Studies of shoot organogenesis in *Chlorophytm borivilianum* Sant. *et* Fernand

A

Dissertation Report

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CANDIDATE'S DECALARATION

I hereby declare that the work presented in the dissertation entitled '**Studies of shoot organogenesis in *Chlorophytum borivilianum***' Sant. *et* Fernand in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala; is an authentic record of my own work carried out during the period January-June 2018, under the supervision and guidance of **Dr. Anil Kumar**, Associate Professossr, Department of Biotechnology, Thapar Institute of Engineering and Technology (TIET), Patiala. I have not submitted this dissertation to any other University for the award of any other degree or diploma.

Place: Patiala

Date: 22-08-2018

Gurjinder Kaur

CERTIFICATE

This is to certify that the thesis entitled **"Studies of Shoot organogenesis in *Chlorophytum borivilianum* Sant *et* Fernand"** submitted by Miss Gurjinder Kaur (Roll no. 301601007) in partial fulfillment of the requirements for the award of the degree of Master of Science in Biotechnology, to Thapar Institute of Engineering and Technology (Deemed University), Patiala, is a record of student's own work carried out by her under the supervisor and guidance. The report has not been submitted for the award of any other degree or certificate to any other University or Institute.

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ABBREVATIONS

%	Percent
°C	Degree centigrade
µg	Microgram
µl	Microlitre
µM	Micromolar
AFLP	Amplified fragment length polymorphism
BAP	6-Benzylaminopurine
cm	Centimetre
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
G	Gram
hrs	Hours
HCl	Hydrochloric acid
IBA	Indole-3 –butyric acid
IAA	Indole -3-acetic acid
ISSR	inter Simple Sequence repeat
STS	Sequence Tagged Site
M	Molar
mg	Milligram
min	Minute
ml	Mililitre
mM	Millimeter
mM	Millimolar
MS	Murashige and Skooge (1962) medium
N	Normal
PGR (s)	Plant growth regulator (s)
RAPD	Random amplification of polymorphic DNA
SE	Standard Error
V	Volt
v/v	Volume by volume
w/v	weight by volume

Fig.	Figures
TDZ	Thidiazuron
CTAB	Cetyltrimethyl ammonium bromide
PCR	Polymerase Chain Reaction
s	Seconds
DNTP	Deoxynucleotide triphosphate
Ft	Feet
bp	Base pair
UV	Ultraviolet

ABSTRACT

Medicinal plants especially herbs are utilized by locals for the treatment of various diseases. The advances in the field of phytochemistry and pharmacology led to discovery of modern drugs from plants. Thus, the interest for *Chlorophytum borivilianum* Sant. *et* Fernand (Safed musli) is rising rapidly due to its medicinal properties. As a consequence, its demand is limited by traditional methods of propagation. Thus, tissue culture seems to be an alternative which results in the conservation of its natural stock. The present work was focused to induce shoot organogenesis form the leaf explants of *C. borivilianum*. Maximum shoot regeneration in *C. borivilianum* was achieved on MS medium fortified with 5.0 µM BAP.

Moreover, sucrose as carbohydrate source in MS medium played an important role on growth of shoot clumps in *C. borivilianum*. The rooting response of *C. borivilianum* varied significantly with increasing concentrations of IBA. IBA enriched MS medium resulted in 100% root induction. Moreover, the effect of 2, 4-D in combination with BAP & TDZ was studied for the induction of shoot organogenesis on leaf segments excised from microshoots of *C. borivilianum*. Direct shoot organogenesis was achieved on MS medium supplemented with 5.0 µM BAP and 2.5 µM 2, 4-D. Another important finding was the induction of shoots from the leaf bases on MS medium containing only IBA. Morphology of regenerated plants was found to be identical with their mother plants. Regenerated plantlets from leaf bases were checked for any somaclonal variations using PCR based molecular marker such as RAPD and ISSR. The regenerated shoots were found true to type. Thus, present study resulted in the optimization of regeneration protocol which possesses the great potential for carrying out trait specific modifications in *C. borivilianum*.

INTRODUCTION

India is home of large number of medicinally important plants & herbs growing naturally at altitudinal range of 800-4500m amsl (Nadeem et al. 2002). The ever increasing demand of the drug and pharmaceutical industries for the natural products has significantly increased their consumption, leading to increased level of exploitation (Cragg and Newmann 2013). Moreover, limited growing period and harsh climatic conditions limits their expansion at higher altitudes (Kumar et al. 2011). The unsustainable collection resulted in population decline indicating their elimination from natural environment if immediate steps for its conservation are not taken. Therefore, there is an immediate need for conservation steps to be taken up along with promotion of cultivation of medicinal plants. The limitation of in situ conservation of plants involves slow regeneration, over exploitation and destruction of habitat (Chen et al. 2016). Therefore, tissue culture method is seen as promising alternative for the multiplication of medicinal plants (Sidhu 2010; Jain et al. 2012). One such plant identified is *Chlorophytum borivilianum* known for its various medicinal properties like anti-inflammatory, antiageing, anti-ulcer, anti-microbial and anti-stress activities (Deore & Khadabadi 2008; 2009; Acharya et al. 2009; Mujeeb et al. 2009)

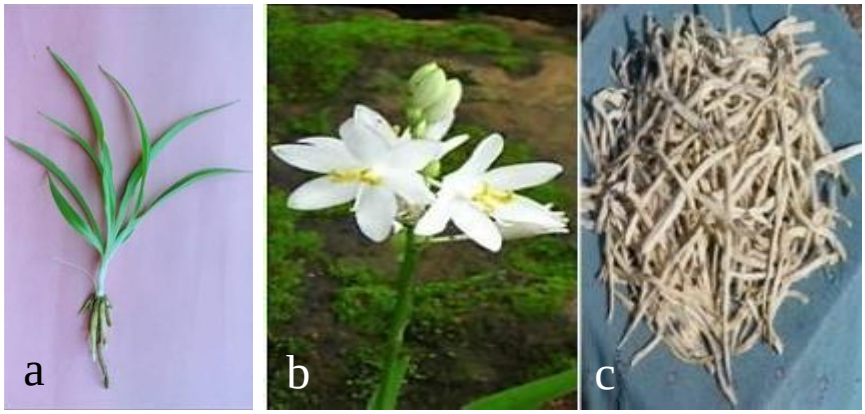

Figure 1. Morphological features of *Chlorophytum borivilianum,* a) Full grown Safed musli plant; b) Flowers of Safed musli; c) Roots of Safed musli

Chlorophytum borivilianum Sant. *et* Fernand a traditional medicinal plant, also called as safed musli. It is a member of Liliaceae family (Khanam et al 2013). This plant is endemic to India (Maiti and Geetha 2005) and widely distributed in many states such as Rajasthan, Western Madhya Pradesh, Karnataka and few parts of Madhya Pradesh (Panda et al. 2007). Among 300 species of *C. borivilianum*, 13 species are found in India (Santapau and Fernandes 1955). It is a very popular aphrodisiac agent, with negligible side effects and serve

as an integral component of various ayurvedic formulations (Bathoju and Giri 2012). It is also used for arthritis, improving sexual performance, diabetes and for several other uses (Singh et al. 2012).

Since, both sexual and asexual methods are involved in the propagation of Safed musli (Debnath et al. 2006), vegetative method is preferred over seed propagation because the seeds remain dormant for about 10-11 months (Sharma et al. 2006) and seedlings also show heterogeneity. Furthermore, poor regeneration in the natural habitat, unsustainable collection practice and declining population could results in its extinction in near future (Singh et al. 2012). Micropropagation could be useful for the conservation of this herb. Hence, it is crucial to develop an efficient *in vitro* propagation protocol for the conservation of important herb. Moreover, an efficient regeneration protocol will be useful for mass production and trait specific genetic improvement. Thus, the *in vitro* plant regeneration through shoot organogenesis and or somatic embryogenesis needs to be optimized (Rizvi et al. 2010). Though, plant regeneration via callus phase is not a preferred approach to produce uniform planting material (Pardee 1989; Nakasha et al. 2016). It often contains polypoid cells and are genetically uniform. Further, the plants regenerated from direct shoot organogenesis are genetically stable. Somatic embryogenesis can be defined as formation of bipolar structures from somatic cells called somatic embryos which eventually form complete plantlets (Saini et al. 2010). Somatic embryogenesis offer potential advantages over organogenesis such as it allows the generation of large numbers of propagules (Jimenez 2001). Moreover, somatic embryos often originate from single cells which results in the low probability of getting cameric and somaclonal variants among regenerates as compared to callus mediated regeneration pathway (Krishna et al. 2016).

In vitro responses of explants depend on the exogenous supply of PGRs in the culture medium (Satyavathi et al. 2004). Auxins to cytokinin ratio determine the shoot or root regeneration (Bhojwani and Razdan 1981). Higher auxin concentration results in root formation whereas high cytokinin is reported to form shoots (Aloni et al. 2006). Auxins such as 2,4-D and NAA are widely used for the induction of callus (Dodeman 1997, Rizvi et al. 2010). However, studies are also available on the use of auxins like picloram, dicamba, IAA and IBA (VonArnold et al. 2002). Apart from auxins, cytokinins like BAP can also be used with combination of auxins for the stimulation and maintenance of callus (Verma et al. 2016). Furthermore, the embryogenic calli have the potential to differentiate into somatic embryos on medium containing lower auxin levels, but further development requires medium devoid of auxin (Kukreja et al. 2010). In addition to PGRs, the type and age of explant, medium pH,

various physical factors such as intensity of light, photoperiod and temperature have also been reported to influence the callus induction (Kumari et al. 2018).

In vitro regenerated plants have been tested for their genetic uniformity using many molecular markers such as Random amplification of Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeat (ISSR) etc. (Arcade et al. 2000; Mahatma et al. 2017). Both RAPD and ISSR markers are extensively used for the confirmation of clonal fidelity of micropropagated plants (Rani et al. 1995; Rizvi et al. 2012).

REVIEW OF LITERATURE

Mankind had been surviving from the past 300 decades based on the therapeutic properties of plants (Petrovska 2012). It has been well documented that 1100 plant species are regularly used in present healthcare and medicine system. One such plant identified is *Chlorophytum borivillianum* Sant. *et* Fernand of Liliaceae family. It is one of the most important, endangered valuable plants and holds place in Ayurveda and allopathic system of medicine (Deore & Khadabadi 2009). It is a supplementary therapy for blood purification, nervous disorder and some gynaecological problems (Ramawat et al. 1998). In past, it has been used as an anti-inflammatory, anti-ageing and as a general health promotive, cardiac and brain tonic (Sharma et al. 2014). It is also involved in improving sexual performance (Thakur and Dixit 2006), post-biological time syndrome, albuminorrhoea-leucorrhoea, menorrhea etc. (Ramawat et al. 1998; Oudhia et al. 2000). Safed Musli also find its uses in supplements for body building as it helps in the strengthening of bones and muscles (Acharya et al. 2009). In the Unani system of medicine, its formulations are utilized in curing respiratory diseases, ophthalmic conditions, vomiting, dyspepsia, and lumbago, pain (Sahoo 2018).

Being economically valuable plant, efforts have been directed towards the need of plant tissue culture methods for effective conservation of the herb (Sidhu 2010). Since micropropagation offers rapid multilplication, numerous attempts have been made in *C. borivilianum* regarding the optimization of micropropagation protocol. These are discussed below:

Purohit et al. (1994) initiated *in vitro* culture in *C. borivilianum* from stem discs used as explants on MS medium containing a mixture of different plant growth regulators (auxins & cytokinins). Within 25 days of inoculation the inoculated shoot bases produced root primordia. Maximum shoot regeneration was achieved on MS medium supplemented with 22.2 µM BA whereas kinetin was found to be less effective. All the shoots induced rooting when transferred on MS medium with salts and 9.8 µM IBA. The *in vitro* raised plantlets when transferred to pots resulted in 67% survival rate.

The effect of various explants such as seedlings, root and leaf base on *in vitro* clonal propagation in *C. borivilianum* was studied by (Gaikwad et al. 2003). Along with different

concentrations of hormones and vitamins were analyzed for callus development, shoot and root regeneration. The leaf base showed best response as compared to other explants.

Micropropagatiosn of *C. borivilianum* was reported by (Dave et al. 2003). The highest shoot number was recorded on MS medium containing 22.2 µM BAP and 3% (w/v) sucrose. However, better response was observed when Phytagel 0.2% (w/v) was used in place of agar. Hardening under greenhouse conditions showed 90% survival than in the open field.

Samantaray et al. (2009) studied *in vitro* response of young inflorescences of *C. arundinaceum* and *C. borivilianum.* Shoot bud regeneration was achieved on half-strength MS medium augmented with cytokinin (BAP) and auxins (IAA & NAA). More number of shoot buds were achieved from apex portion. However, shoot bud initiation delayed by 28 days from terminal segment the shoots showed 81.66 and 86.33% rooting response for *C. arundinaceum* and *C. borivilianum* respectively. 90% survival rate was observed in the soil from the *in vitro* raised plantlets.

Kemat et al. (2010) recorded shoot induction on medium containing basal salts of MS along with BAP and KIN as growth hormones in various concentrations. Maximum number and length of shoots were observed on BAP enriched basal MS medium. The regenerated shoots followed by rooting were transferred to pots containing vermiculite.

Garima & Shruthi (2012) reported the effect of various carbon sources on the multiplication of shoots on MS medium fortified with cytokinin BAP. Higher multiplication rate (3.5 folds) was observed when sucrose was used as carbohydrate source as compared to other sources such as maltose, glucose and fructose. Rooting was observed in more than 80% microshoots on medium containing 9.8 µM IBA. After hardening and acclimatization, the rate of plantlet survival ranged from 87-90% under open field and green house conditions.

Somatic embryogenesis/shoot organogenesis

The initial reports on somatic embryo induction in *C. borivilianum* were developed from immature zygotic explants by Purohit et al. (1994). Callus induction was observed within 2 weeks of inoculation on MS medium supplemented with different concentration of 2, 4-D ranging from 4.52 -22.6 µM. The best response was achieved at concentration of 4.52 µM. The regular sub culturing on MS medium containing 1.13 µM 2, 4-D and 5.67 µM ABA

resulted in faster growth of these embryonic cultures. Furthermore, mature somatic embryos germinated in the presence of light on MS medium containing PGRs.

Singh et al. (2006) studied various biochemical changes during the differentiation of shoots from the callus cultures of *C. borivilianum.* Callus was derived from bud pedicel on MS medium supplemented with 1 mg/l BAP and 1 mg/l NAA. The starch content, total soluble sugars, free amino acids, total soluble proteins and total phenols increased significantly in shoot differentiating cultures as compared to control. The activities of various enzymes like – α-amylase, acid protease, acid phosphatase and peroxidases increased till the emergence of green patches (8-12 days) and reached maximum after two weeks due to shoot appearance. However, the activity of the acid invertase decreased till the appearance of shoots.

Kukreja et al. (2010) achieved somatic embryogenesis in *C. borivilianum*. The addition of constituents like proline and polyethylene glycol in modified MS medium containing 2,4-D, 2-isopentenyladenine stimulated somatic embryo production and maturation.

Table 1: **Various reports of *C. borivilianum* pertaining the micropropagation and regeneration protocols**

Explants used	Medium used	Response	References
Young leaf and leaf base	MS + NAA (5.37 µM) + Sucrose (3% w/v)	Callus induction Field trials (96.85% survival)	Purohit et al. (1994)
Shoot buds	MS + BAP (22.2µM) + Sucrose (3% w/v)	Maximum shoot multiplication Field trials (87-90% survival)	Dave et al. (2003)
Microshoots	MS+2,4-D(4.53 µM)+BAP(4.44 µM)+KIN(2.32 µM)	100% callus induction and embryogenic response	Prasad et al. (2007)
Embryo	MS +TDZ (2.23 µM) + KIN (2.32 µM)	Shoot induction (60%)	
Germinated Seeds	MS + KIN(1.16 µM) + 2,4-D (1.13 µM)	Maximum callus induction (80%)	Rizvi et al. (2010)
Immature zygotic embryo	MS+2-Isopentenyladenine (7.3 8µM)	Somatic embryogenesis	

Young shoot buds	MS+BAP (3.0 µM) + KIN (3.0 µM).	Multiple shoot proliferation from shoot buds Field trials (70-80% survived)	Kemat et al. (2010)
Stem discs with shoot buds	MS + sucrose (58-116 mM + 174mM)	Increased shoot growth was observed following heat shock on inhibited shoot growth and rooting frequency. Field trials (>95% survived)	Kumar et al. (2010)
Shoots	MS + BAP + KN(0.5-12.5µM)	Shoot multiplication	
Shoots	BAP(>2.5µM)	Shoot multiplication	
Shoots	KIN(<1.0 µM)	Shoot elongation	
Shoots	MS + BAP (22.2µM) + (3% w/v) sucrose	Maximum shoot multiplication Field trials (87-90% survival)	Shruthi et al. (2012)
Tuberous roots	MS+BAP (4.4µM)	Shoot multiplication response (90%)	Jana et al. (2012)
Isolated shoots	MS+IBA (2.4µM)	Rooting response (90%)	

Clonal fidelity of regenerated plants

Recent progress in the field of molecular markers such as RAPD confirms the homogeneity of micropropagated plantlets at genetic level (Piccioni et al. 1997). Thus molecular markers find application for the quick recognition of cultivars when the morphological differences are limited.

Debnath et al. (2011) studied salt and water stress effect on *C. borivilianum*. The regenerated plantlets were subjected to different stress conditions such as NaCl (0- 171.0 µM) and mannitol (0 - 53.4 µM). Both the salt and water stress affected the morphology resulting in the decline of growth at higher concentrations. Moreover, chlorophyll content decreased, while increase in proline and protein content was observed in stressed plants. These stressed plants were further analysed using 24 random decamer primers.

Polymorphism was observed with 15 RAPD primers. Plants under draught stress showed similarity with control, while salt stressed plants showed least similarity.

Katoch et al. (2010) established phylogenetic relationship among five different species of *Chlorophytum* using RAPD markers. Out of 60 random decamer primers, polymorphism was achieved with 9 primers with a size ranging from 0.1-2 kb. UPGMA dendogram was constructed using RAPD data and the study resulted in low homogeneity between medicinal and garden plants.

Kumar et al. (2010) studied genetic similarity among *in vitro* regenerated plants of *C. borivillianum* using molecular markers. The banding pattern of regenerated plants when compared with mother plants was found to be identical from various primers tested for amplification. Thus, RAPD & ISSR markers possess great potential for true to type identification of *Chlorophytum* species.

OBJECTIVES

The key objectives of the study are:

(a) Optimization of micropropagation protocol of *Chlorophytum borivilianum*

(b) Development of shoot organogenesis protocol using leaf explants

(c) To test the genetic uniformity of regenerated plants using RAPD & ISSR markers

MATERIAL AND METHODS

Chemical and Glassware

All chemicals (AR grade) were purchased from HiMedia laboratories (Mumbai). The experiments were performed in 300 ml culture bottles (Kasablanka Corporation, Mumbai). Glassware like measuring cylinders, beakers etc. were purchased from Borosil Glass Works Ltd., Mumbai, India.

Plant Material

The stem discs of *Chlorophytum borivilianum* Sant. *et* Fernand at TIFAC- CORE of this institute were used as explant source for the establishment of *in vitro* cultures. Explants were rinsed under running tap water for 30 min to remove adherent particles. The explants were treated with neutral detergent (Tween 20 and savlon solution for 5 min) followed by antifungal agent 0.1% (w/v) for 15 min. Under sterile conditions, the explants were rinsed with autoclaved water twice followed by treatment with 0.1% (w/v) mercuric chloride. Again the explants were washed with autoclaved distilled water for 4-5 times. These were then dried on autoclaved blotting paper and inoculated on MS medium after the removal of small portion from the ends. After 1 month, shoot multiplication was observed on MS media supplemented with increasing concentration of BAP.

Medium Preparation

Mineral nutrients and water are important for the growth of plant. Sucrose is a rich carbon source. Plant growth regulators act as an organic constituent. A gelling agent, agar is added to make the culture media semi-solid. For MS medium (MS, 1962 Detailed in Annexure 1) preparation, the required quantities of concentrated stocks of different constituents such as macronutrients, micronutrients and vitamins were added and solution was mixed properly. After mixing well final volume was raised using distilled water, pH of media was titrated to 5.8 using 0.1N NaOH and 0.1N HCl. Thereafter 0.8% (w/v) agar was added to each culture vessel. The medium was poured into these vessels (50 ml each). The mouth of culture vessels was closed with plastic caps and was labelled. Then the vessels containing media was autoclaved at temperature 121 °C (250 °F) at 15 psi (100 kPa) for 15-20 minutes. Depending on the sensitivity of PGRs to high temperature, PGRs were added before/after autoclaving the media to prevent their activity. After this the media was allowed to cool and stored in dust free environment inside the culture room.

Culture conditions

The plantlets after inoculation on fresh media were maintained under the culture lab conditions i.e.; 25±2°C Temperature, 70% Relative Humidity, 16/8 of Light/Dark period and 42 μmol m^{-2} s^{-1} Light intensity.

Effect of BAP on shoot multiplication

The effect of MS medium fortified with various concentrations of BAP (0, 1, 2.5, 5.0 μM) was examined on the multiplication of shoots. Four shoot clumps of equal sizes were cultured per bottle. Number and length of shoots (in cm) per inoculum was recorded after 3 weeks.

Effect of Sucrose Concentration on shoot multiplication

The effect of MS medium supplemented with different concentrations of sucrose (0, 2, 4, 6, 8, 10 % w/v) was also examined. Four shoot clumps of same sizes were cultured in each bottle. Number of shoots & roots induced per inoculum and along with the length of shoot & roots were recorded after three weeks.

Root Induction

For root induction in *C. borivilianum, in vitro* microshoots were used as source of explants. Rooting media was tested on IBA (0, 1, 2.5, 5.0 μM) enriched MS basal medium. Data was scored after 21 days of culture.

Shoot organogenesis

The leaf explants excised from microshoots were cut into small segments (approx. 1cm long) and injured with surgical blade then were used for the induction of shoot organogenesis. 8 small leaf segments were cultured per flask and 3 replicates for each combination were performed. MS medium supplemented with different PGRs was used. The combinations performed were:

- IBA(0-5.0 μM)
- 2,4-D (1-12.5μM) and BAP (0-12.5 μM)
- 2,4-D (1-12.5μM) and TDZ (0-2.5μM)

Clonal fidelity of regenerated plants

Regenerated plants for any somaclonal variations were tested by PCR based molecular markers RAPD (Random amplification of polymorphic DNA) and ISSR (Inter simple sequence repeat). The microshoots regenerated were multiplied and considered as lines for testing clonal fidelity. DNA was isolated from the regenerated shoots by CTAB method proposed by (Doyle and Doyle 1990).

Quantification and Qualitative estimation of DNA

The quality of the isolated genomic DNA was evaluated on agarose gel (0.8 % w/v) containing ethidium bromide. The samples were quantified using Nanodrop 1000 Spectrophotometer (Thermo Scientific). 1 µl of each sample was used for the measurement against TE buffer used as blank and after blanking the nanodrop with 1 µl of distilled water.

PCR amplification using RAPD & ISSR makers

Amplification of DNA sample of *C.borivilianum* was carried out using PCR - Veriti 96 well Thermocycler (Applied Biosystems). The selected primers were used for amplification each of RAPD and ISSR using the standardized amplification protocol (Kumar et al. 2010) given below:-

	Steps	Temperature (°C)	Period	Cycles
I	Initial denaturation	94	4 min	
II	Denaturation	94	1 min	
III	Annealing	36-RAPD 55-ISSR	45 sec	41
IV	Extension	72	1.30 min	
V	Final Extension	72	5 min	
VI	Store	4	∞	

Amplified fragments were separated on 0.8% (w/v) agarose gel and visualized under UV trans-illuminator.

Stastical analysis

All experiments were conducted in triplicates. The calculations for mean, standard deviation & standard error were performed in MS-Excel (2013). Further, data was analysed with the help of Least Square difference test using the software *Graphpad Prsim 5.1*.

RESULTS & DISCUSSION

In vitro propagation technique offer great potential for the conservation of endangered herbs like *Chlorophytum borivilianum* (Sant. *et* Fernand). Thus, aseptic cultures of *C borivilianum* were established on MS medium with an efficiency of 80% and further multiplied on basal medium containing 2.5 µM BAP and subcultured at an interval of 21 days before carrying out the experimentation work. The *in vitro* studies on Safed musli conducted by Dave et al. 2003; Panchal & lngle 2011 reported the use of various explants for *in vitro* culture establishment. Though the use of vegetative tissue like stem disc is important for the micropropgation (George et al. 2008) as it lacks variations which are likely to occur if seeds were utilized as explants (Skirvin et al. 1994).

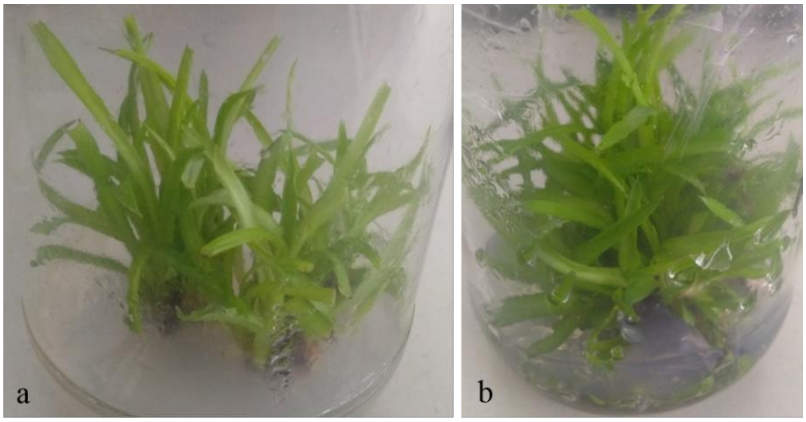

Figure 2: 21 day old cultures of *Chlorophytum borivilianum* growing on MS medium containing 2.5 µM BAP

Effect of BAP on shoot multiplication

Literature indicated that cytokinins such as BAP promoted shoot multiplication (Mazri 2015; Cronauer & Krikorian 1984). In the present work, the various concentration of BAP (0-5 µM) were tested. The increase in the concentration of BAP in MS medium result in a significant decrease in shoot length after 3 weeks (Figure 2). The higher concentration of BAP results in shoot multiplication whereas lower concentration promoted shoot elongation. Further, present study revealed that the shoot number per inoculum increased with increase in BAP concentration (Table2)

Table 2: The effect of BAP concentration in MS basal medium on growth and multiplications of *C. borivilianum* microshoots.

Concentration of BAP (µM)	Average Shoot length (cm) Mean±SE	Number of Shoots per inoculum Mean±SE
0	3.4±0.11[a]	3.8±0.28[c]
1	2.64±0.08[b]	4.69±0.66[c]
2.5	1.49±0.15[c]	6.88±0.46[b]
5	1.38±0.13[d]	10.75±0.27[a]

*Values having distinct alphabets within the column are significantly different at P<0.05

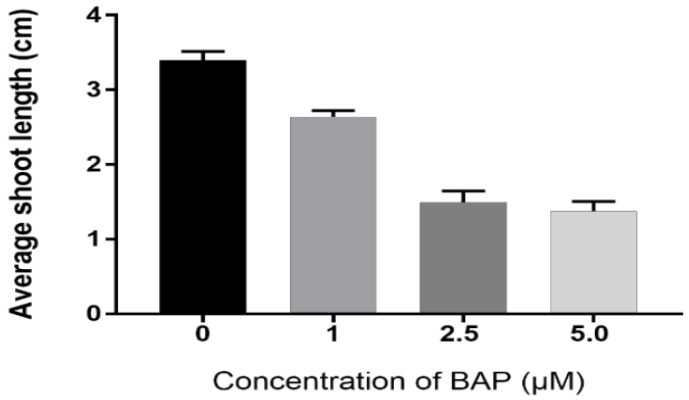

Figure 3: The effect of BAP concentration on Shoot length (cm) of *C. borivilianum*

Various growth aspects such as shoot multiplication, shoot length, root length of *C. borivillanum* were affected by the amount and type of PGRs used in (Shrivastav & Banerjee 2008; Jahan & Mohammad 2009). Since the utility of BAP for shoot multiplication had been established in various plant species (Mathur et al. 2008; Thakur et al. 2009). Cytokinins such as BAP, Kinetin are involved in reducing apical dominance and the induction of both axillary and adventitious shoots from various explants in *C. borivillanum* (Kumar et al. 2010). Thereafter, efforts were made to determine the optimal concentration for shoot multiplication in *C. borivillanum*. The highest shoot number per explant was achieved on MS medium fortified with 5.0 µM BAP.

Effect of sucrose on shoot multiplication and rooting

Sucrose is widely used carbohydrate source on growth optimization and proliferation in MS medium (Samoylov et al. 1998; Haddadi et al. 2010; Cuenca et al. 2000). Sucrose influences shoot multiplication, growth and rooting of microshoots (Sharma et al. 1992; Pabna 2003; Hazarika et al. 2003). In this study the effect of Different concentrations of sucrose 2-10% (w/v) in the basal medium was studied on shoot multiplication. The medium without sucrose served as control. The well sprouted shoots were observed in the presence of different concentration of sucrose but medium devoid of sugar results in the browning of shoots although they achieved growth and proliferation. Highest percentage of explants responded to shoot proliferation along with maximal growth and rooting response was observed at 2% (w/v) level of sucrose. The decline of growth was observed at high concentration of sucrose due to osmotic stress (Table 3). As per the growth profile of shoot cultures, 2% (w/v) sucrose concentration was optimized for further studies (Figure 4).

Table3: Effect of different concentrations of sucrose in MS medium on growth and proliferation of microshoots and roots of microshoots of *C. borivilianum.* Values having distinct letters within the column are significantly different at P<0.05

Sucrose Concentration (%w/v)	Length of shoots (cm) Mean±SE	Shoot number per clump Mean±SE
0	$2.66\pm.0.11^{c}$	3.10 ± 0.16^{e}
2	4.38 ± 0.25^{a}	3.8 ± 0.19^{de}
4	3.33 ± 0.18^{b}	4.3 ± 0.14^{cd}
6	2.46 ± 0.09^{d}	4.9 ± 0.2^{c}
8	1.95 ± 0.14^{cd}	5.1 ± 0.4^{b}
10	1.12 ± 0.12^{e}	5.4 ± 0.08^{a}

Sucrose Concentration (%w/v)	Length of roots (cm) Mean±SE	Number of roots per shoot Mean±SE
0	2.95 ± 0.09^{d}	2.3 ± 0.17^{e}
2	5.2 ± 0.21^{a}	3.2 ± 0.16^{de}
4	4.35 ± 0.205^{b}	3.8 ± 0.13^{cd}
6	3.07 ± 0.17^{c}	4.2 ± 0.17^{c}
8	2.15 ± 0.34^{cd}	4.74 ± 0.07^{b}
10	0.9 ± 0.07^{e}	4.9 ± 0.11^{a}

Apart from other nutritional requirements, carbohydrates are considered as important carbon and energy sources influencing the physiology, growth and differentiation of cells (Kaur & Debnath 2015; Sharma et al. 2017). The incorporation of carbohydrates to plant tissue culture medium is essential as *in vitro*-grown tissues generally have low photosynthetic activities (Farshad et al. 2013; Schenk et al. 1972). They also act as osmotic agents in the culture medium (Mathur et al. 2008; Rout et al. 2000). Therefore, amount and type of carbohydrate source needs to be optimized. In the present study, sucrose at 2% (w/v) was foremost carbohydrate source for shoot and root multiplication. And furthermore our results are predictable with earlier reports where sucrose had been proved to be a superior carbon source than other sugars involved in the multiplication of shoots and roots (Lane 1978; Kavyashree. 2009).

Effect of IBA on root induction

The microshoots when transferred to MS medium supplemented with various concentrations of IBA resulted in root induction after 3-4 weeks. The best rooting response was achieved in higher concentration of IBA (5 µM) after 20–25 days of culturing (Table 3). Number and length of roots per shoot increased significantly with increasing concentrations of IBA (Figure 3).

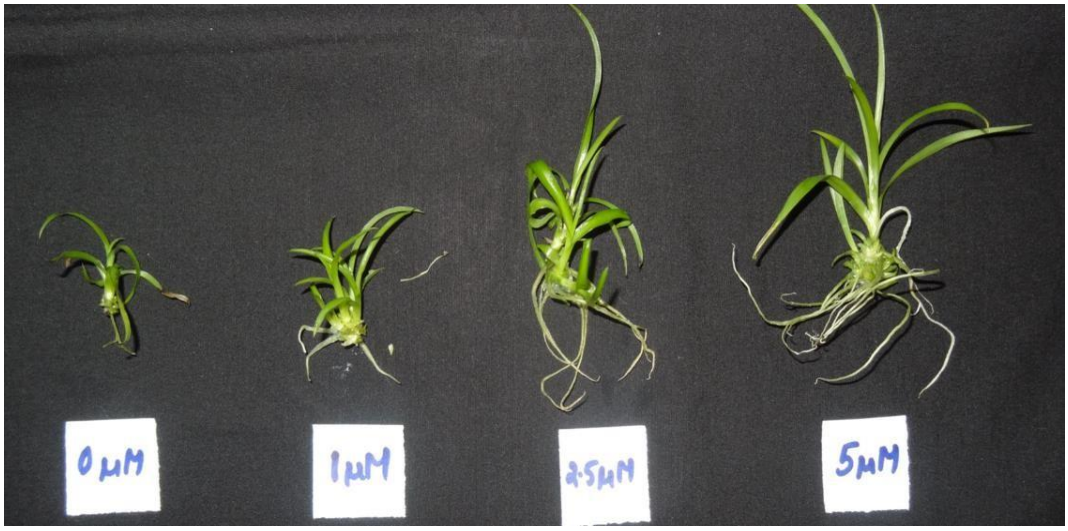

Figure 4: The effect of different concentrations of IBA enriched MS medium on the induction of roots in *C. borivilianum*

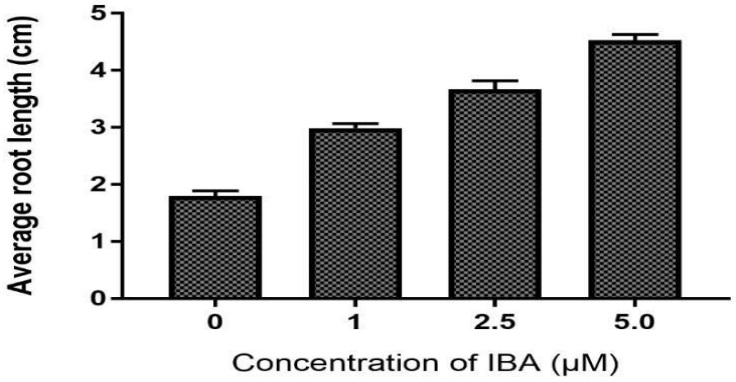

Figure 5: The effect of IBA concentrations on the length of roots (cm) of microshoots of *C.borivilianum*

Table 4: Rooting response of *C. borivilianum* microshoots on IBA enriched MS media. Values are represented as Mean±SE. Values sharing a distinct letter within the column are significantly different at P<0.05

Concentration of IBA (µM)	% of shoots rooted	Average Root length (cm)	No. of roots per Shoot
0	50 ± 5.77^{b}	1.8 ± 0.09^{c}	2.3 ± 0.16^{d}
1	80 ± 8.12^{b}	2.99 ± 0.08^{bc}	4.89 ± 0.08^{b}
2.5	95 ± 5^{ab}	3.67 ± 0.15^{b}	6.4 ± 0.18^{c}
5	100^{a}	4.53 ± 0.10^{a}	7.2 ± 0.13^{a}

In many plant species, root formation is considered as crucial step and is affected by various physiological, biochemical and genetic factors (Pawlicki and Welandar 1995). The addition of auxin either alone or in combination resulted in root induction of many plant species (Sharma et al. 2006; Debnath et al. 2006). Thus, IBA plays a stimulatory role in root formation and most commonly used auxin for root initiation (Rout et al. 2006). In our study, the rooting capacities of *C. borivilianum* vary depending on the concentration of IBA and the roots started emerging after two weeks of culturing. Maximum root formation along with roots with maximum length in *C. borivilianum* was observed from the shoot bases as explants when cultured on MS medium supplemented with 5.0 µM IBA.

Shoot organogenesis

Leaf bases of *C. borivilianum* were cultured on to study the effect of BAP (0-12.5 µM), TDZ (0-2.5 µM) in combination with 2, 4-D (1-12.5 µM) and the observations were recorded after 28 days. In the present study, the presence of (BAP) alone the medium did not promoted shoot organogenesis. However, increasing the concentration of TDZ in the medium resulted in the formation of nodular compact callus. When BAP in combination with 2, 4- D was used, it resulted in the formation of red-green colored compact callus. The highest frequency of callus was achieved on medium fortified with BAP (12.5 µM) in combination with increasing concentration of 2, 4-D (2.5-12.5 µM). However, it was interesting to note that direct shoot bud initiation was observed from leaf segments after six weeks of culture on medium supplemented with 5.0 µM BAP and 2.5 µM 2, 4-D. In contrast, TDZ alone did not promoted shoot organogenesis. Further, TDZ in combination with 2,4-D resulted in compact callus formation whereas the morphology of callus changes from green to white- green colour with increasing concentration of TDZ in combination with 12.5 µM 2, 4-D. However, in most of the combinations tested, 100% explants responded positively to callus formation with minor morphological variations. Moreover, direct shoot organogenesis was achieved on MS medium fortified with 5.0 µM IBA within 21 days of inoculation (Figure 7e2f).

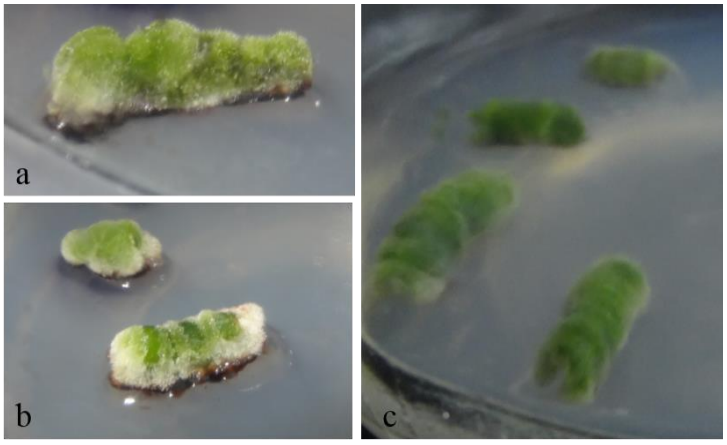

Figure 6: Callus Induction from the leaf segments of *C. borivilianum* on MS media supplemented with **(a)** 1 µM TDZ and 12.5 µM 2, 4 –D; **(b)** 2.5 µM TDZ and 12.5 µM 2,4 –D; **(c)** 12.5 µM BAP and 5.0 µM 2,4-D

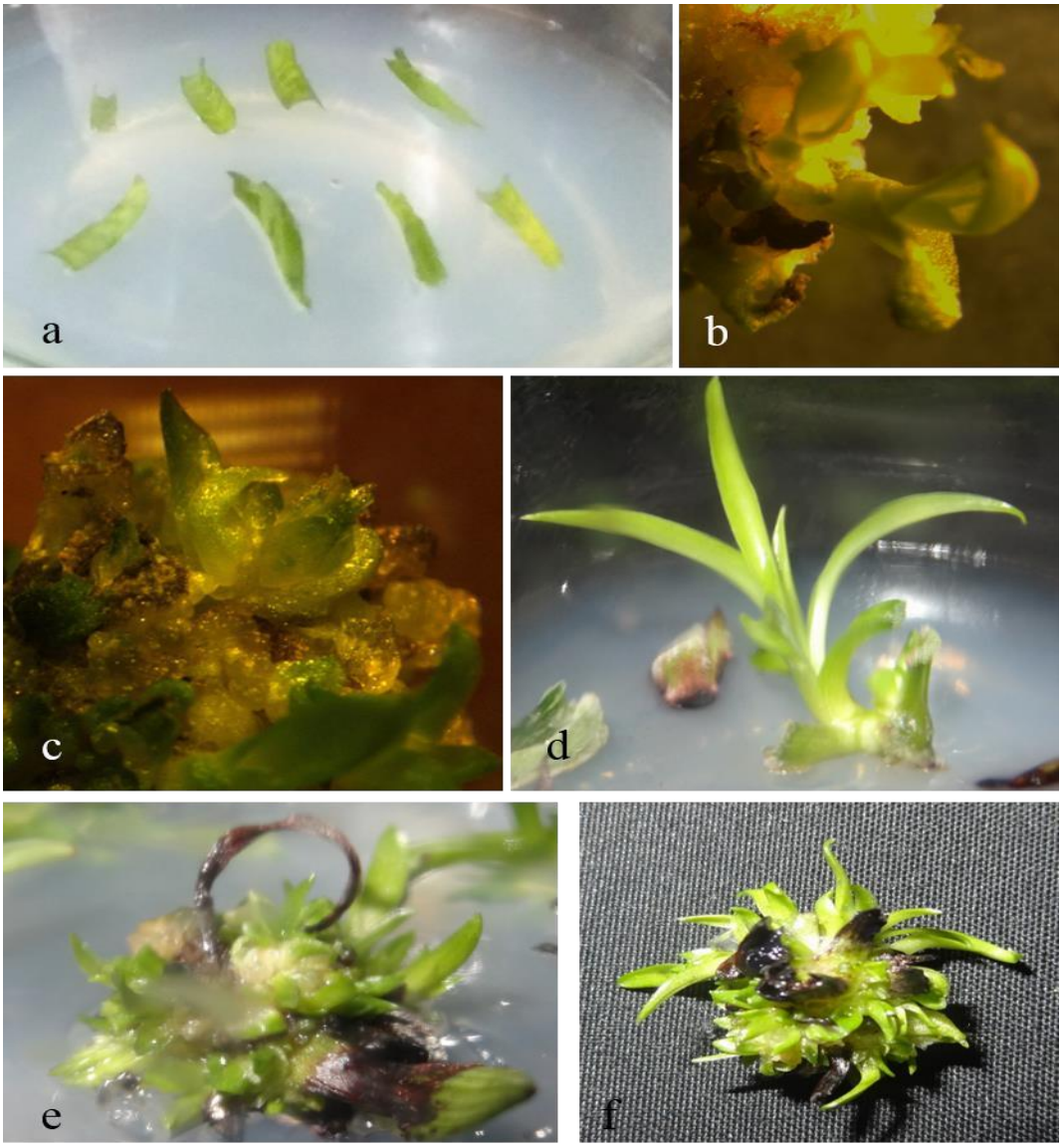

Figure 7: Shoot organogenesis in *C. borivilianum* **(a)** Leaf segments taken from microshoots cultured on MS medium supplemented with BAP & 2, 4-D; **(b-d)** Shoot bud initiation on MS medium supplemented with BAP (5.0 µM) & 2,4-D (2.5 µM); **(e-f)** Shoot organogenesis achieved on IBA (5.0 µM) enriched MS medium

Table 5: Effect of BAP and 2,4-D on shoot organogenesis from the leaf bases of *C.borivilianum*

S.no	BAP (µM)	2,4-D (µM)	% shoot callus induction	% Shoot organogenesis	Type of Response
1	1	0	0	0	NR
2	1	1	0	0	NR
3	1	2.5	25	0	Callus
4	1	5.0	0	0	NR
5	1	12.5	0	0	NR
6	2.5	0	0	0	NR
7	2.5	1	0	0	NR
8	2.5	2.5	33.3	0	Callus
9	2.5	5.0	0	0	NR
10	2.5	12.5	54.6	0	Red green callus
11	5.0	0	0	0	NR
12	5.0	1	0	0	NR
13	5.0	2.5	0	100	Regeneration
14	5.0	5.0	0	0	NR
15	5.0	12.5	100	0	Green colour
16	12.5	0	0	0	NR
17	12.5	1	4.1	0	Green colour
18	12.5	2.5	100	0	Green
19	12.5	5.0	100	0	Dark green
20	12.5	12.5	100	0	Red green

*NR= No response

Table 6: Effect of Thidiazuron and 2,4-D on shoot organogenesis from the leaf bases of *C.borivilianum*

S.no	TDZ (µM)	2,4-D (µM)	% callus induction from explants	Callus Morphology
1	0	0	0	NR
2	0	1	0	NR
3	0	2.5	0	NR
4	0	5.0	0	NR
5	0	12.5	29.1	Green
6	0.1	0	0	NR
7	0.1	1	50	Light green

8	0.1	2.5	54.1	Green
9	0.1	5.0	66.6	Green
10	0.1	12.5	87.5	Green
11	1	0	0	NR
12	1	1	95.8	Green
13	1	2.5	100	Green
14	1	5.0	100	Green
15	1	12.5	100	Green
16	2.5	0	0	NR
17	2.5	1	100	Green
18	2.5	2.5	100	Green
19	2.5	5.0	100	White
20	2.5	12.5	100	White green

*****NR= No response

Table 7: Effect of IBA on shoot organogenesis from the leaf bases of *C.borivilianum*

Concentration of IBA (µM)	**% Shoot organogenes Mean±SE**	**No. of shoots per explant Mean±SE**
0	0	0
1.0	27.6^{c}	2.3 ± 0.11^{c}
2.5	53.4^{b}	4.7 ± 0.22^{b}
5.0	68.8^{a}	12.4 ± 0.18^{a}

The type and amount of PGR present in the medium was considered as significantly for callus formation, organogenesis and regeneration from the leaf explants etc. (Arora et al. 1999; Zang et al. 2004). Different species respond differentially to PGRs in terms of both concentration and combination with other PGR (Kaur et al. 2013). Therefore, it is critical to find the optimal concentration of PGR in the medium influencing shoot organogenesis and/or somatic organogenesis. Thus, the present study resulted in direct shoot organogenesis from leaf bases on MS medium fortified with 5.0 µM BAP and 2.5 µM 2, 4-D. Furthermore, TDZ in combination with 2, 4- D resulted in the generation of compact green coloured callus where 100% explants responded.

Clonal Fidelity of Regenerated Plants

The regenerated shoots from leaf bases of *C. borivilianum* after 3-4 repeated subculturing on MS medium supplemented with 2.5 µM BAP were checked for somaclonal variations using RAPD & ISSR markers. The various molecular markers tested for amplification revealed genetic uniformity of regenerated plants with their mother plants. All the bands were found to be monomorphic indicating true to type nature of regenerated plants.

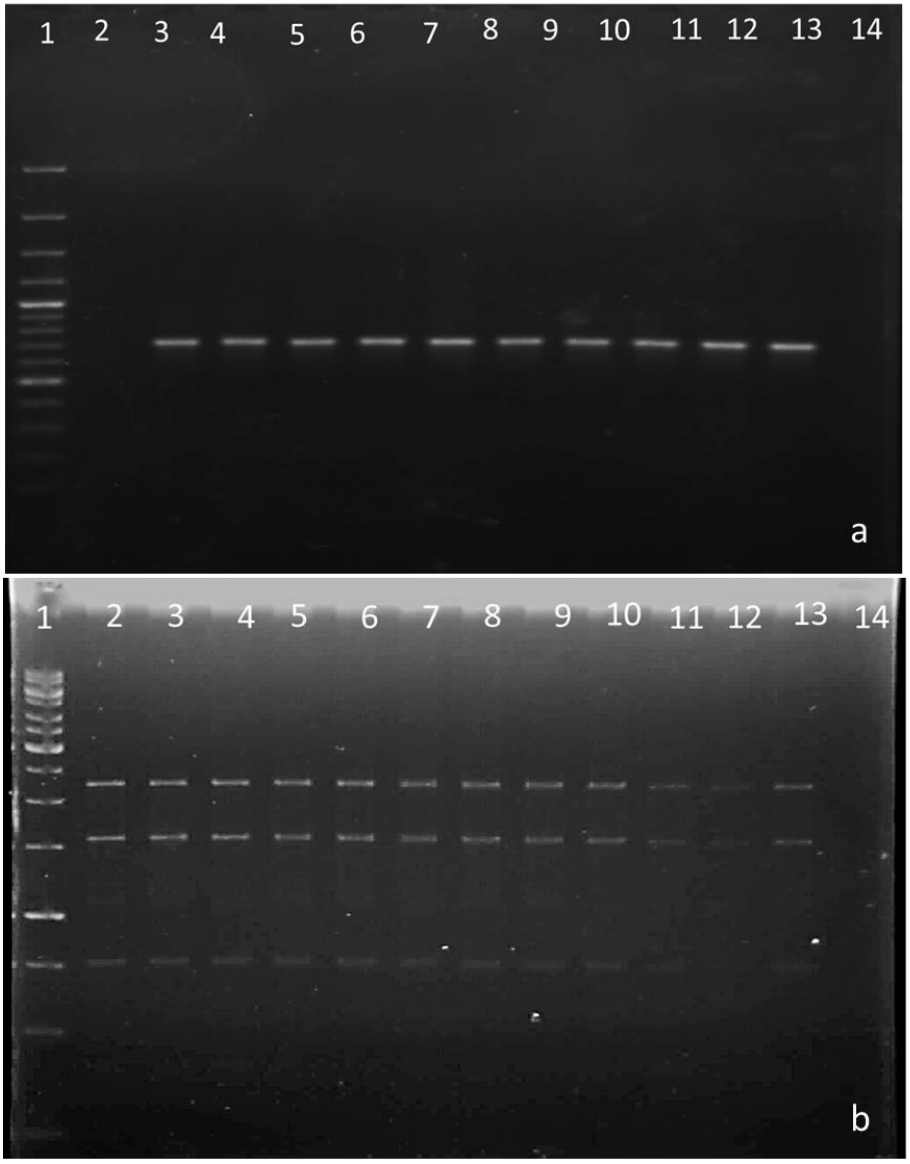

Figure 8: Amplification of RAPD markers (a-RAPD-12; b-RAPD-7) of regenerated plants from the leaf bases of *C. borivilianum*. (a) Lane 1- 1 Kb ladder, Lane 2- Negative control; Lane 3-14- regenerated plants ; (b) Lane 1- 1 Kb ladder, Lane 2-13- regenerated plants, Lane 14- negative control

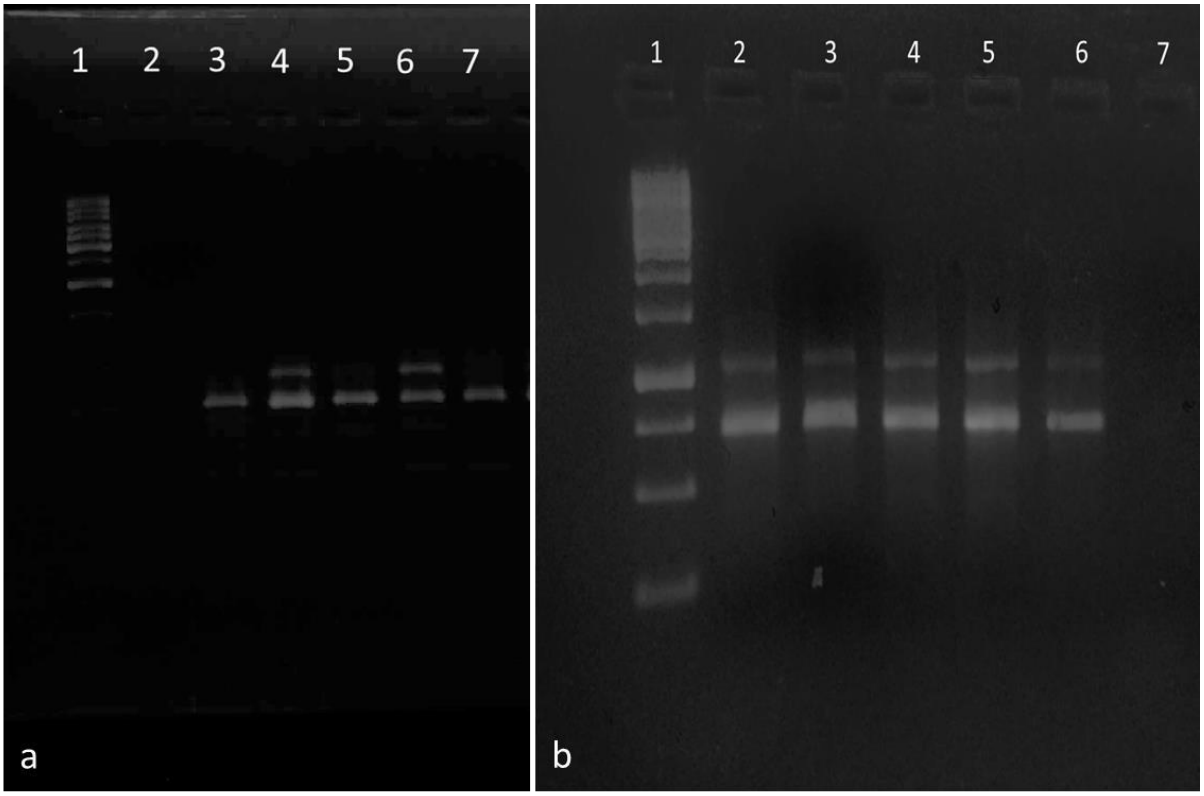

Figure 9: Amplification of ISSR markers (a-ISSR-1; b-ISSR-2) of regenerated plants from the leaf bases of *C. borivilianum*. (a) Lane 1- 1 Kb ladder, Lane 2- Negative control; Lane 3-7- regenerated plants; (b) Lane 1- 1 Kb ladder, Lane 2-6- regenerated plants, Lane 7- negative control

Since both shoot organogenesis and somatic embryogenesis develop from callus phase, testing the clonal fidelity of regenerated tissue is mandatory. Therefore, the use of various molecular markers such as RAPD & ISSR markers for molecular identification of micropropagated plants had been well studied in *C. borivilianum* (Latto et al. 2006; Katoch et al. 2010; Kumar et al. 2010; Debnath et al. 2011).

CONCLUSION

In present study, attempts were made to optimize the micropropagation and regeneration protocol in *Chlorophytum borivilianum.* The effect of various plant growth regulators (PGR's) was observed on shoot multiplication & rooting, callus induction and shoot organogenesis.

The results obtained are summarized as follows:

- Higher shoot number per clump was accomplished on MS medium fortified with 5.0 µM BAP.
- The regeneration of shoots from leaf segments were observed on MS medium supplemented with (a) 5.0 µM BAP and 2.5 µM 2, 4-D; (b) 5.0 µM IBA.
- MS medium supplemented with IBA resulted in 100% root induction.
- The study managed to find the optimal concentration for callus induction among different concentrations of TDZ and 2, 4-D tested on MS medium.
- Regenerated plantlets were tested for clonal uniformity and all the bands were found to be monomorphic thus, indicates the clonal uniformity of regenerated plants.

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Annexure 1

Media preparation

Composition of Murashige and Skoog medium (1962)

MACRO SALTS

MACRO SALT	(mg/l)
NH_4NO_3	1650
KNO_3	1900
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170

MINOR SALTS

MINOR SALTS	(mg/l)
$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_20$	0.025
Na_2Fe-EDTA	30

VITAMINS

VITAMINS	(mg/l)
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo – inositol	100
Sucrose	30000

ANNEXURE II

Stock preparation

Composition of stock preparation:-

STOCKS	mM
BAP	2.5
2,4-D	2.5
TDZ	1
IBA	2.5

Composition of CTAB buffer

2% CTAB	20 gm/l CTAB
20mM	40 ml EDTA stock (0.5M)
10mM TRIS-Hcl	100 ml TRIS-Hcl stock (1M)
1.4M NaCl	20 ml NaCl stock (5M/l)