In vitro regeneration of Allophylus serratus. Roxb (Kurz), an important medicinal plant

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Research Article

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Abstract

Background

Allophylus serratus Roxb (Kurz.) is a medicinal plant with various therapeutic properties. It has been used for the treatment of various alignments such as elephantiasis, edema, fractured bones, in bone dislocations, wound, several gastrointestinal disorders such as dyspepsia, anorexia, and diarrhea. The aim of this study is to establish protocol for surface sterilization, *in vitro* callus induction and plant regeneration from leaf and nodal segment explants of *Allophylus serratus.*

Results

From the tested media, the maximum callus induction frequency (81.11%) from leaf and 84.44% from nodal segment explants were achieved on MS medium supplemented with 3 mg l^{-1}BAP) combined with 0.5mgl^{-1} NAA. The highest shoot regeneration frequency (57.78%) and root regeneration frequency (65.55%) were achieved on growth regulators free MS medium and MS medium supplemented with 1.5 mg l^{-1}IBA respectively. Acclimatization of the regenerated plantlets was established successfully (100%) in a pot containing sand, soil and compost in 1:2:2 ratios.

Conclusions

In this study successful callus induction protocol from leaf and nodal explants of *A. serratus* using MS media supplemented with different concentrations and combinations of plant growth regulators (2,4-D, NAA, and BAP). MS media containing 3mg/l BAP and 0.5mg/l NAA was the most efficient for callus induction. these callus induction and plant regeneration protocols are very important for mass propagation of the species and also opens a new way to facilitate secondary metabolites production and isolation of pharmaceuticals from callus rather than harvesting the plant.

1. Introduction

Allophylus serratus (Roxb) Kurz, locally known as Tippani in Hindi is a medicinal, dicotyledonous plant of the family Sapindaceae. It is an evergreen, low branching small tree or a large shrub about 3-6m tall. Theplantis found distributed in India, Sri Lanka, and South East Asia. In India; it is found to be growing on the site of Andhra Pradesh, West Bengal, Arunachal Pradesh, Tripura, Orissa, Karnataka, Tamilnadu and Kerala, scrubby forest of Mahabaleshwar, scrubby forests of Kolhapur, Satara, Borbet, Barki, Manoli, Panhala, Patgaon of Kolhapur District (Kero et al. 2015). According to Selvam (2007), the plant is tolerant to drought and high salinity. It is adapted to grow in fine sandy, coarse and nutrient poor soil and it can also grow well in shade too.

Allophylus serratus has strong ethnobotanical and ethnopharmacological background. The plant has been used in Indian system of medicine (Ayurveda) for the treatment of various alignments such as elephantiasis, edema, fractured bones, in bone dislocations, wound, several gastrointestinal disorders such as dyspepsia, anorexia, and diarrhea (Gupta and Tandon 2004). This plant is also used as an anti-inflammatory and as a carminative drug. The plant has edible fruits that are sweet having cooling and nourishing tonic. The leaves are used to reduce

fever, to relieve rashes, promote lactation, to treat colic, to relieve stomach aches, as antiulcer and to reduce piles (Maurya and Srivastava 2011; Devi et al. 2013). The roots of this plant contain tannin and are considered astringent and used for treating nose bleeding, diarrhea and rheumatic pains (Maurya and Srivastava 2011).

Due to overexploitation, depletion of habitat and misuse, the world is facing the problem of losing valuable plant resource. This situation is an alarm for efficient and in time conservation measures to enrich our lives with the services of plants. Various research groups across the world have attempted conservation of plants to protect biodiversity (Parabia et al. 2007; Nadeem et al. 2000). Different techniques for conservation of plants have been practiced worldwide, the most important being tissue culture (Pola 2005; Parabia et al. 2007). Plant tissue culture is an important method to overcome the problem connected with medicinal plants conservation and utilization (Bajaj et al.1988).

In vitro conservation of medicinal plants by tissue culture is advantageous in producing multiple copies of a plant species within minimum time and space. This *in vitro* conservation consists of different techniques such as selection of explants, sterilization of explants, aseptic culture establishment, multiple shoot production; rooting of the shoots followed by acclimatization of the plantlets. From these stages of *in vitro* conservation techniques, standardization of surface sterilization of explants is fundamental for aseptic culture establishment. In the majority of plant tissue culture laboratories, on average there are 3-15% losses due to contamination (Leifert et al. 1989) the majority of which is caused by bacteria and fungi (Leifert et al.1994). Various factors such as plant species, the source of explants, the age of explants and other climatic conditions are the reasons for explant contaminations (Srivastava et al. 2010). In addition to surface sterilization, standardization of media (concentration and combinations of plant growth regulators) for the callus induction is critical in tissue culture technique (Pola et al. 2007 and 2008). Callus cultures are used to study cell development and to obtain primary and secondary metabolites through cell suspension cultures. In addition suspension cultures can be used to obtain different novel products through bio-transformation. Plant regeneration protocol development through callus has also applications in production of novel products through genetic transformation.

Various studies have been carried out on the biochemical (Priya et al. 2012), ethnobotanical (Kero et al. 2015, Binu and Rajendran 2013 and Trivedi 2006) and medicinal (Dharmani et al.2005 ; Gaikwad and Chavan 2013; Vinay et al. 2005) aspects of *Allophylus serratus*. Almost all researchers have focused on its medicinal properties. It is sensible to mention that there are no research reports on tissue culture issues (surface sterilization, callus induction and in vitro plant regeneration studies) before in this plant species. Surface sterilization and callus induction studies are the prerequisites for tissue culture and genetic manipulation studies and conservation of this medicinal plant. Therefore, the objectives of this study were to establish surface sterilization and in *vitro* regeneration protocol through callus induction of this plant.

2. Methods

2.1 Plant materials

Young, green and healthy leaves and nodal segments of *Allophylus serratus* were used as explants for both surface sterilization and callus induction protocol studies. Explants were collected from mature mother plant from the field at Andhra University compound and Biodiversity park at Visakhapatnam, India. The plant species was authenticated by Dr. Bodaih Padal, taxonomist, department of botany, Andhra University, Visakhapatnam.

The voucher specimens (21921) were deposited in the herbarium, college of Science and Technology, Department of Botany, Andhra University.

2.2 Surface sterilization of leaf and nodal segment explants

The explants were collected from the field and taken to the laboratory in a bottle containing water. Once in the laboratory, they were washed under running tap water for 30 minutes. Then the explants were washed with standard detergent powder and kept under running tap water for 30 minutes. They were then washed with Tween -20 and rinsed with sterile double distilled water 3-5 times. In a laminar hood (inside surface was first sterilized by 70 % ethanol and then sterilized by an ultraviolet light for at least 20 min before use), the explants were surface sterilized by using Ethanol, NaOCl and $HgCl_2$. In the first method, the explants were dipped into 70% Ethanol for 1minute followed by $HgCl_2$ at concentrations of 1% and 0.1% for 3, 4 and 5 minutes. In a second way, 70% Ethanol for one minute followed by NaOCl at 1.0% and 2.0% concentrations for 3, 4 and 5 minutes were used for surface sterilization. The third way involves surface sterilization of explants with70% Ethanol for one minute followed NaOCl at concentrations of 1.0% and 2.0% for 3, 4 and 5 minutes and $HgCl_2$ (1.0% and 0.1%) for 2 and 3 minutes. After each chemical sterilants treatment, the explants were washed with sterile double distilled water three to four times with gentle shaking under sterile conditions to remove the chemical sterilants and reduce their effect on explants.

2.3 Callus Induction

After sterilization, the explants were cut into the suitable size, dried on sterile tissue paper and were inoculated onto MS (Murashigie and Skoog 1962) medium with 3.0% sucrose and 0.80% bacteriological grade agar (Hi-Media, India) and supplemented with different concentrations and combinations 2,4-D (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l), BAP (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l) NAA and (0.5 mg/l) alone and in combination. The pH of the medium was adjusted to 5.80 using 1N HCl or 1N NaOH before autoclaving. The media was autoclaved at 121^{o}C at 1.50 kg cm-2 for 20 minutes. The plant growth regulators were added to the media by filter sterilization (pore size 0.22 micron) after the media was autoclaved and cooled to 50-60 ^{o}C. Fifteen to Twenty milliliters of the medium was dispensed into culture tubes or Petri dishes under an aseptic condition in a laminar hood. Cultures were then kept at a temperature of 27 ± 3 ^{o}C, air humidity of 55% and in dark. Growth regulators free MS medium was used as a control. Data on, contamination, survival, and a loss percentage of explants, the rate of callus response, nature of the callus (callus texture, callus color), the day of callusing, callus fresh weight and dry weight were recorded.

2.4 Shoot regeneration from callus

For shoot regeneration, the calli induced on callus induction MS media were sub cultured on same medium every four weeks two times. Then the healthy and embryonic calli were cut into small pieces and inoculated on to shoot regeneration MS media supplemented with BAP (1, 2, 3, 4 mg/ l) and Kinetin (1, 2, 3, 4 mg /l). Plant growth regulators free MS media was used as control. All the cultured calli were incubated under 16/8 hrs photo period (light/dark) with light provided by white fluorescent tube lights (1000 lux) at 27±3 °C for four weeks. After four weeks the data were recorded for percent of shoot formation, (number) and length of shoots.

2.5 Root regeneration

Healthy regenerated shoots with two or more leaves and about 1-3 cm in length were cultured on root induction MS media supplemented with NAA (0.0, 0.5, 1.0, 1.5, 2.0 mg/l) or IBA (0.0, 0.5, 1.0, 1.5, 2.0 mg/l). Hormone free MS media was used as control. The cultures were kept in 16/8 hrs photo period (light/dark) at 27±3 °C temperature. After four weeks of culture, the data were recorded for percent of root induced, number and length of roots.

2.6 Acclimatization

Regenerated plantlets in good conditions (good looking shoot and root) were carefully removed from culture tubes and washed with sterile distilled water to remove the agar. The plantlets were then planted in pots containing sand, soil, and compost in 1:2:2 ratios. The potted plantlets were covered with polyethylene bags with small holes and kept in tissue culture room for 10 days. The polyethylene cover was removed little by little after 7 days. After 30 days the number of survived plants was recorded.

2.7 Experimental Design and Data Analysis

Completely Randomized Design (CRD) with three replicates per treatment and thirteen explants per replicate were arranged. The data of explants surface sterilization (rate of contamination, survival, and loss) were recorded as percentage. The data of effect of different concentrations and combinations of growth regulators on callus induction, shoot and root regeneration were recorded and subjected to one-way ANOVA using statistical data analysis software SPSS version 20.0 at 5% probability level.

3. Results

3.1 Explant Surface Sterilization

The data obtained on surface sterilization of leaf and nodal segment explants was carried out using Ethanol (70%), NaOCl (1% and 2%) and $HgCl_2$ (0.1 % and 1%) in different combination at varying length of time intervals given in Table 1. The data reveals that the highest percent survival of leaf explants (80.00%) was achieved with treatment (70% ethanol for 1minute, 1.0% NaOCl for 4 minutes and 0.1% $HgCl_2$ for 3 minutes) and the highest percent survival of nodal segment explants (90.00%) was with treatment (70% ethanol for 1min, 2% NaOCl for 3 minutes and 1.00% $HgCl_2$ for 2 minutes. Percentage of contamination was brought down to 30% from 100% for leaf explants and 10% from 100% for nodal segment explants. The least leaf explants survival (10.00%) was recorded with 70% Ethanol for 1 minute followed by 1.00% NaOCl for 3 minutes and for nodal segment explants the least survival (14.4%) was recorded with 70% ethanol for 1 minute followed 1.0% NaOCl for 5 minutes. The result of our study showed that the leaf and nodal segment explants of *Allophylus serratus* have shown diverse sterility response for different concentrations and combinations of chemical sterilants at the different duration of time.

Table 1. Effect of different Chemical Sterilants on surface sterilization of leaf and nodal segment explants of *Allophylus serratus* after treatment with 70% Ethanol for 1 min.

Concentrations of Sterilizing Agents (%)		Exposure time (Minutes)	Leaf explants			Nodal segment explants		
			Contaminated %	Survival %	Lost %	Contaminated %	Survival %	Lost %
----	0.00	0.00	100	0.00	0.00	100	0.00	0.00
$HgCl_2$	0.10	2.00	38.89	53.33	7.78	52.22	36.67	11.11
		3.00	37.78	33.33	28.89	44.44	47.78	7.78
$HgCl_2$	1.00	2.00	58.89	25.56	15.56	42.22	34.44	23.33
		3.00	56.67	24.44	18.89	62.22	22.22	13.33
NaOCl	1.00	3.00	72.22	10.00	17.78	60	23.33	16.67
		4.00	58.89	32.22	20.00	57.78	24.44	17.78
		5.00	41.11	38.89	20.00	70.00	14.44	15.56
NaOCl	2.00	3.00	38.89	44.44	16.67	63.33	33.33	3.33
		4.00	41.11	48.89	10.00	52.22	31.11	16.67
		5.00	40.00	50.00	10.00	53.33	32.22	8.89
NaOCl	1.00+0.10	3.00+2.00	3.33	38.89	50.00	51.11	38.89	10.00
+		4.00+2.00	3.67	30	57.78	51.11	38.89	10.00
$HgCl_2$		5.00+2.00	4.33	25.56	60.00	43.33	38.89	17.78
		3.00+3.00	2.33	37.78	54.44	37.78	47.78	13.33
		4.00+3.00	0.67	40.00	57.78	33.33	44.44	20.00
		5.00+3.00	3.00	21.11	68.89	37.78	48.89	13.33
NaOCl	2.00+ 0.10	3.00+2.00	20.00	72.22	7.778	30.00	54.44	15.56
+		4.00+2.00	18.89	68.89	12.22	25.56	57.78	16.67
HgC_{l2}		5.00+2.00	12.22	70.00	17.78	33.33	53.33	13.33
		3.00+3.00	18.89	68.89	11.11	33.33	57.78	8.89
		4.00+3.00	15.56	80.00	4.444	23.33	65.56	11.11
		5.00+3.00	13.33	70.00	16.67	15.56	73.33	11.11
NaOCl	1.00+1.00	3.00+2.00	20.00	72.22	7.778	27.78	60.00	12.22
+		4.00+2.00	40.00	42.22	17.78	18.89	68.89	8.89
$HgCl_2$		5.00+2.00	33.33	55.56	11.11	20.00	67.78	12.22
		3.00+3.00	23.33	66.67	10.00	17.78	70.00	12.22
		4.00+3.00	25.56	47.78	26.67	13.33	72.22	14.44
		5.00+3.00	26.67	57.78	15.56	16.67	71.11	11.11

NaOCl + $HgCl_2$	2.00 +1.00	3.00+2.00	23.33	62.22	14.44	6.667	90.00	3.33
		4.00+2.00	22.22	67.78	10.00	11.11	72.22	14.44
		5.00+2.00	23.33	51.11	25.56	16.67	61.11	22.22
		3.00+3.00	20.00	47.78	34.44	27.78	56.67	15.56
		4.00+3.00	11.11	50.00	38.89	24.44	61.11	14.44
		5.00+3.00	15.56	53.33	31.11	17.78	71.11	11.11

3.2 Callus induction

The initiation of callus induction from leaves and nodal segment explants of *Allophylus serratus* was carried out after successful sterilization of the explants. The successfully surface sterilized leaves and nodal segment explants were inoculated onto callus induction media (MS medium supplemented with different concentration and combinations of 2, 4-D, NAA and BAP. Different hormonal regime induced callus from both leaf and nodal segment explants differently. Callus induction frequency and callus type induced varied depending on the type of explant used, type and level of plant growth regulator used.

3.2.1 Effect of 2, 4-D on callus induction

Leaf and nodal segment explants cultured on MS medium supplemented with different concentrations of 2, 4-D responded differently. The highest response of callus induction in leaf explants (68.89 ± 2.94 %) and nodal segment explants (42.22 ± 2.94 %) were observed on the medium supplemented with 3.00mg/l 2, 4-D. Whereas the minimum callus induction response 8.89 ± 4.01% and 10.00 ± 1.92% were observed on MS media with 4.00 mg/l 2,4-D in leaf and nodal segment explants respectively. At low concentration of 2,4-D (below 2.00 mg/l), no callus induction was observed and as the concentration of 2,4-D increases above 3.00mg/l, the percentage of callus induction was declined in both explants (Table 2). When the concentration of 2,4-D is increased from 2.00mg/l to 2.50 mg/l and then to 3.00mg/l, rate of callus induction increased from 42.22 ± 5.88% to 62.22 ± 4.84% and 68.89 ± 2.94% in leaf explant and 30.00 ± 3.83% to 35.56 ± 2.94% and 42.22 ± 2.94% in nodal segment explant.

The calli started to induce at the major veins in leaf explants and the cut end and on upper surface of nodal segment explants then continues to grow until it covered the entire explants. Callus formation was observed earlier in nodal segment explants (15-20 days) than the leaf explants (20 to 25 days). The calli were soft, friable and creamy white in leaf explant but soft, compact and white green in nodal segment explant (Fig 1). In this species, young explants were more likely to produce callus than older explants.

Table 2. Effect of 2,4-D on callus induction from leaf and nodal segment explants of *Allophylus serratus*

Concentration of 2,4-D (mg/L)	Leaf explants	Nodal Segment explants
	Callus induction (%) (Mean ± SE)	Callus induction (%) (Mean ± SE)
0.00	0.00	0.00
2.00	42.22 ± 5.88	30.00 ± 3.83
2.50	62.22 ± 4.84	35.56 ± 2.94
3.00	68.89 ± 2.94	42.22 ± 2.94
3.50	35.56± 2.94	18.89 ± 2.94
4.00	8.89 ± 4.01	10.00 ± 1.92

The highest callus fresh weight 1.30g and dry weight 0.05g (leaf explants) were produced on 2mg/l 2, 4-D containing medium. For the nodal segment explants, the highest callus fresh weight 1.63g and dry weight 0.06g was produced on 2.50mg/l 2,4-D supplemented MS medium (Fig 2). The lowest callus fresh weight (1.00g) were observed with 3.00 mg/l of 2,4- D in both leaf and nodal explants (Fig 2).

3.2.2 Effect of of BAP in combination with NAA on callus induction

From various concentrations of BAP combined with NAA tested, maximum percentage of callusing (81.11%) in leaf explants and (84.44 %) in nodal explants were observed on 3.00mg/l BAP and 0.50 mg/l NAA (Table 3). This medium also frequently produced callus in a shorter period (9.3 days) than that of other hormonal combinations in both leaf and nodal segment explants.

When the concentration of BAP increased from 3.00mg/l to 3.50 mg/l the degree of callus induction reduced to 78.89% in leaf explant and 81.11% in nodal segment explant. Overall, increasing or decreasing the concentration of BAP from 3.00 mg/l keeping the concentration of NAA constant (0.50mg/l) reduces the percentage of callus formation. The nature of the calli produced was soft, compact and light green to white in color (Figure 3).

In both leaf and nodal segment explants, the calli induced on media containing BAP at different concentration combined with NAA at 0.5mg/l are good quality and growth rate is also higher next to the calli produced on BAP alone (Fig 6). This is in agreement with Mohajer et al (2012) who reported that MS medium supplemented with BAP in combination with NAA was the best for callus induction due to high callus percentage and high callus fresh weight. This study also presents nodal segment as the best explant for induction of callus for this species which expressed maximum (84.40%) callusing at 3mg/l BAP and 0.5 mg/L NAA compared to leaf explants (81.10%).

Table 3 Effect of BAP in combination with NAA on callus induction from leaf and nodal segment explants of *Allophylus serratus*

Concentration (mg/l)		Leaf explant	Nodal Segment explant
BAP	NAA	Callus induction (%) (Mean ± SE)	Callus induction (%) (Mean ± SE)
0.00	0.00	0.00	0.00
1.00	0.50	34.40±2.94	38.90± 2.22
1.50	0.50	45.60±4.01	45.60± 4.01
2.00	0.50	70.00± 3.85	70.00± 1.92
2.50	0.50	68.90± 2.94	70.00± 5.09
3.00	0.50	81.11± 2.22	84.44± 2.94
3.50	0.50	78.89± 1.11	81.11± 2.22
4.00	0.50	72.22± 2.22	77.78± 1.11

The average fresh weight and dry weight of calli from leaf and nodal segment explants were measured after four weeks. The highest callus fresh weight (5.20 g in leaf and 5.80g) and dry weight (0.26 g and 0.26g) in leaf and in nodal segment explants was obtained on media containing 3mg/l BAP + 0.5mg/l NAA (Figure 3 and 4).

3.3.3 Effects of BAP on callus induction

When explants were cultured on MS medium containing BAP alone at various levels, the highest callus induction frequency (71.10% and 73.30% in leaf and nodal segment explants respectively) was obtained on 3mg/l concentration (Table 4). The least callus induction was observed in 1.00mg/l BAP in leaf explant. When the concentration of BAP is lower or higher than 3.00mg/l the formation the percentage of callus induction and callus growth rate decreased in both leaf and nodal segment explants. Overall, better percentage of callus induction on BAP fortified media observed when nodal segment explant used than leaf explants.

The length of time it takes for callus induction is about 15 days in leaf explants and 12 days in nodal segment explants. The nature of calli formed were soft, compact and white gray (Fig 5). Data illustrated in Fig 6, showed the fresh weight and dry weight of callus induced with different concentrations of BAP from leaf and nodal segment explants. In both explants, 3.00mg/l BAP provided better value of callus fresh weight (5.30 g and 6.00g) and the callus dry weight (0.26 g and 0.29g) from leaf and nodal explants respectively than all the other BAP treatments (Fig 6).

Table 4. Effect of different concentrations of BAP on callus induction from leaf and nodal explants of *Allophylus serratus*

Concentration of BAP(mg/L)	Leaf explant	Nodal Segment explant
	Callus induction (%) (Mean ± SE)	Callus induction (%) (Mean ± SE)
0.00	0.00	0.00
1.00	46.60 ±6.67	60.00±1.92
2.00	57.70±2.94	63.30±1.93
3.00	71.10±2.94	73.30±1.93
4.00	64.40±2.94	66.60±0.00

3.3 Shoot regeneration from callus

For shoot regeneration small piece of green and embryonic calli were transferred to shoot regeneration MS medium containing different concentrations of BAP and Kin. MS medium devoid of growth regulators was used as control. There was shooting response in all the tested BAP and Kin containing media as well as MS media devoid of plant growth regulators (Table 5). The calli cultured on shoot regeneration media gradually turned green as they grow and gradually start to develop shoot buds and then shoots within four weeks (Fig 7). Calli from BAP in combination with NAA were superior in shoot regeneration as compared to calli from BAP alone or 2,4-D alone.

Growth regulators free MS media was more effective in obtaining shoots from callus as compared to MS media supplemented with cytokines. The highest percentage 57.78% of shoot regeneration was achieved on growth regulators free MS medium (Table 5). Maximum shoot length (3.30 ± 0.24 cm) and shoot number (1.33±0 .71) were also observed on growth regulator free full strength MS medium. MS media supplemented with BAP and Kin also influenced shoot regeneration from callus. Among all the concentrations of BAP and Kin, BAP (2 mg/l) and Kin 1mg/l were suitable for shoot induction (37.00%) and (28.89%) respectively from callus Table 5. The least shoot regeneration (3.33%) was occurred on MS medium with 3.0mg/l Kin.

Table 5. Effect of BAP and Kinetine on shoot regeneration from callus of *Allophylus serratus*

Concentration of BAP and Kin (mg/L)		Response		
BAP	Kin	% Regeneration	Mean number of shoot/ callus	Mean length of shoot (cm)
0.00	0.00	57.78%	3.33±0.00	1.30±0.24
1.00	0.00	31.11%	1.00 ±0.47	0.90±1.12
2.00	0.00	37.77%	1.33±0.94	1.13±0.93
3.00	0.00	16.67%	1.67±1.14	0.70±1.93
4.00	0.00	12.22%	1.00±0.00	0.60±0.00
0.00	1.00	28.89%	1.00±0.00	1.10±0.81
0.00	2.00	10.00%	1.00±0.00	0.62±0.91
0.00	3.00	3.33%	1.00±0.00	0.82±2.11

3.4 Root regeneration

The small shoots (about 1-3 cm in length) regenerated on shoot induction media were transferred to root induction MS media (MS media supplemented with IBA and NAA at different levels) to test the response for root induction. The data obtained on root induction from *in vitro* regenerated shoots is given in Table 6. From all root induction media tested, 1.5 mg/l IBA containing MS medium produced maximum root induction percentage (65.55%). The maximum mean number of roots per shoots (3.33 ± 1.17) and maximum mean root length (4.00 ± 0.00 cm) were also obtained on the same media.

NAA also affect the root induction from shoot. Among all concentrations of NAA tested, 0.50 mg/l NAA produced best root induction (51.00%), mean number of roots (2.75 ± 1.66) and mean root length (2.76 ± 0.13 cm). The least root induction percentage (2.00%) was observed on MS medium supplemented with 2.00mg/l NAA (Table 5).

Table 6. Effect of IBA and NAA on root induction from *in vitro* shoot of *Allophylus serratus*

Concentration of IBA and NAA (mg/L)		Response		
IBA	NAA	% Regeneration	Mean number of root/ shoot	Mean length of root (cm)
0.00	0.00	0.00	0.00	0.00
0.50	0.00	31.11%	2.33 ±0.67	0.91±1.92
1.00	0.00	36.67%	2.70±1.34	0.93±1.93
1.50	0.00	65.55%	3.33 ± 1.17	1.00 ± 0.00
2.00	0.00	52.22%	1.33±0.94	0.82±0.10
0.00	0.50	51.11%	2.33 ± 1.66	0.76 ± 0.09
0.00	1.00	40.00%	1.33 ± 0.76	0.46 ± 0.11
0.00	1.50	33.33%	2. 67 ± 1.13	0.42 ± 0.15
0.00	2.00	2.22%	0.67 ± 0.36	0.51 ± 0.91

3.5 Acclimatization

The result of acclimatization showed that all plantlets (100%) survived after transferred to a pot containing sand, soil and compost in 1:2:2 ratio. The plantlets were grown normally and no any morphological abnormalities were observed (Fig .8).

4. Discussion

4.1 Explant Surface Sterilization

In this study surface sterilization of leaf and nodal segment explants of *A. serratus* was developed using different chemical sterilants. The highest percent survival of leaf explants (80.00%) was achieved with treatment (70% ethanol for 1minute, 1.0% NaOCl for 4 minutes and 0.1% $HgCl_2$ for 3 minutes) and the highest percent survival of nodal segment explants (90.00%) was with treatment (70% ethanol for 1min, 2% NaOCl for 3 minutes and 1.00% $HgCl_2$ for 2 minutes. Our result indicates that different tissue type requires different concentration of sterilants and a time exposure which is in agree with Rezadost et al. (2013) report and with Sharma et al. (2014), which indicated that the exposure time of sterilization depends on the type of tissue; for example, leaf explants require a shorter sterilization time than seeds with a tough seed coat. In this study, leaf explants need shorter sterilization time and a lower concentration than nodal segment explants. When the exposure time becomes shorter or longer, the death of explants was higher which may be due to microbial contamination or effect of chemical sterilants (Tola et al. 2015).

4.2 Callus induction

Callus induction frequency and callus type induced varied depending on the type of explant, type and level of plant growth regulator used. The highest response of callus induction in leaf explants (68.89 ± 2.94 %) and nodal

segment explants (42.22 ± 2.94 %) were observed on the medium supplemented with 3.00mg/l 2, 4-D. Callus induction frequency increased as the concentration of 2, 4-D increased. This is in agreement with the report of Xing et al., (2010) who achieved optimum callus induction with increase in 2,4-D. Further increase in concentration of 2,4-D decreased callus production. Many other researchers have been also reported that MS medium supplemented with 2.4-D is suitable for callus induction (Baskaran et al., 2005; Faisal and Anis, 2003; Rani et al., 2003). Liu et al. (2006) also revealed that 2.4-D alone or in combination with cytokinins is commonly used to induce and maintain callus.

From the medium supplemented with BAP and NAA in combination tested for callus induction, highest percentage of callusing was recorded in leaf explants (81.11%) and in nodal explants (84.44 %) on 3.00mg/l BAP and 0.50 mg/l NAA.Akram and Aftab, (2015) also revealed highest callus formation (93.00%) in *Tectona grandis* on media supplemented with BAP combined with NAA. Similarly, callus induction on MS medium containing BAP combined with NAA were also observed in several plants (Tomar and Tiwari 2006; Panigrahi et al. 2006; Rahmatullah et al. 2012). The nature of the calli produced was soft, compact and light green to white in color. Similar results were also reported by Rajeshwari and Paliwal (2008), Patel and Shah (2009) and Isikalan et al. (2010).

From the different concentrations of BAP alone, the highest callus induction frequency (71.10% and 73.30% in leaf and nodal segment explants respectively) was obtained on 3mg/l concentration. In all tests, leaf and nodal segment explants of *A. serratus* cultured on MS basal medium without hormone did not induce callus which is in line with previous reports by Zare et al. (2010) and Devendra et al. (2009) on F*. asafoetida* and cucumber respectively. This confirms that the presence of plant growth regulators is necessary for callus formation from leaf and nodal explants of *A. serratus*.

Overall, MS media supplemented with BAP combined with NAA is the best for callus induction from leaf and nodal segment explants in *Allophylus serratus* which confirmed that both cytokinin and auxin are necessary for callus induction. The percentage of callus induction was lower for nodal explants on media with 2,4-D when compared to BAP alone and in combination with NAA (Table 1). The results of this study concluded that callus induction by BAP (3.00mg/l + NAA (0.50 mg/l) gave best results for callus induction from leaf and nodal segment explants. Other reports such as that of Tola et al. (2015) also found that the combination of auxin with cytokinin (combination of BAP and 2,4-D) gave the high frequency of callusing in *Coccinia abyssinica*. MS media supplemented with 2, 4-D alone and BAP alone initiated callus induction less frequently than that supplemented with BAP in combination with NAA. BAP alone gave lower percentage of callusing than when it combined with NAA. This indicated that the presence of NAA is important to improve the callusing efficiency of the explants.

Among the leaf and nodal segment explants, nodal segment explants were found to be suitable for callusing in this species. Leaf derived callus showed late induction and retarded growth relatively. Sarin and Bansal (2011) also reported the superiority of nodal segment explant in callusing over leaf explant in *Adhatoda vasica* and *Ageratum conyzoides.*

In all tests, leaf and nodal segment explants of *Allophylus serratus* cultured on MS basal medium without hormone did not induce callus which is in line with previous reports by Zare et al. (2010) and Devendra et al. (2009) on F*. asafoetida* and cucumber respectively. This confirms that the presence of plant growth regulators such as 2,4-D, NAA and BAP alone or in combination in the medium is necessary for callus formation from leaf

and nodal explants of *Allophylus serratus*. This is because of both hormones auxin (2, 4-D and NAA) and cytokinin (BAP) are essential for stimulating cell division, cell elongation and cell growth in plants.

4.3 Shoot regeneration

In this study, calli produced on MS medium supplemented with BAP in combination with NAA gave highest shoot regeneration as compared to calli from BAP alone or 2,4-D alone which is in agreement with (Teshome and Feyissa, 2015). But growth regulators free MS media was more effective in obtaining shoots from callus as compared to MS media supplemented with cytokines. This is in agreement with Guruchandran and Sasikumar, (2013) and Sen et al., (2014) who reported shoot regeneration on BAP and Kin supplemented media in Stevia *rebaudiana* and *Achyranthes aspera* respectively. Al-Taha *et al* (2012) and Miah *et al*., (2002) also confirmed our findings.Overall, BAP is better than Kin for shoot regeneration from callus in *Allophylus serratus* similar to Zare et al. (2010) reporton*Ferula assa Foetida*. Our result, shoot regeneration on hormone free MS media, is in contrast with other reports on shoot regeneration on medium supplemented with plant hormones (Teshome and Feyissa, 2015 on *Glinus lotoides ;* Guruchandran, and Sasikumar, 2013 on *Stevia rebaudiana,* Kumar et al,2014 on *Swertia chirayita* and (Sen et al. 2014) on *Achyranthes aspera).*

4.4 Root induction and Acclamatization

From all root induction media tested, 1.5 mg/l IBA containing MS medium produced maximum root induction percentage (65.55%), maximum mean number of roots per shoots (3.33 ± 1.17) and maximum mean root length (4.00 ± 0.00 cm). Therefore, IBA was found to be better than NAA for root induction in *Allophylus serratus*. Other studies also reported that IBA is more effective in root induction than NAA (Fracaro and Echeverrigaray, 2001; Bera et al. 2009; Oluk and Çakyr, 2009; Jitendra et al. 2012; Pandey et al. 2013).

The efficiency of IBA in root induction was also reported in other medicinal plants including *Psoralea corylifolia* (Pandey *et al*., 2013), *Stevia rebaudiana* (Jitendra *et al.,* 2012), *Solanum nigrum* (Rathore et al., 2013) and *Operculina turpethum* (Sebastinraj *et al*., 2013). IBA also gave higher response in other parameters such as mean root number, and mean root length than in NAA. But, in contrast to our result, the highest root induction ability of NAA was reported in other plants such as *Thymus satureioides* (Aicha *et al*.,2013) and *Solanum surattense* (Rahman *et al*., 2011). As the concentration of auxins (IBA and NAA) increased, there was decline in the induction and development of root as previous report (Teshome and Feyissa, 2015).

The rooted plantlets were acclimatized in a pot containing sand, soil and compost in 1:2:2 ratio. The plantlets were grown normally and no any morphological abnormalities were observed.

5. Conclusion

In general, surface sterilization of explants should guarantee the explants sterility and regeneration capacity which are known to be affected by disinfectant concentration and length of sterilization period. The *in vitro* surface sterilization protocol reported in this study can be used for surface sterilization of explants from leaf and nodal segments of *Allophylus serratus*. This result also suggests successful callus induction protocol from leaf and nodal explants of *Allophylus serratus* using MS media supplemented with different concentrations and combinations of plant growth regulators (2,4-D, NAA, and BAP). MS media containing 3mg/l BAP and 0.5mg/l NAA was the most efficient for callus induction. Callus induction frequency depends on the composition of callus

induction media, plant organs, and callus type. Maximum means of shoot and root were observed on MS medium containing 0.5 mg/l BAP and 1.5 mg/l IBA respectively. All the plants were survived after acclimatization in greenhouse. Production of callus from medical plants and regeneration of plant from callus are important for mass propagation of the plant and to facilitate production of secondary metabolites. *Allophylus serratus* is a medicinal plant which contains different groups of phytochemicals. Therefore, these callus induction and plant regeneration protocols are very important for mass propagation of the species and also opens a new way to facilitate secondary metabolites production and isolation of pharmaceuticals from callus rather than harvesting the plant itself.

List Of Abbreviations

ANOVA ; Analysis of Variance

BAP; Benzyl amino Purine

CRD; Completely Randomized Design

IBA; indole-3-butyric acid

MS; Murashige and Skoog

NAA; naphthalene acetic acid

SPSS ;Statistical Package for the Social Sciences

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

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Competing interests

The authors declare that they have no competing interests

Authors Contribution

The authors K.J, designed, conducted the study, interpreted the results and analyzed the data and wrote and reviewed the manuscript

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Figures

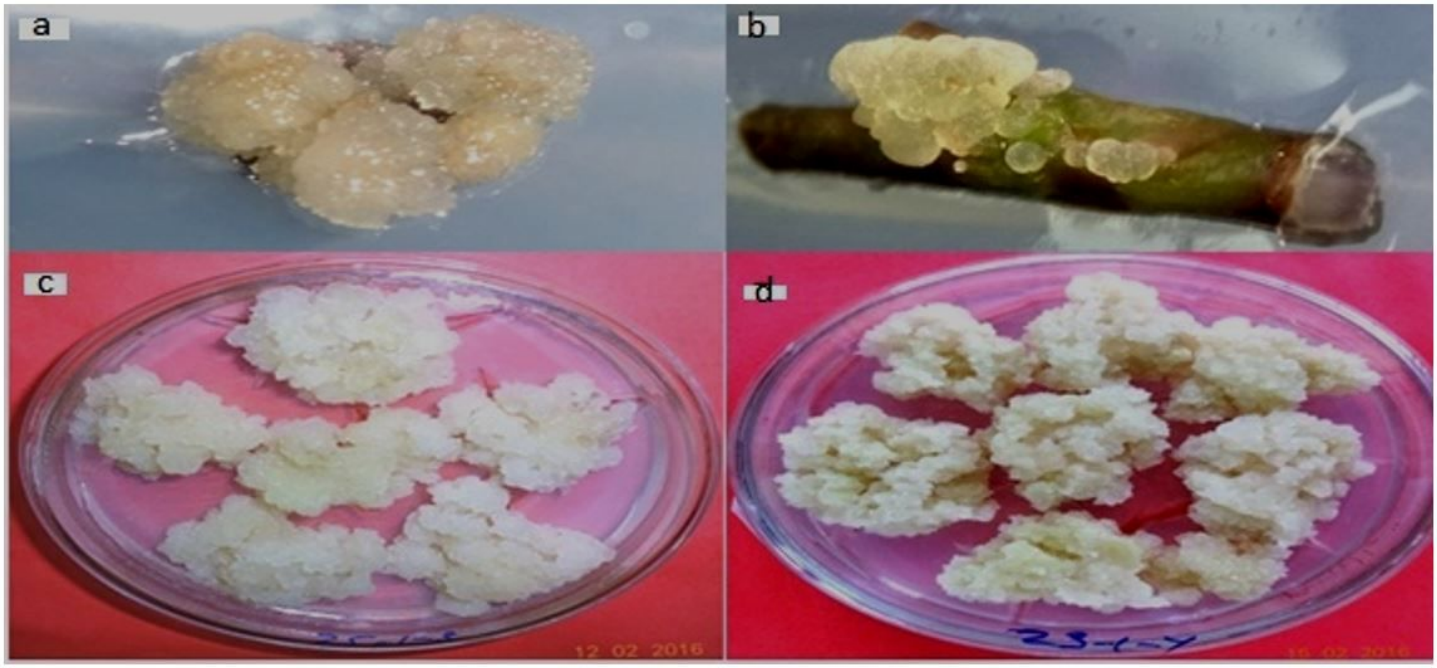

Figure 1

Callus induction from leaf and nodal segment explants. (a) Leaf explant 3.0 mg/l 2,4-D; (b) Nodal segment explants, 3.0 mg/l 2,4-D; (c) Callus induced from leaf explants subcultured on same media after 30 days. (d) Callus induced from nodal segment explants subcultured on same media after 30 days

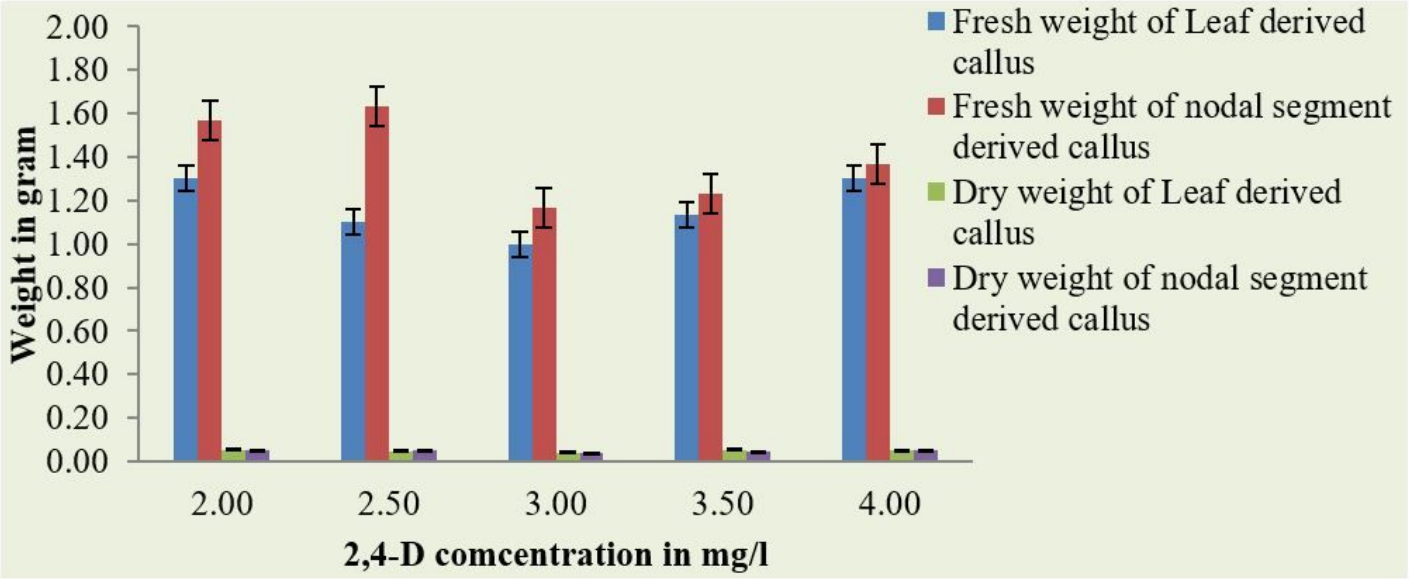

Figure 2

Callus fresh weight and dry weight of different concentrations of 2,4-D

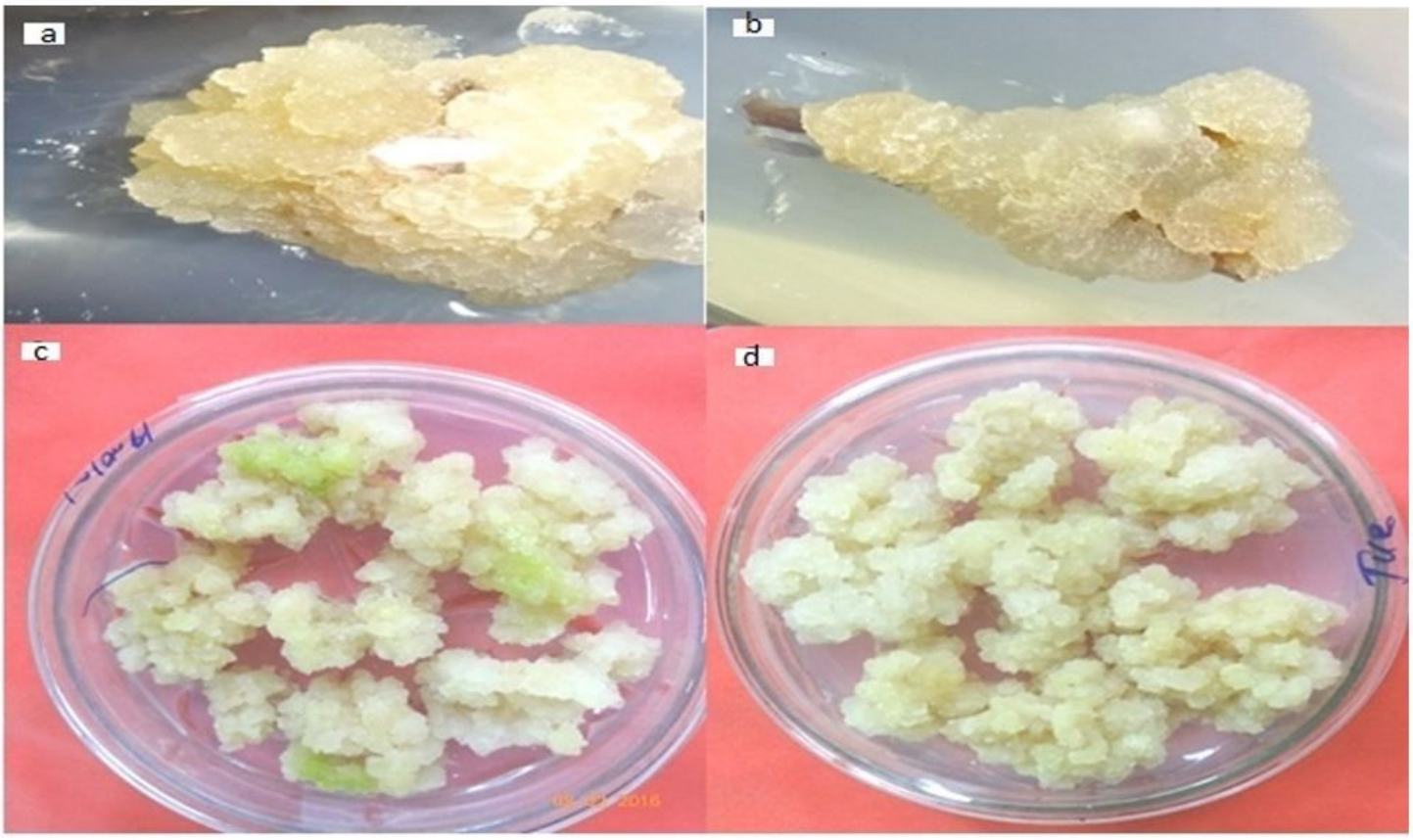

Figure 3

Callus induction from leaf and nodal segment explants. (a) Callus induced from leaf explants on MS + 3.0 mg/l BAP + 0.5mg/l NAA; (b) callus induced from nodal segment explant on MS + 3.0 mg/l BAP + 0.5mg/l NAA; (c) Callus induced from leaf explants subcultured on same media after 40 days . (d) Callus induced from nodal segment explant subculturedon same media after 40 days

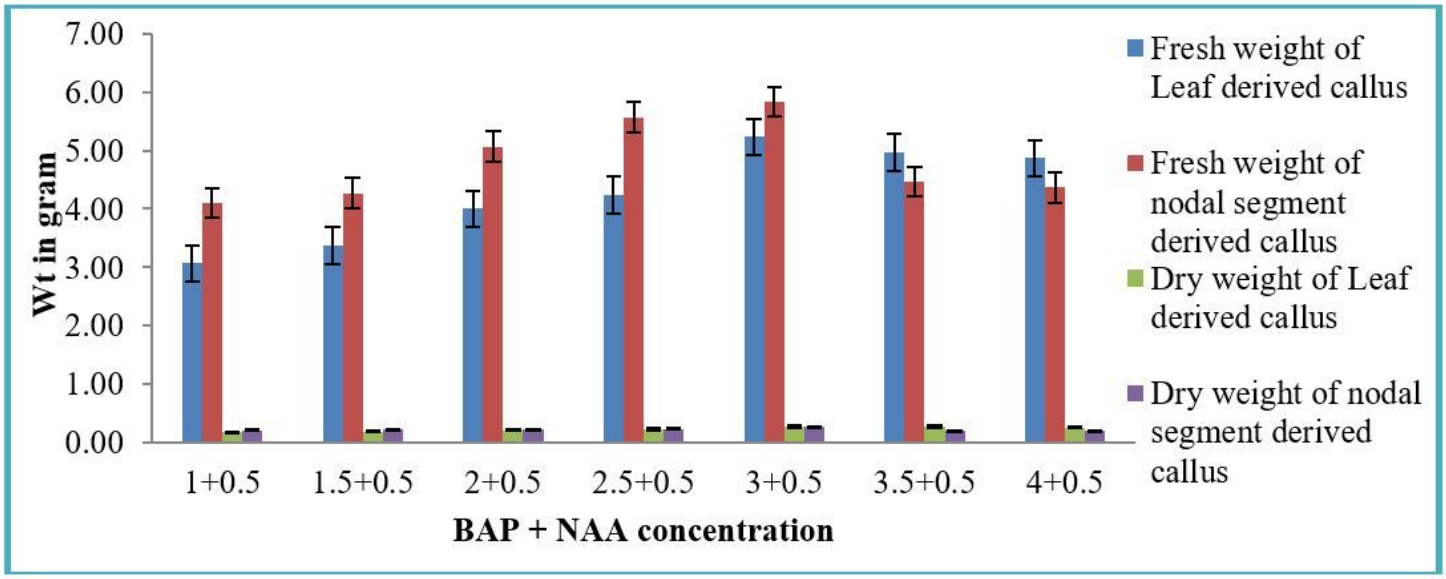

Figure 4

Fresh and dry weights of callus induced with BAP + NAA

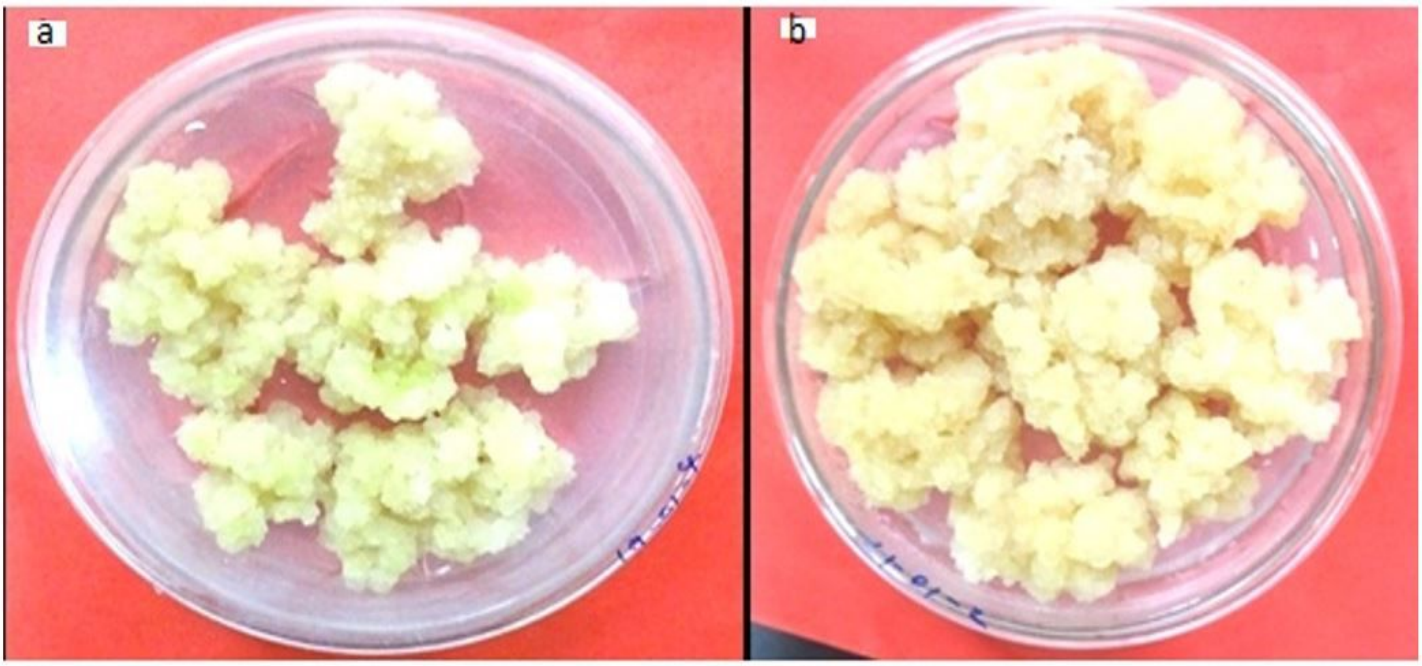

Figure 5

Callus induction from leaf and nodal segment explants. (A) Leaf explants callus induced on MS + 3.0 mg/l 2,4-D and subculturedon same media after 40 days (B) Nodal segment explant callus induced on MS + 3.0 mg/l 2,4-D and subculturedon same media after 40 days.

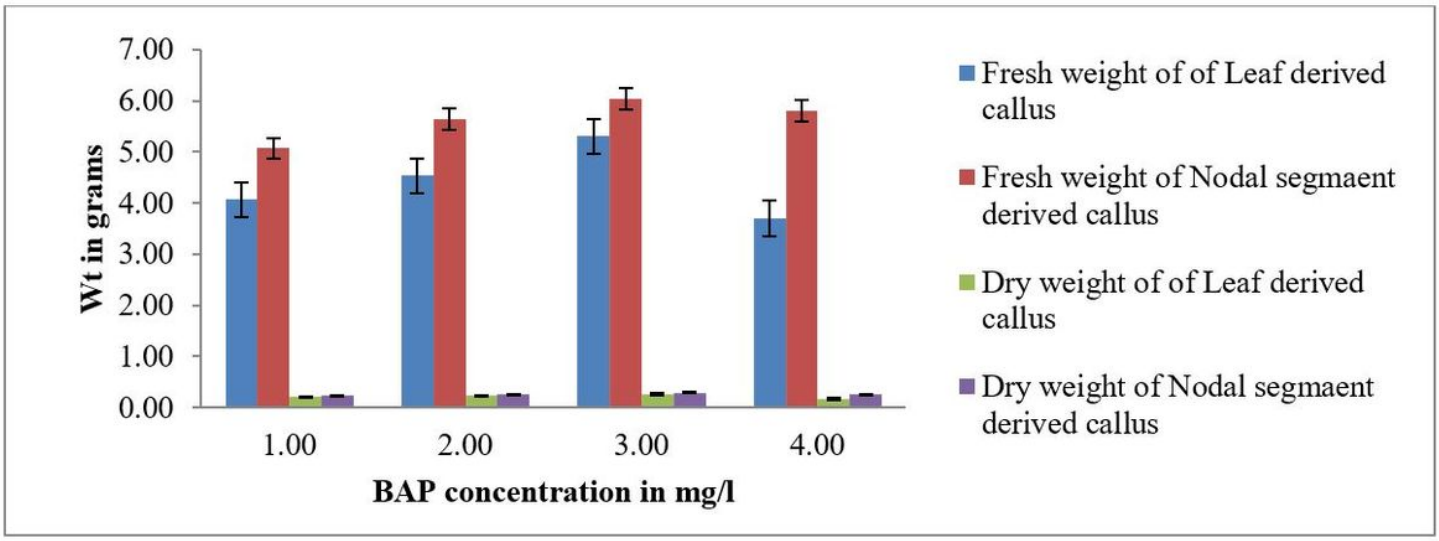

Figure 6

Freshweight and dry weight of callus induced withdifferent concentrations of BAP.

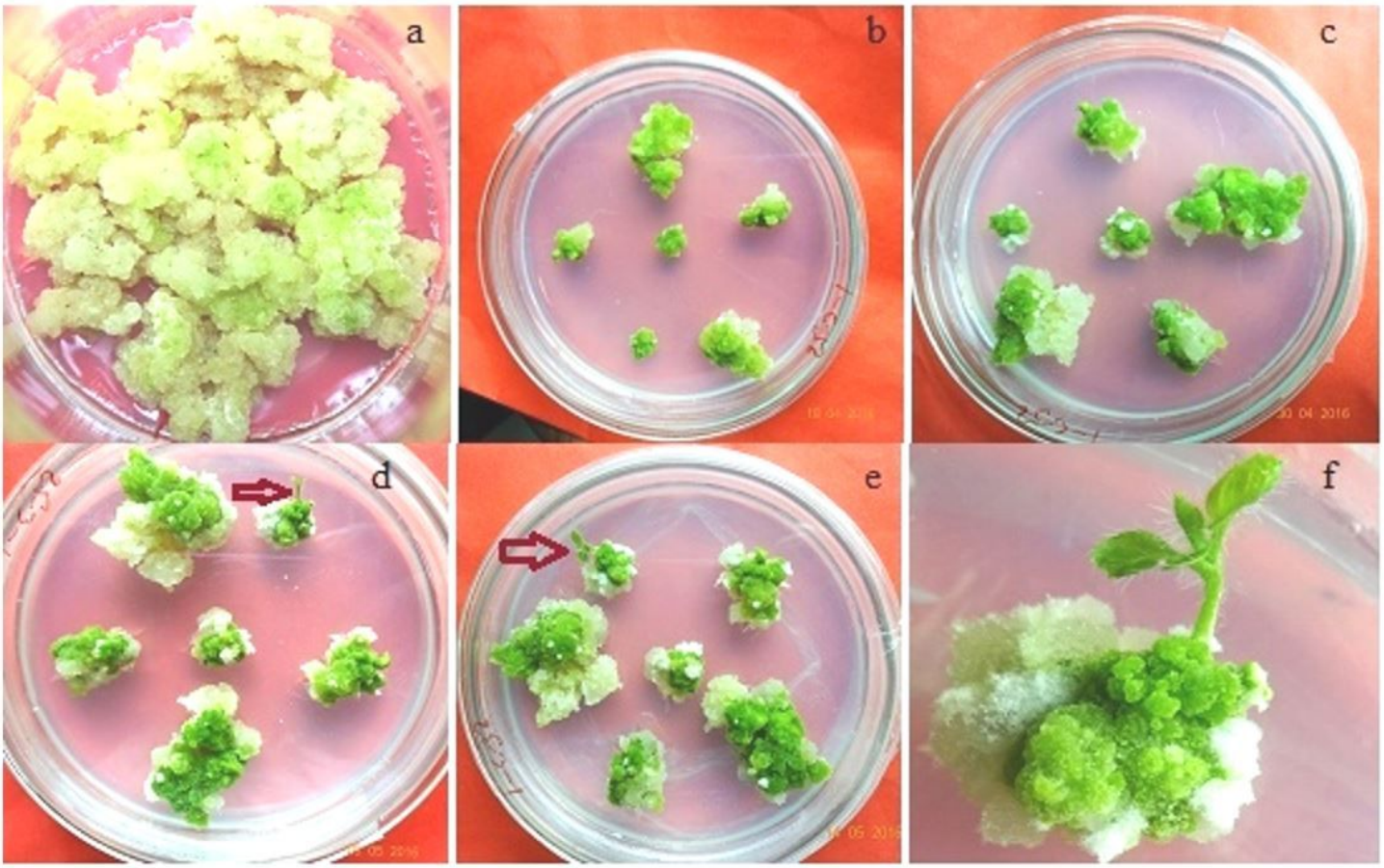

Figure 7

Shoot regeneration from callus. a) Callus induced on MS medium supplemented with 3mg/l BAP + 0.5 mg/l NAA. b) Callus sub cultured on growth regulator free MS medium after three weeks having green shoot buds. c, d and e) shoot growing from shoot buds of callus after four weeks.

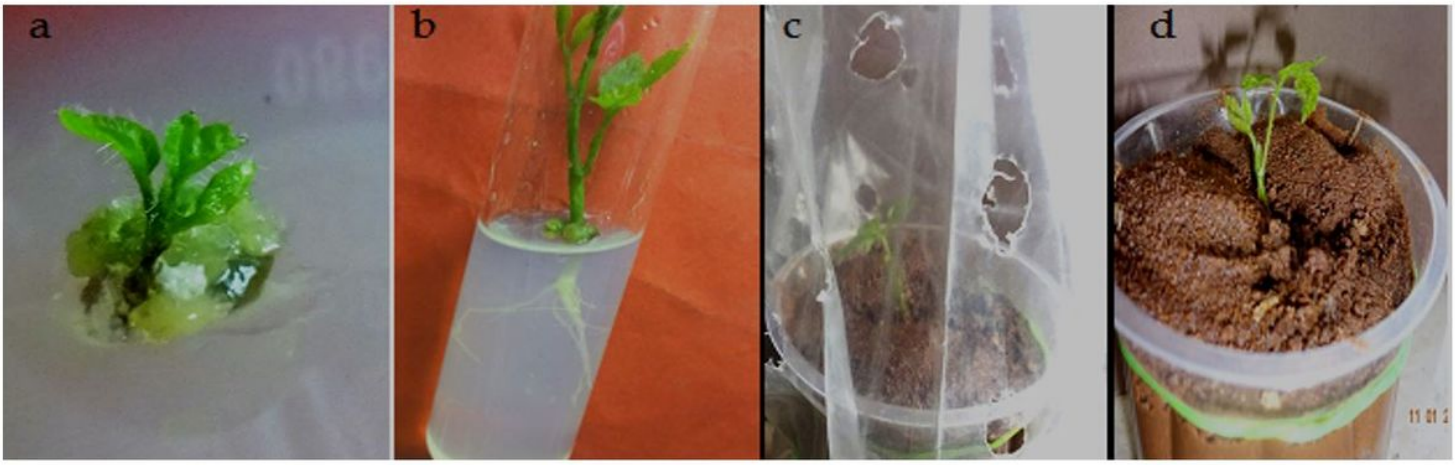

Figure 8

Root regeneration from shoot and acclimatization of the regenerated plant. a) shoots inoculated on root induction media. b) Root induced on MS medium supplemented with 1.5mg/l IBA c) Rooted plantlet transferred planted on sand soil and compost mixture in a pot and covered by polyethelene bag. and d) acclimatized plantlet uncovered and exposed to the environment.