 1.0	7.6 ± 0.6	60.0 ± 5.7	38.3 ± 3.5	18.2 ± 1.4	57.6 ± 5.2
BAP 1.0 + IBA 0.5	-	-	2.1 ± 0.2	-	15.2 ± 1.2
BAP 1.0 + IBA 1.0	-	-	7.3 ± 0.6	-	20.4 ± 1.9
BAP 2.0 + IBA 0.5	-	-	16.2 ± 1.4	-	28.5 ± 2.6
BAP 2.0 + IBA 1.0	-	-	24.5 ± 2.2	-	33.5 ± 3.1
BAP 3.0 + IBA 0.5	-	-	30.1 ± 2.9	-	40.1 ± 3.7
BAP 3.0 + IBA 1.0	-	-	33.5 ± 3.2	-	48.3 ± 4.5

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

3.2 Organogenic callus induction:

For callus mass propagation and organogenic callus production initial callus cultures were subcultured on MS medium supplemented with (BAP, 2,4-D, IAA, NAA and

IBA) alone or in combinations of BAP (1.0-3.0 mg/l) with NAA (0.5-1.0 mg/l), BAP (1.0-3.0 mg/l) with IAA (0.5-1.0 mg/l) and BAP (1.0-3.0 mg/l) with IBA (0.5-1.0 mg/l). Internode explants were respond different frequencies in all hormone treatments. In MS medium supplemented with 2, 4-D (1.0-4.0 mg/l) showed best callus induction. For maximum organogenic callus (65.2%) developed in medium supplemented 2, 4-D (3.0 mg/l) followed by 52.5% in 2, 4-D (2.0 mg/l) and 40.5% in 2, 4-D (4.0 mg/l). Minimum percentage 44.6% of organogenic callus induction was observed in 2, 4-D (1.0 mg/l). In combination of hormone treatment, organogenic callus induction was higher (81.2%, 60.0%) in medium supplemented with BAP (3.0 mg/l) with NAA (1.0 mg/l), BAP 3.0 mg/L with IAA 1.0 mg/l, followed by 62.4% and 56.1% in BAP (3.0 mg/l) with NAA (0.5 mg/l) and BAP (3.0 mg/l) with IAA (0.5 mg/l), 58.1% and 42.2% in BAP (2.0 mg/l) with NAA (1.0 mg/l), BAP (2.0 mg/l) with IAA (1.0 mg/l), 46.7% and 31.3% in BAP (2.0 mg/l) with NAA (0.5 mg/l), BAP (2.0 mg/l) with IAA (0.5 mg/l), 37.3% and 21.0% in BAP (1.0 mg/l) with NAA (1.0 mg/l), BAP (1.0 mg/l) with IAA (1.0 mg/l), respectively. Low level of organogenic callus was noticed on both 22.6% and 14.1% in BAP (1.0 mg/l) with NAA (0.5 mg/l), BAP (1.0 mg/l) with IAA (0.5 mg/l) respectively ("Table 1"). Percentage of callus and organogenic 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 indirect multiple shoots or roots induction.

3.3 Multiple shoot induction

Multiple shoot initiation and establishment were occurred on proximal cut end of internodal explants on MS medium supplemented with various concentrations of BAP alone or in combination with NAA, IAA and IBA is presented in "Table 1". Initial cultures internodes were induced multiple shoot developed from cut surface as well as from callus cultures. The multiple shoot induction and multiplication was observed in medium supplemented with BAP, 2, 4-D (1.0-4.0 mg/l) and NAA, IAA and IBA (1.0 mg/l) alone. Except BAP none of the other hormone treatments does not induce multiple shoot. In MS medium supplemented with BAP (1.0-4.0 mg/l) showed best multiple shoot induction. For maximum multiple shoot (20.1%) developed in medium supplemented BAP (3.0 mg/l) followed by 16.3 % in BAP (4.0 mg/l) and 12.8% in BAP (2.0 mg/l) ("Figure 1D, E"). Minimum percentage 8.2% of multiple shoot induction was observed in BAP (1.0 mg/l). In combination of hormone treatments for multiple shoot induction was observed in all three combinations of BAP with NAA, BAP with IAA and BAP with IBA. The BAP with NAA combination was showed the best followed by BAP with IAA and BAP with IBA. Highest number of multiple shoots (80.1%) was noticed on medium supplemented with BAP (3.0 mg/l) with NAA (1.0 mg/l), followed by 72.4% in BAP (3.0 mg/l) with NAA (0.5 mg/l), 52.1% BAP (2.0 mg/l) with NAA (1.0 mg/l), 40.2% in BAP (2.0 mg/l) with NAA (0.5 mg/l), 31.4% in BAP (1.0 mg/l) with NAA (1.0 mg/l), respectively. Lowest noumer of multiple shoots formation was observed on 18.6% in BAP (1.0 mg/l) with NAA (0.5 mg/l) ("Figure 1G, H" "Table 1").

3.4 Root induction

Shoot developed intermodal explants were readily induce roots at the distal cut end ("Figure 1J"), at the same time when intermodal explants were inoculated upside down, the distal cut ends facilitate to produce numerous roots ("Figure 1K") on MS medium supplemented with NAA alone. For root induction, NAA IAA and IBA (1.0 mg/l) was supplemented alone. Rooting was significantly affected with the increasing concentration of (1.0 mg/l). The percentage of root induction, number of roots and mean of root length were calculated after 28 days. Maximum root induction 63.5% was observed in MS medium supplemented with NAA (1.0 mg/l) followed by 32.3% in medium supplemented with IAA (1.0 mg/l). Minimum root induction 25.8% was observed in MS medium supplemented with IBA (1.0 mg/l) ("Table 1"). Similarly maximum root induction (39.4%) was also achieved in combination of BAP (3.0 mg/l) with NAA (1.0 mg/l), followed by 31.0% in BAP 3.0 mg/l with NAA 0.5 mg/l, 28.4% in BAP 2.0 mg/l with NAA 1.0 mg/l, 20.8 in BAP 2.0 mg/l with NAA 0.5 mg/l and 13.2% in BAP 1.0 mg/l with NAA 1.0 mg/l, respectively. Very minimum root induction (5.2%) was observed on medium supplemented with BAP (1.0 mg/l) + NAA (0.5 mg/l) ("Table 1").

3.5 *In vitro* Plantlets regeneration

In vitro developed plantlets were produced from well-developed shoots (3.0-7.0 cm) were removed from culture tubes and sub cultured on MS medium supplemented with BAP with NAA, IAA and IBA combination at different concentration for plantlet regeneration. All three combinations were facilitated plantlet regeneration. Among all concentrations tested MS medium with BAP (3.0 mg/l) with NAA (1.0 mg/l) showed maximum plantlets regeneration (80.8%) followed by 67.4% in BAP 3.0 mg/l with NAA 0.5 mg/l, 60.1% in BAP 2.0 mg/l with NAA 1.0 mg/l, 55.9 in BAP 2.0 mg/l with NAA 0.5 mg/l and 47.5% in BAP 1.0 mg/l with NAA 1.0 mg/l, respectively. Very minimum root induction (3.1%) was observed on medium supplemented with BAP (1.0 mg/l) + NAA (0.5 mg/l) ("Table 1").

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 85-90% 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.

3.6 Histology

The longitudinal section of internode explants showed the proximal cut end the callus formation. The promimal portion

internode revealed the presence of 2 distinct regions, inner zone and outer zone. These 2 zones contain numerous meristamatic regions called primary thickening meristem (PMT) (Figure 1F). The first change, i.e. the onset of active cell division of PTM/apical meristem, observed after 5-10 days on regeneration medium. Continuous meristamatic activity in these cells resulted in the formation of adventitious shoot primordia that ruptured the explant proximal cut surface. The longitudinal and transverse sections of the multiple shoot induced aerial stem showed that the shoots regenerated from apical meristem and PTM of the explant ("Figure 1I"). The root primordial was regenerated from internode distal cut end ("Figure 1L").

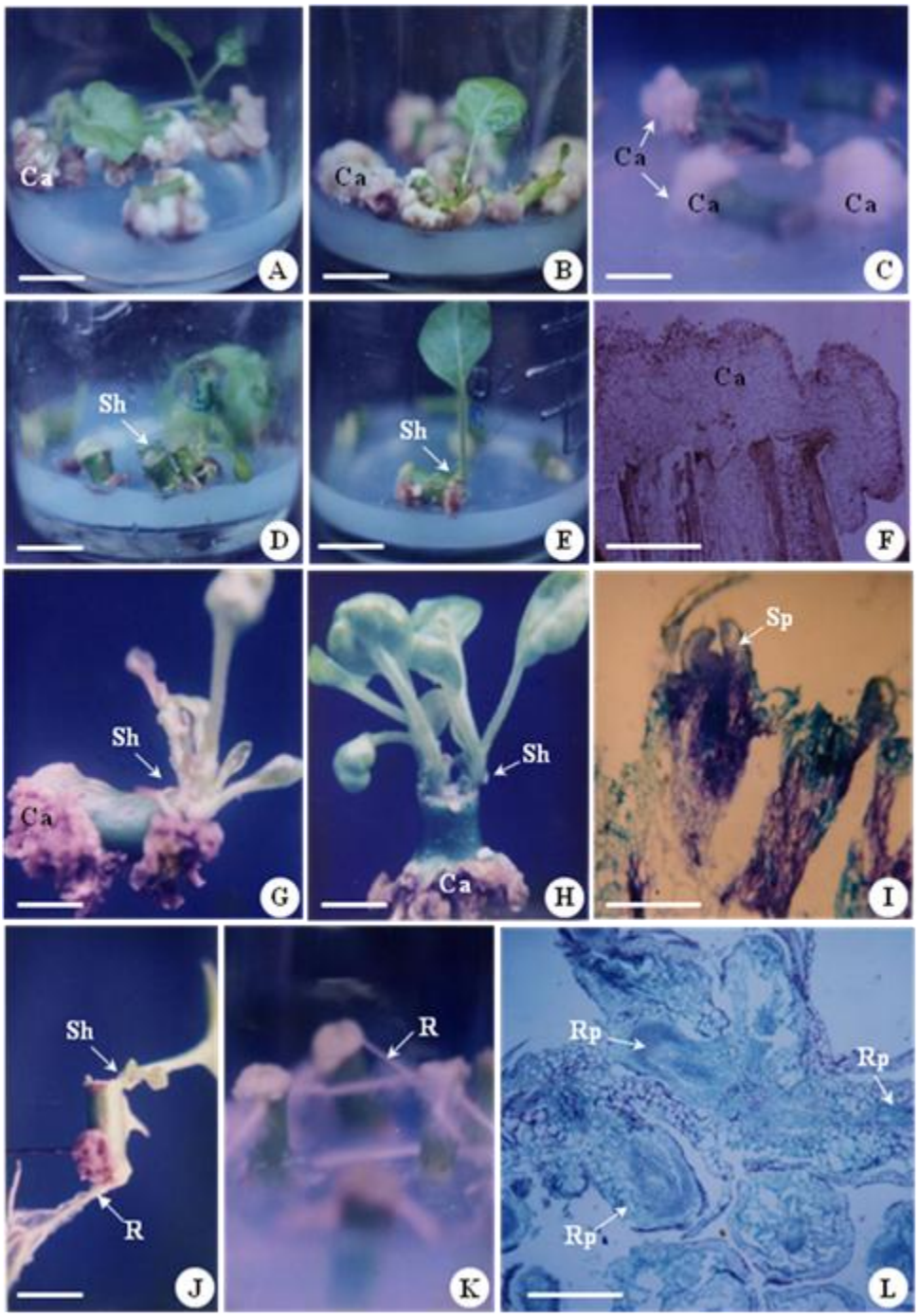

Fig 1: Hisological development of internodal explants of *Solanum trilobatum* L. during organogenesis. A-B. Internodal explants showed callus induction on the surface. C. Callus induction on proximal cut end. D-E, Explants showed direct multiple shoots and callus induction. F. Microtome section of internode with callus at apical portion (200 µm). G-H. Explants showed direct multiple shoot proliferation at proximal cut ends and callus induction at distal end. I. Longitudinal section showed origin of multiple shoot primordial tissue development (200 µm). J. Explants showed shoot and root development. K. Explants directly produce roots on distal cut end. L. Microtome section showed root pole development (200 µm). (Bar = 1 cm).
Ca - Callus; Sh - Multiple shoot; Sp-Shoot primordia; R-Root.

4. Discussions

In the present study, organogenesis of *Solanum trilobatum* L. 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 NAA or BAP with IAA also showed callus proliferation from internode explants. The hormone BAP with NAA 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. The color of calli was mostly greenish white. The results obtained by Shahzad [18] were similar to our findings. Various types of explants such as leaf segments, leaf segments with petiole, and internodal segments were used for callus initiation [19, 20]. 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 [21]. The auxin commonly used for callus induction is 2, 4-D, but NAA and IAA are also used [22]. The results of present study showed 2, 4-D and combination of BAP with 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 [23]. Endogenous hormone level in internode explants responded well in the formation of callus, direct multiple shoots and direct roots from the cut surfaces [24, 25]. Moreover, depending on 2, 4-D concentration there was a range of variations in callus initiation, percentage of explants developed from callus formation was noticed. Callus initiation on cut ends of *in vitro* cultured explants could be observed in all 2, 4-D and BAP with NAA levels after 21 days. Similar findings were reported by Yasmin *et al*., [26]. 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 [27].

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 [28]. The higher concentration of BAP and NAA was previously found to promote callus formation from the leaf explant in *C. asiatica* [29]. 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 [30, 31, 32]. Individual seedlings were subcultured on MS medium supplemented with various concentrations of BAP and NAA, with or without activated charcoal (AC), for maximum growth [33]. The MS medium containing 3 mg/l of BAP and 0.5 mg/l 2, 4-D was found to be the most effective for multiple shoot formation in Indian barley (*Hordeum vulgare*) cultivar that could produce 12 shoots per explants [34]. Primordial initiation and development of shoot-buds has been accomplished by using shoots derived from *Castanea sativa* seedlings cultured with BAP.

Germination of chestnut seeds in the presence of BAP stimulated varying numbers of shoot-buds in those areas of the main axis that were favorably altered [35]. Multiple shoot induction in *Sapindus emarginatus* has been achieved by two methods by addition of BAP (3.0 mg/l) supplemented MS liquid medium and supported on a filter-paper bridge and in plants treatment for axillary bud regions under sterile conditions [36]. All the treatments that contained 3 mg/l BAP plus 1 mg/l NAA were found to be the best hormonal treatment for shoot regeneration from leaf-derived callus [37]. Shoot formation in treatment containing 3 mg/l BAP plus 0.2 mg/l NAA or in that containing 2 mg/l BAP plus 1 mg/l NAA were more suitable than other treatments. Muthukumar *et al*. [38] also reported *in vitro* plant regeneration from nodal explants of *Datura metal* cultured on MS medium with BAP (0.5 to 3.0 mg/L) and NAA (0.5mg/L). Jabeen *et al*. [39] reported root initiation from in vitro raised shoots of *Solanum nigrum* cultured on MS-medium containing different concentration of NAA, IAA and IBA separately. The percentage of shoots forming roots and the number of roots per shoots were significantly varied with different concentration of NAA, IAA and IBA. These findings were similar to our study. They found that among the three auxins tested NAA at 1.0 mg/L induced maximum roots when compared to other concentrations of IAA and IBA tested [40].

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 [41, 42, 43] were also noticed similar results in developing callogenesis and somatic embryogenesis of *Solanum tuberosum.* Similar kind of results organogenic green callus obtained from *Eclipta alba* [44]. The role of IAA as an effective root inducing auxin had also been in *Solanum tuberosum* [45]. Similarly direct regeneration of the same species of plant was reported by Jawahar *et al*. [46] 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* an important medicinal plant of India using leaf explants.

5. Conclusion

In the present study clearly demonstrated that the approach of direct callus, multiple shoots and roots have 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*. The histological investigation revealed that the callus, direct multiple shoots and roots in organogenesis steps. All stages of development during organogenesis were observed in internodes of *Solanum trilobatum*. The information described herein could be used

for regeneration protocol optimization for *Solanum* species and for the production of plants through genetic transformation.

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