In vitro propagation of *Chlorophytum borivilianum*

DISSERTATION

By

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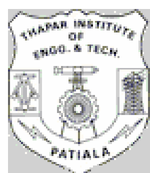
Submitted in partial fulfillment of the requirement
for the award of the degree of
Masters of Science in Biotechnology



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// ABSTRACT

Chlorophytum borivilianum (Safed musli) is an important medicinal plant and used world wide in drug industry. Although *Chlorophytum* propagates through tubers in its natural state, but propagation rate is too slow to meet demand of high quality planting material for commercial cultivation. Micropropagation protocol from selected, elite plants of *Safed musli* using nodal segment as explant was standardized. Shoot cultures were initiated on MS medium (1962, MS) containing BA (2.5 μM) and NAA (0.5 μM). Shoot proliferation was achieved on medium containing BA (5 μM) within 21 days of culture. Heat shock (50 °C for 1 h after 7 days of culturing) enhanced shoot proliferation at high sucrose concentration (232 and 290 mM). Heat shock also affected shoot length significantly in medium with 174 mM sucrose. 'Compound A' (a bioactive compound) was found to have beneficial effect on shoot proliferation at lower sucrose concentrations. Effect of two gelling agent (agar and phytagel) was compared in the present study. It was found that the phytagel has beneficial effect on the shoot proliferation and growth. However, agar was found to be beneficial for rooting of shoots. 95% rooting of microshoots was obtained on MS medium containing 290 mM sucrose and supplemented with IBA (5 μM). Regenerated plants after hardening were transferred to soil and they showed 24% survival in polyhouse while 90% survival was observed when directly transferred to shade house, thereby reducing the cost of acclimatization of plants. FYM and vermicompost were found to have beneficial effect on acclimatization of *in vitro* produced plants. The regenerated plants were morphologically similar to control plants and preliminary study indicate that they are also genetically identical.

CANDIDATE'S DECLARATION

I hereby declare that the work presented in the dissertation entitled *'In vitro* propagation of *Chlorophytum borivilianum'* 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 of five months from January 2006 to May 2006,under the supervision of Dr. Anil kumar, Research Scientist, **TIFAC-CORE(Center of Relevance and Excellence) in Agro and Industrial Biotechnology,** Thapar Institute of Engineering and Technology (TIET), Patiala. I have not submitted embodied in this dissertation for the award of any other degree or diploma.

Place: Patiala
Date: Preeti Gupta

CERTIFICATE

This is to certify that the thesis entitled '***In vitro* propagation of *Chlorophytum borivilianum*'** submitted by Ms Preeti Gupta 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 my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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(Preeti gupta)

ABBREVIATIONS

%	- Percent
°C	- Degree centigrade
μg	- Microgram
μl	- Microlitre
μM	- Micromolar
AFLP	- Amplified fragment length polymorphism
BA	- N^6-Benzyladenine
cm	- centimeter
DNA	- Deoxyribonucleic acid
EDTA	- Ethylenediaminetetraacetic acid
g	- Gram
h	- hour
HCl	- Hydrochloric acid
IBA	- Indole-3-butyric acid
IAA	- Indole-3-acetic acid
iSSR	- inter Simple Sequence repeat
KOH	- Pottasium hydroxide
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	- Rapid amplification of polymorhic DNA
SE	- Standard error
UV	- Ultraviolet
V	- Volt
v/v	- Volume by volume
w/v	- Weight by volume
X g	- Multiplied by force of gravity

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INTRODUCTION

Indian herbal industry is at a blooming stage now-a-days. There is an increasing awareness towards consumption of herbal medicines. The world health organization has estimated that more than 80% of the world population in developing countries depends primarily on herbal medicines for basic healthcare needs (Canter *et al.,* 2005). Large number of plants have medicinal properties like Aloe, Jatropa, Satavari, *Piper methystium*, Ginkgo etc. One of such important medicinal plant is *Chlorophytum borivilianum* Sant. et Fernand.

Chlorophytum borivilianum, commonly known as Safed musli (Fig. 1) is a traditional medicinal plant which belongs to family liliaceae. The genus includes about 300 species, which are distributed throughout the tropical and subtropical parts of the world. Tropical and subtropical Africa are the probable centre of origin of the genus, where about 85% of the species are found. In India *C. borivilianum* is mainly distributed in Southern Rajasthan, North Gujrat and Western Madhya Pradesh (Maiti and Geetha, 2005).

Thirteen species of *Chlorophytum* have been reported from India (Shariff and Chennaveeraiah, 1972). All these species differ in appearance, native species are sold as 'Safed musli' in the Indian drug market. Amongst these, *Chlorophytum borivilianum* produces the highest yield and highest saponin content (Shariff and Chennaveeraiah, 1972). Other important indigenous species are : *C. arundinaceum, C. tuberosum, C. laxum,* and *C. breviscapum* .

Chlorophytum borivilianum is a small perennial herb with a full crown of radical leaves appearing over the ground with the advent of summer rain. Its root tubers are fleshy, fascicled and directly originates from the stem disc devoid of any fibrous structure. They are cylindrical and 5 -20 in number. It has 6 -13 radical leaves spirally imbricate at the base, sessile in nature, linear or ovate with acute apex and slightly narrowed at the base. The leaves spread horizontally, with smooth surfaces, wavy margins and parallel venation.

Flowers of *Chlorophytum* are small, white, bracteate, pedicillate, zygomorphic, usually arranged in alternate clusters, each cluster comprising of 3 flowers. The

flower clusters are dense on the upper part of the scape; bracts are linear, papery and purplish, 1.0 -10.5 cms long; pedicle whitish and 6 -10 mm long. It bears green to yellow coloured fruit which is almost equal in length and breadth. Seeds are endospermic, onion-like, black coloured and angular in shape.

Major biochemical constituents of Safed musli are Carbohydrates 42%, Protein 80 - 90%, Fibres 3 - 4%, saponins 2 -17% and Alkaloids 15 - 25%. (Bordia *et al.,* 1995). Primarily saponins and alkaloids impart medicinal value.

Chlorophytum borivilianum has therapeutic application in Ayurvedic system of medicine (Kirtikar and Basu, 1975). Generally, it is considered very good to increase General Body Immunity. Its aphrodiasiac properties has proved very much useful for the people suffering from Erectile Dysfunction & and to increase male potency. It has spermatogenic property and helpful in curing impotency as they are rich in glycosides.

Safed musli is considered as a curative of Natal and Post Natal Problems and a cure for Diabetes and Arthritis. Its root powder is fried in the ghee and chewed in case of apthae of mouth and throat. It is effective in curing Rheumetism and Joint Pains. It is an essential part of a Traditional Diet of Mothers (after delivery) in the form of "**Laddoos**". Efforts in countries like U.S.A and England are also on to convert it into chips/flakes to use it as a nutritious breakfast. Gujarat State Forest Development Corporation launched a potency drug by name **NAI CHETNA** (The Indian Express, 1st December 1999) that has been enjoying widespread publicity with increasing acceptance as an alternative to “**Viagra**”.

Safed musli is traditionally used for lack of libido male impotency, oligospermia. It is also widely used as a general health promotive tonic and for delaying the ageing process. Varying its common use for health promotion, it is also used for increasing lactation, treating various gynecological disorders, arthritic conditions and to control diabetes melitus. As such safed musli has no adverse effect if taken in a proper dose while hyper dosing may lead to gastrointestinal disorders.

Fig. 1. Plant of Safed musli growing in the field

Fig. 2. Dried roots of Safed musli

It grows naturally in most parts of Central India where climatic conditions are suitable. This plant can grow well in a range of temperature and rainfall conditions. A sandy loamy soil with adequate drainage is ideal for its production. Normal pH range, higher dose of super phosphate, decomposed farmyard manure and good drainage system facilitates better tuber growth. It is usually found in soils rich in organic matter and requires bright sunlight (Oudhia *et al.,* 2001).

Cultivation of Safed musli usually begins with land preparation in the months of April/May. After the field is ploughed, available farmyard manure or any form of compost is applied and mixed well with the soil. The soil can also be enriched by growing a green manure crop such as sunhemp and incorporating it at the flowering stage. If the soil is too dry, the field can be irrigated to ensure easy decomposition of green manure.

Safed musli is sown with the onset of the monsoon. Fingers are separated from the bunch of tubers with the crown and disc kept intact. They are planted at a distance of about 25 cm within the row. The planting density is about 80,000 fingers per ha, weighing approximately 400-500 kg. Before planting, the fingers can be treated with 50 g of Bavastin, that is mixed with 15 litres of water to prevent fungal attack (Kothari and singh, 2003; Maiti and Geetha, 2005).

Leaves turn yellow and fall off after 3.0 to 3.5 months, but they should be left in the field for some more time for ripening which increases their medicinal properties. During this period, the soil moisture status must be maintained. The skin of the tubers turns dark brown by January-February when they are ready for harvest. Mature tubers should be dug out at this stage while the smaller ones are left untouched, to be used as planting material for the next season.

Long, healthy fingers that detach naturally from the tubers are processed by peeling off the skin of the fingers with a stainless steel knife and sun drying for 3 - 4 days (Fig. 2). They are then packaged in polythene bags and transported for marketing.

Although Indian forest are rich in Safed musli demand is increasing rapidly in Indian and international drug market. Foreign demand has been estimated as 300-700 tonnes annually (Bordia *et al.,* 1995), a quantity that Indian forest cannot sustain. Moreover obnoxious weeds like *Parthenium* and *Lantana* are taking its place (Oudhia, 1996). This has created a pressure on Indian forests and it is predicted that if steps for timely conservation are not taken, the Indian forests will lose this valuable plant (Oudhia, 2001 b).

Therefore, to avoid the pressure on the natural forests, attempts have been made to cultivate Safed musli (Kothari and Singh, 2003; Maiti and Geetha, 2005). However to undertake mass scale cultivation large quantity of quality planting material is required. The tuberous roots of Safed musli are the only propagule which can either be sold in the market for economic gains or saved for commercial cultivation year after year.
This has created a severe shortage of quality planting material for cultivation. Also the seed germination has been reported to be very poor (14-16% only) (Jat and Bordia, 1990). So to fill the gap of demand and supply, and to provide genetically uniform planting material from a known source, micropropagation is one of the most desirable option.

The technique of tissue and organ culture is being used for rapid multiplication of elite plants. In Comparison to conventional propagation, micropropagation has the advantage of mass scale propagation in limited time and space, maintenance of disease free germplasm and round the year propagation of quality planting material (Kumar *et al.,* 2000; Kumar 1996).

Attempts have been made to develop *In vitro* propagation protocol for safed musli (Purohit *et al.,* 1994; Joshi *et al.,* 2004; Dave, 2003) wherein some of the parameters have been worked out. However, in order to propagate the quality planting material from the selected elite plants, further studies are required to investigate the various factors which influence large scale multiplication and subsequent acclimatization.

Micropropagation being a clonal method has the potential for large-scale propagation of elite trees, but there is a risk of getting somaclonal variation. Therefore, a method

has to be evolved to characterize deviant phenotypes and to determine the level of clonal fidelity (true-to-type plants) among *in vitro* propagated plants. Various technologies which can be applied to test the clonal fidelity are phenotypic studies, protein based markers (protein patterns and isoenzymes) and DNA based markers. Amongst the DNA based technology such as RAPD, iSSR, AFLP etc. has the potential to find out the variants if any amongst the regenerated plants at the genetic level.

RAPD is the amplification of genomic DNA using at least one short oligonucleotide (random) primer, in low stringency conditions, results in multiple amplification products from loci distributed throughout the genome (Williams *et al.,* 1990; Welsh and McClelland, 1990). The attraction of RAPD is that there is no requirement of hybridization or of sequence information. It is considered the most easy and simple method for detecting variations in regenerated plants. This technique is based on the use of a single arbitrary primer (mostly 10 mers) in a PCR reaction to synthesize multiple copies of random genomic DNA regions.

In iSSR (Inter Simple Sequence Repeat), iSSR primers anchored at 5' or 3' are used. Micro satellite or SSR'S consist of tandemly repeated 2-7 base pair units which are distributed widely throughout the genome and the region flanking the SSR are generally conserved among genotypes of same species. PCR primers similar to the flanking regions are used to amplify the iSSR -containing DNA fragments. Variability is due to the the difference in the number of repeat units.

Keeping in view the huge need for the quality planting material, the present study was
designed on the following objectives:

- Development of micropropagation protocol for the selected elite germplasm.
- To investigate the factors influencing micropropagation.
- To work out the factors for the acclimatization and survival of *In vitro* produced plants.
- To study the clonal fidelity of *in vitro* produced plants.

REVIEW OF LITERATURE

The use of plants for treatment of various ailments dates back to over 5000 years. A great source of ancient information is contained in the 'Vedas' and more specifically 'Yajur Veda' is the main source of such information. In these 'Vedas' the medicinal importance of many plants has been mentioned (Prakash, 2001). The earliest monumental contribution on 'Ayurveda' is the 'Samhitas' of 'Charak' and 'Sushrut' (1000-700 B.C.), which included 500 plants with their therapeutic properties. Although with the invasion of Greeks, Sagthians, Huns, Mughals and Europeans, the progress of Ayurveda declined, but the plant based drugs caught the attention of the west from the early colonial days. Garcia da Orta's 05 OS Coloquius published (in Portugese) from Goa 1565 is Chronologically the first printed book on Indian plant drugs, which contained the most classical information on the valuable plants of Malabar hills.

Now-a-days world-wide shift towards herbal preparations over synthetic pharmaceuticals has realized the importance of focused research in medicinal plants (Kumar *et al.,* 2004). Indian continent is the repositories of large number of medicinal plants, and most of these are available as wild plants in the forests of hills and planes. Disturbances of the natural habitats of these plants, as a result of anthropogenic activities and invasion of the exotic species has resulted in the drastic decline in the population of these important plant species and many of these species are now listed among the rare, critically rare and endangered category.

Chlorophytum borivilianum (liliaceae), commonly known as safed musli has also been listed as endangered species (Nayar and Shastry, 1988) valued for its fasciculated storage roots. These are reported to have aphrodisiac properties and form an important ingredient of herbal tonics prescribed in the Ayurvedic systems of medicine (Kirtikar and Basu, 1975). It is also known as a curative for diabetes, arthritis and to improve the immune system.

Due to these properties, there has been indiscriminate collection of the roots from the natural habitats, which resulted in the decline in natural populations. Further, seed germination in safed musli is reported to be very poor (14-16%) (Jat and

Bordia, 1990). Therefore, it was realized that to meet the demand of this important plant species steps must be taken to undertake the systematic cultivation. Therefore, subsequently attempts were made to identify superior germplasm and to develop the cultivation practices (Kothari ans Singh, 2003; Maiti and Geetha, 2005).

For planting one hectare of safed musli large amount of roots are required (400-450 kg) which not only adds to the cost but also affects the supply of musli in the market. Furthermore, from the selected elite clones a limited number of propagules are available. In order to undertake large scale cultivation programme; micropropagation is the only option which can provide large number of plants in shorter time frame.

Attempts have been made to propagate *Chlorophytum borivilianum* (safed musli) through tissue culture (Dave *et al.,* 2004; Purohit *et al.,* 2003; Dave *et al.,* 2003; Dave *et al.,* 2002; Purohit *et al.,* 1994) where in many factors influencing *In vitro* multiplication has been studied.

In vitro clonal multiplication of Safed musli has been achieved on Murashige and Skoog, 1962 (MS) medium supplemented with 22.2 μM N_6-Benzyladenine (BA) using young shoot bases as explants (Purohit *et al.,*1994). Shoots multiplication rate of four fold at every three weeks interval was achieved. Rooting of shoots was obtained on ¾ MS medium supplemented with 9.8 μM Indole-3-butyric acid (IBA). Micropropagated plants were successfully established in pots. These authors also studied various factors influencing *In vitro* growth of shoots. Shoot proliferation in more than 95% cases was observed from young shoot buds. BA (5 mg/l) in the MS medium proved to be optimum for obtaining best response. The MS salt formulation was found to be the best among different nutrient media (MS, Gamborg's media) tested during shoot multiplication. It was also noticed that any variation in sucrose concentration from 3% (w/v) taken as control, adversely affected the growth and multiplication of shoots.

Joshi *et al.* (2003) establihed callus cultures of *Chlorophytum* using young shoot bases as explants on MS medium containing various cytokinins and auxins either individually or in combination. They obtained fluffy unorganized, loose and shiny mass of callus, at the base of shoots on MS medium containing 5.0 mg/l BA. When

callus was subcultured on fresh medium, a large number of shoots differentiated from this callus mass. Subsequently shoots were rooted on ¾ strength MS medium containing 1.0 mg/l IBA. They reported that frequency of shoot regeneration from callus cultures was little higher than shoot multiplication obtained during clonal propagation. Although the callus organogenetic pathway is not a preferred method for clonal plant propagation, this can serve as a very good system for genetic manipulation of plants which are highly desirable in medicinal plants especially for metabolic engineering.

Dave *et al.* (2004) succesfully achieved production of plants of Safed musli using encapsulated shoot buds. 4 mm long shoot buds were encapsulated in 3.0% sodium alginate matrix polymerized by 100 mM solution of hydrated calcium chloride. Subsequently plants were produced from these buds. Storage conditions, gel matrix and period in storage influenced the potential of *in vitro* regrowth of these encapsulated shoot buds. This method of micropropagation opens possibilities for storage of shoot buds during off season and facilitates transport of germplasm with ease to far away places.

To cater the growing demand for the planting material, a highly reproducible field tested and cost effective micropropagation protocol was developed by Dave *et al.* *(*2003). They showed that best shoot multiplication was achieved on agar gelled MS medium containing 22.2 μM BA and 3% sucrose. Plantlets were hardened under agroshade net conditions during monsoon months of high humidity showed better survival rate and growth compared to plantlets hardened *in vitro* and subsequently transferred to the green house for acclimatization.

Kukda *et al.* (1994), described a method for somatic embryogenesis and plantlet regeneration in Safed musli. They induced callus from immature embryos inoculated on MS medium containing 1.0 mg/l 2,4-Dichloro Phenoxy Acetic Acid (2,4-D) which was later subcultured on MS medium supplemented with 0.5 mg/l 2,4-D. They further reduced concentration of 2,4-D to 0.1 mg/l for maturation of these embryos. On plant growth regulator free (PGR-free) basal MS medium, about 20% of these embryos were successfully converted into plantlets.

MATERIAL AND METHOD

Chemical and Glassware

All routine chemicals used were purchased from HiMedia Laboratories, Mumbai, plant growth regulators (PGR's) were purchased from Sigma Chemical Co, St. Louis, USA. Unless otherwise mentioned all experiments were conducted in Jam bottles.

Plant material

Elite plants selected on the basis of higher growth and yield of tubers. These were selected from the farmers field. Segments containing vegetative buds were take from the flowering spike and used as explants.

Surface disinfection of Explant

Explants were first washed thoroughly in running tap water for 30 minutes. These were then cleaned with a solution of liquid detergent (Tween 20; Himedia Laboratories, Mumbai) for 10 minutes with vigorous shaking and again washed with running tap water to remove any traces of detergent and kept in 1% (w/v) solution of Bavistin (BASF India Limited) for one hour. A quick rinse was then given in 1% (v/v) solution of savlon (Johnson and Johnson, USA). After these treatments explants were taken inside the laminar flow hood. Here 2-3 washings were given with sterile water. Further explants were surface disinfected with freshly prepared 0.1% (w/v) aqueous solution of mercuric chloride for 5 minutes. They were then thoroughly washed for 3-4 times with sterile water to remove any traces of mercuric chloride

Culture media

Murashige and Skoog medium (MS, 1962. detail in Annexure 1) with sucrose 3% (w/v) and 0.65 % (w/v) agar was used as basal medium throughout the experiment. Growth hormones N^6-Benzyladenine (BA) and Indole-3-butyricacid (IBA) were added to the basal medium either singly or in various combinations, specially as mentioned with each experiment.

The concentrated stock solutions of all the ingredients were prepared and stored under refrigeration. Similarly stock solutions of growth hormones were also prepared. All plant growth regulators (PGR's) were dissolved in few drops of potassium hydroxide (1N) and made to desired volume (2.5 mM) with 70% ethanol and stored at 4 °C.

The medium was prepared by adding required quantities of all the ingredients in the conical flask. After adding all the ingredients in required amounts, the final volume was made up with the help of distilled water. pH of the medium was adjusted to 5.8 using 1N KOH or 1N HCl (Cyberscan 510,Eutech Instruments, Singapore). After adjusting the pH, agar (0.65 %; w/v) was added and melted by heating in autoclave. Medium (50 ml) was poured in jam botlles (unless otherwise mentioned) and capped with platic caps. It was then autoclaved (Equitron, Medica Instruments. India) at 121 ^{0}C for 20 minutes at 15 psi.

Culture conditions

All cultures were incubated under 16 h photoperiod, 2000-2500 lux light intensity (Provided by Polylux XL, GE Britain, 36W) and incubated at 25 ± 1 ^{0}C.

Inoculation of explants

After disinfection explants were trimmed from the exposed ends and cut into individual nodal explants and were inoculated on MS medium supplemented with 2.5 μM BA + 0.5 μM NAA. After vertically inoculating the explants in culture bottle the mouth of bottle is quick flamed and bottles are tightly capped and was properly sealed with kling film.

Shoot proliferation

For shoot proliferation, MS medium was supplemented with different concentrations of BA (0 – 12.5 μM). Three shoot clumps (each clump of 2 – 3 shoots) were inoculated per culture bottle with 3 replicates per treatment.

In another experiment effect of sucrose concentrations (58 – 290 mM) and heat shock (at 50 °C) was studied on shoot proliferation in MS medium supplemented with BA (5 μM). Heat shock (50 °C, 1 h) was given to the cultures after a week of inoculation in a BOD incubator. Data were recorded 2 weeks after the heat shock

treatment of culture and only shoots greater than 1.5 cm were considered for taking data.

In another experiment effect of a novel compound yet not known for any activity on plant tissue was also studied (reffered to as compound A). Different concentrations of this compound (0.0, 0.5, 1.0, 1.5, 2.5 and 5.0 mg/l) were used in both MS basal as well as in MS medium supplemented with BA (5 μM), to see their effect on shoot proliferation. This compound was dissolved in 70% ethanol and added in the medium after autoclaving (at temperature about 40 °C).

Effect of agar and phytagel (0.65% and 0.225% respectively) was also observed on shoot proliferation and rooting.

Rooting of microshoots

Newly formed shoots measuring 3-4 cm in length were excised individually from the parent explant and transferred to rooting media. Two types of rooting media were tested; one MS basal media without phytohormone and other basal MS media supplemented with IBA (5 μM). In both these media combinations, the effect of different sucrose concentrations (58 – 290 mM) and heat shock on rooting was also studied. This experiment was also conducted in 250 ml conical flask. Ten to fifteen shoots per culture flask were inoculated and 3 flasks were used for each treatment. Data were recorded after 21 days of culture.

Acclimatization

After 15 days of culture on rooting media, the plantlets were shifted to polythene bags filled with various soil mixes for hardening prior to final transfer to natural conditions. Plants with newly formed roots were carefully taken out from the culture bottles with the help of forceps and dipped in warm water (not hot) to remove any traces of solidified agar media. After removing media, plants were dipped in 1% (w/v) solution of Bavistin to prevent any fungal infection to newly developed plants. These were carefully planted in plastic pots containing different soil mixtures having vermicompost, perlite, FYM and soil in varying ratios. After planting the plants were watered and kept under polyhouse having 80% humidity for ten days. Subsequently,

plants in polyhouse were shifted to shade house with less humidity level and indirect sunlight. In shade house also plants were watered two times a day i.e. morning and evening to prevent wilting (if any).

In another experiment, plants after transfer to polybags were divided into two halves, one half of the plants were kept in the polyhouse (80% humidity) and other half of the plants were kept directly in shade house (about 40% humidity).

DNA isolation

For the DNA isolation, CTAB method was used.

Procedure

1. The young and soft leaves (~1.0 g) were pulverized to a fine powder using liquid nitrogen and put in the centrifuge tubes (2.0 ml capacity). Immediately preheated CTAB buffer was added to make slurry. The tubes were incubated at 65 ˚C for 1 h. with occasional mixing, after every 15 min.

2. Equal volume of chloroform: iso-amyl alcohol (24:1) was added to it. The solution was mixed well by inversion for 7 min and then centrifuged at 8000 rpm for 5 minutes.

3. The upper aqueous layer was recovered in a separate centrifuge tube.

4. DNA was precipitated from these samples by adding 0.66 volume of cold iso-propanol.

5. The samples were centrifuged at 10,000 X g for 15 minutes.

6. Supernatant was discarded and pellet was dissolved in 1 ml TE buffer and solution was taken in microfuge tube.

7. To the samples, 2 μl pre heated (60 °C for 5 minutes) RNAse solution (10 mg/ml stock) was added and incubated at 37 °C for one hour.

8. After 1 h, 0.5 ml each of phenol: chloroform (1:1, v/v) was added and samples were thoroughly mixed by shaking. These were then centrifuged (1000 X g, 10 min) and aqueous phase was retained.

9. To the aqueous phase 0.3 volume of 3 M sodium acetate and 0.6 volume of chilled isopropanol was added and incubated for few minutes at room temperature.

10. These were then centrifuged (10,000 X g , 8 min) and pellet was retained. Pellet was washed with 70% ethanol and air dried.

11. Pellet was dissolved in TE buffer and stored at –20 °C.

Reagents needed:

1. CTAB buffer

2% CTAB	20 gm CTAB
20 mM EDTA	40 ml EDTA stock (0.5 M)
100 mM Tris-HCl pH 8.0	100 ml Tris-HCl stock (1M)
1.4 M NaCl	280 ml NaCl stock (5 M)

   made upto 1 litre with distilled water, pH 7.5-8.0, and autoclaved

   0.2% Mercaptoethanol was added into buffer just before use.

2. Isopropanol
3. Chloroform
4. Isoamyl alcohol
5. Saturated phenol
6. Sodium acetate 3 M
7. TE buffer

20 mM EDTA	EDTA stock (0.5 M)
100 mM Tris-HCl pH 8.0	Tris-HCl stock (1M)

AGAROSE GEL ELECTROPHORESIS

Gel Loading dye (6X)

Sucrose	35% (w/v)
EDTA	50.0 mM (pH 8.0)
Bromophenol blue	0.2% (w/v)
Xylene cyanol	0.2% (w/v)

TBE Buffer (5X)

Tris base	54 g
Boric acid	28 g
EDTA	3.8 g

The pH of the buffer was set at 8.0

Submarine agarose gel electrophoresis was performed using standard procedures. 0.7 % agarose gel was made in 0.5 X TBE buffer and after melting in microwave oven gels were casted in a gel tray. The DNA samples were loaded after mixing with the gel loading dye (bromophenol blue). Electrophoresis was carried out at 65 V till the tracking dye (bromophenol blue) covered 2/3rd of the gel length. Gels were then stained with water containing ethidium bromide for 10 minutes. Finally, the DNA bands were visualized under UV light.

Checking clonal fidelity using RAPD and iSSR markers

Testing of clonal fidelity of *In vitro* raised plants was carried out by performing rapid amplified polymorphic DNA (RAPD) or Inter Simple Sequence Repeat (iSSR). PCR was carried out by using single random 10mer primer (RAPD) or single iSSR primer. The amplified segments were separated on 1.2% agarose gel and documented with gel documentation system and viewed for any variant based on banding pattern.

PROTOCOL:

1. Preparation of reaction mixture

The stocks were mixed by inversion and spin to collect solution. Reaction mixture were prepared by mixing the following components in a PCR tube :

Components	Stock concentrations	Vol/Rxn
dNTPs	2.5 mM each	2.0 µl
PCR buffer	10 x	2.0 µl
$MgCl_2$	25 mM	2.0 µl
Taq polymerase	5 µ/µl	0.3 µl
Primer	10 µM	1.0 µl
Sterile H_2O		10.7 µl
DNA	7.5 ng/µl	2.0 µl

2. PCR conditions

PCR tubes were placed in a thermal cycler and amplified using the following temperature profile :

Temperature	Time	No. of cycles
94	4 min	1
94	1 min	
37	45 sec	41
72	1.30 min	
72	5 min	1
Hold Temperature: 4 ^{o}C		

After amplification PCR tubes were removed from the thermal cycler and 4.0 µl of 6x loading dye (bromophenol blue and xylene cyanol) was added to each tube. Samples were then subjected to short spin to collect the mixture. This mixture was loaded on the 1.2% (w/v) agarose gel. Electrophoresis was carried at 60 V till the bromophenol reaches the front. Gels were then stained with ethidium bromide and viewed under UV-transilluminar and photographed. Banding pattern was then compared to find out the clonal fidelity of the plants.

Statistical analysis

All experiments were carried out in triplicates and repeated twice. Means and standard error were calculated by using statistical formulae.

RESULTS

Shoot culture Initiation

Explants (nodal segments) following surface disinfestations were inoculated on MS medium supplemented with 2.5 μM BA and 0.5 μM NAA. After three weeks about 50% of the explants showed sprouting from the nodes which later on resulted in the formation of shoot clumps after 2-3 weeks of subculturing. Following successful initiation of the culture, newly formed shoots were excised individually from the proliferated explant and further cultured on the same medium to increase the number of shoots for further work.

Shoot proliferation

Shoot cultures inoculated on MS medium supplemented with different concentrations of BA, showed increase in the multiplication rate with increasing BA concentration. Best shoot growth and multiplication was observed on MS medium supplemented with 5.0 μM BA (Table 1). At higher concentration of BA (>5.0 μM) shoot growth was severely inhibited and shoots showed the signs of hyperhydricity.

Table 1. Effect of BA on shoot proliferation of *Chlorophytum borivilianum* on MS medium containing 3% (w/v) sucrose.

BA concentration (μM)	Shoot Multiplication	Average shoot length (cm)
0.0	+	5.3
1.0	++	3.2
2.5	++++	2.1
5.0	+++++	1.8
12.5	++++	0.6

Data were recorded after 21 days of subculture. In each flask 3 clumps (each with 3 – 5 shoots) were inoculated in triplicates. Each '+' sign indicates doubling of original inoculated cultures.

The effect of different sucrose concentrations (58 – 290 mM) and heat shock treatment was also studied on basal MS medium supplemented with 5.0 μM BA. Following heat shock the shoot proliferation and average shoot length, both increased with increasing sucrose concentrations upto 232 mM (Table 2; Fig. 3 and 4).

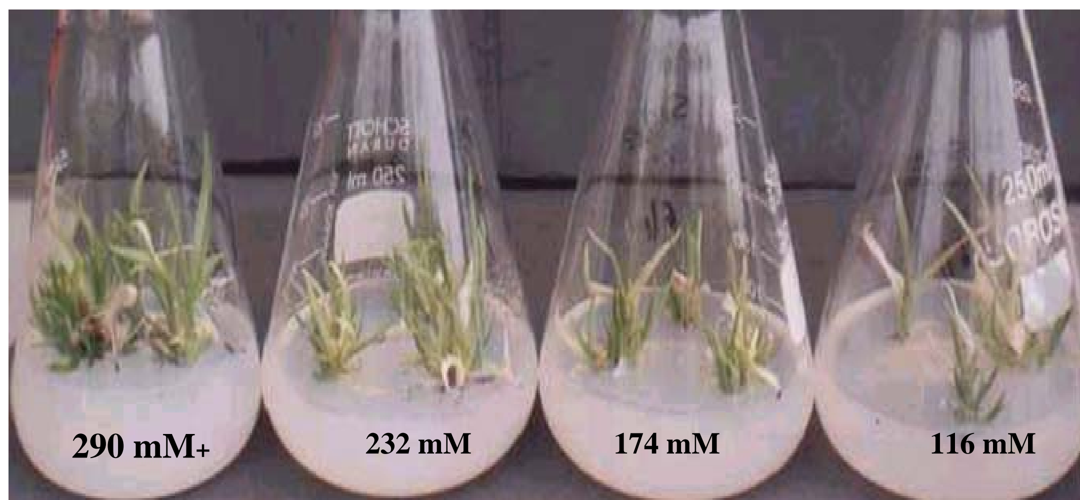

Fig. 3. Effect of different concentrations of sucrose in MS medium supplemented with BA (5.0 µM) on shoot proliferation of *Chlorophytum borivilianum* following the heat shock treatment.

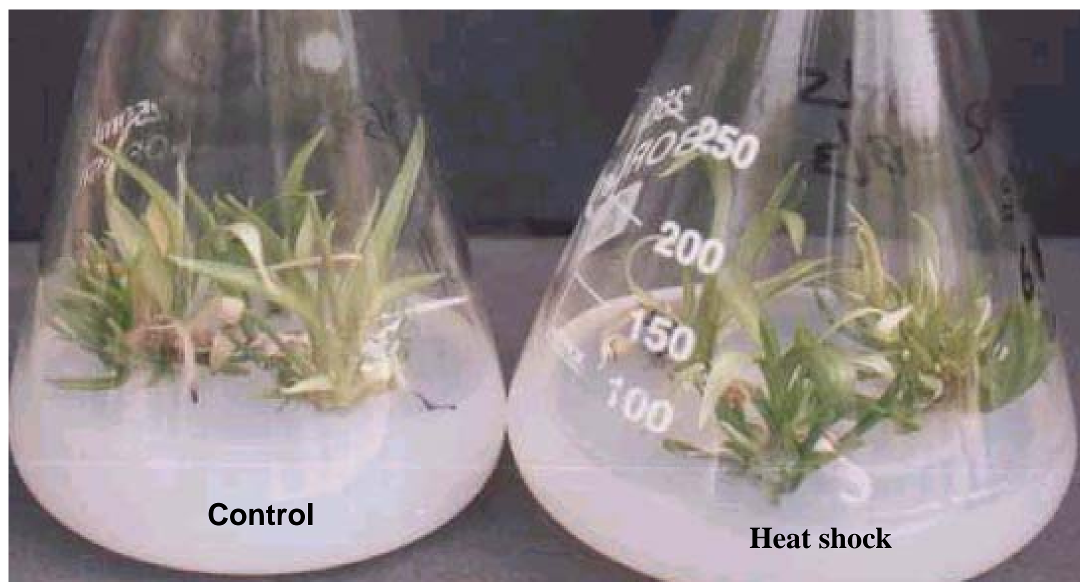

Fig. 4. Effect of heat shock on shoot proliferation of *Chlorophytum borivilianum* cultures inoculated in MS medium supplemented with BA (5.0 µM) with 174 mM sucrose.

Table-2. Effect of different sucrose concentrations and heat shock on shoot proliferation in *Chlorophytum borivilianum* on MS medium supplemented with BA (5.0 μM).

Medium composition MS medium + BA (5μM)	Number of shoots per shoot clump (Mean ±SE) n=3		Average shoot length (cm) (Mean ±SE) n=3	
Diff. Sucrose conc. mM	Without heat shock	With heat shock	Without shock heat	With heat shock
58	6.66 ± 0.32	6.0 ± 0.57	1.60 ± 0.20	1.83 ± 0.08
116	6.33 ± 0.66	6.66 ± 0.32	1.86 ± 0.404	2.06 ± 0.12
174	6.0 ± 0.57	7.33 ± 0.32	2.0 ± 0.20	2.24 ± 0.12
232	5.33 ± 0.66	7.66 ± 0.32	2.16 ± 0.120	2.23 ± 0.12
290	5.33 ± 0.32	7.00 ± 0.57	1.66 ± 0.21	1.83 ± 0.08

Data were recorded after 21 days of subculture. In each flask 3 clumps (each with 3 – 5 shoots) were inoculated in triplicates. Three shoot clumps (3 – 5 shoots each) were inoculated in each flask. Each treatment consisted of three replicates. Heat shock (50 °C, 1 h) was given to the cultures after one week of subculture and data were recorded two weeks after the heat shock.

Maximum shoot proliferation (7.66 shoots per explants) and shoot growth (average length 2.23) was observed on medium containing 232 mM sucrose following heat shock. At lower sucrose concentrations beneficial effect of heat shock was seen on shoot length (2.24 cm) cultured in medium containing 174 mM sucrose. It was noticed that heat shock did not had any significant effect on shoot proliferation in medium having low sucrose concentration specifically 58 mM and 116 mM (Table 2).

Effect of an bioactive compound ('Compound A') was tested on the growth and proliferation of cultures. It was observed that at lower concentrations, incorporation of this compound into the medium has beneficial effect on shoot proliferation whereas higher concentrations were lethal (>5 mg/l) in *Chlorophytum borivilianum* (Table 3; Fig. 5, 6 and 7).

Table-3. Effect of 'Compound A' on shoot proliferation in *Chlorophyutum borivilianum* after four weeks of culture.

Medium composition	Number of shoots per explant (Mean ±SE) n=4		Average shoot length` (Mean ±SE) n=4	
Compound A mg/l	MS Medium (SM)	MS Medium +BA(5µM) (SMB)	MS Medium (SM)	MS Medium +BA(5µM) (SMB)
(control) 0	8.75 ± 0.54	9.25 ± 0.54	1.65 ± 0. 28	1.17 ± 0.09
0.5	9.25 ± 0.54	14.0 ± 0.46	2.1 ± 0.10	3.05 ± 0.17
1.0	16.75 ± 0.54	13.75± 0.54	2.47 ± 0.09	2.32 ± 0.18
1.5	9.75 ± 0.54	8.75 ± 0.54	1.57 ± 0.144	1.65 ± 0.28
2.5	12.75 ± 0.54	6.75± 0.54	1.62 ± 0.12	1.25 ± 0.12
5.0	5.00 ± 0.46	6.00 ± 0.46	1.22 ± 0.09	1.82 ± 0.144*

Data were recorded after 21 days of subculture. In each flask 3 clumps (each with 3 – 5 shoots) were inoculated in triplicates.
* All cultures died after four weeks of subculture.

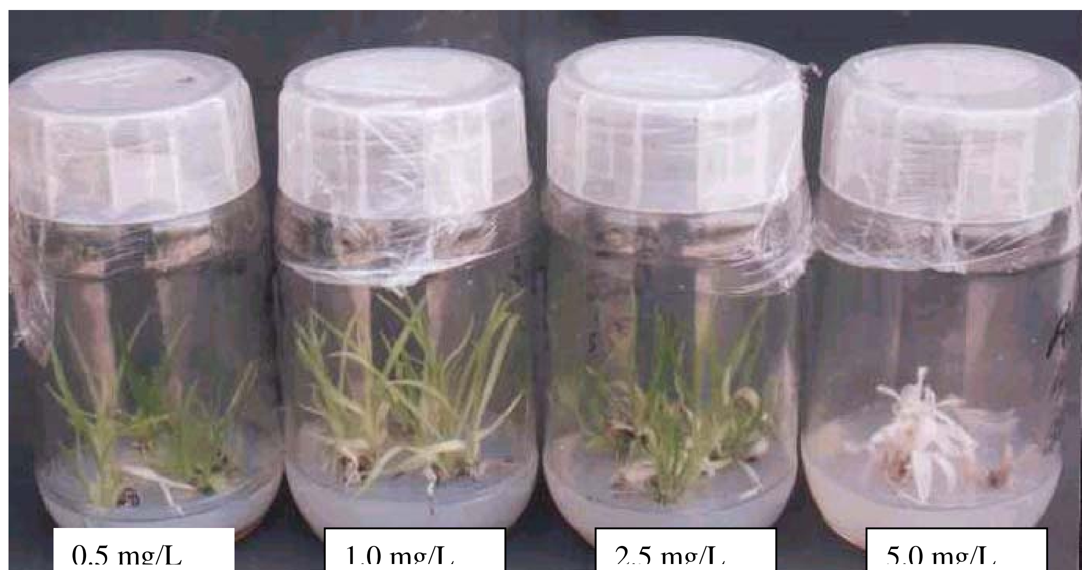

Fig. 5. Effect of 'Compound A' (increasing concentration) on shoot proliferation in Chlorophytum borivilianum after four weeks of culture in basal MS medium.

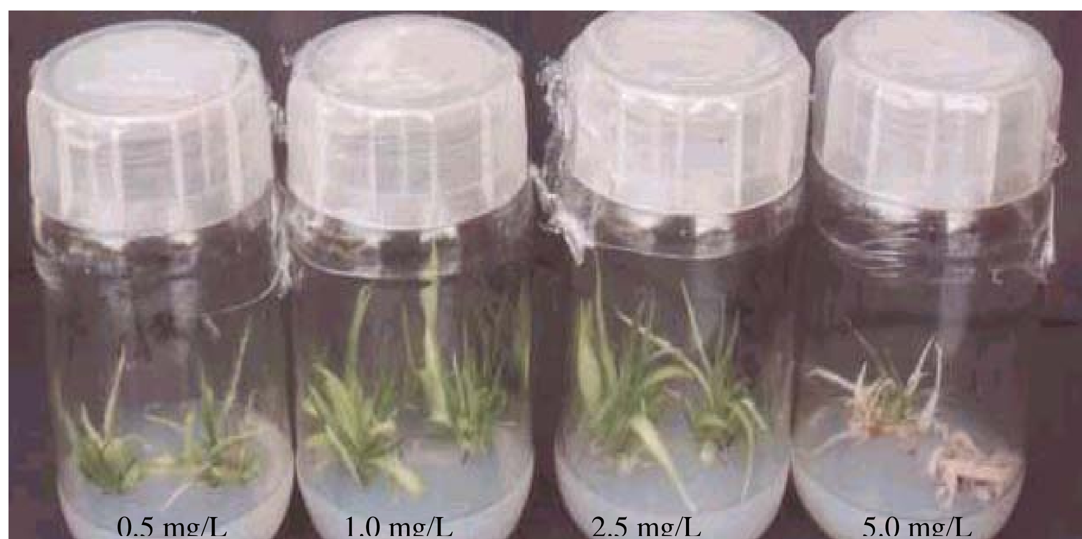

Fig. 6. Effect of 'Compound A' (increasing concentrations) on shoot proliferation in Chlorophytum borivilianum after four weeks of culture in MS medium supplemented with BA (5 μM).

It was observed that explants in medium having 1.0 mg/l ‘Compound A’ showed maximum shoot proliferation i.e. 16.75 ± 0.54 in basal MS medium (SM). It was observed that with the increase in concentration of ‘Compound A’ there was decrease in both shoot number and shoot length (Table 3). In case of MS suuplemented with BA, the positive effect on proliferation was observed with only 0.5 mg/l of this compound whereas higher concentrations are inhibitory for shoot proliferation. It was observed that medium with BA showed higher rate of multiplication in comparison to basal medium, while shoot elongation was better on basal medium.

Effect of Agar and Phytagel on shoot multiplication

Experiments were conducted to compare the effect of agar (0.65%) and phytagel (0.225%) on MS basal medium. It was observed that as compared to agar, phytagel was beneficial for shoot proliferation and elongation. The average number of shoots in medium with 0.225% phytagel was 4.7 ± 0.41 whereas, in case of agar it was 3.37 ± 0.24 (Table 4). It was noticed that there was significant increase in shoot length (13.75 ± 0.47) on medium containing phytagel in comparison to agar (Table 4). In case of agar gelled medium approximately 60% rooting of shoots was observed.

Table-4. Effect of agar and phytagel on shoot proliferation in *Chlorophyutum borivilianum* after four weeks of culture.

Medium composition MS medium	Number of shoots per explant (Mean ± SE) n= 4	Average shoot length (Mean ± SE) n= 4	Percent shoots rooted (%)	Number of explant rooted (Mean ± SE)
Agar (0.65%)	3.37 ± 0.24	7.25 ± 0.47	60	11.25 ± 0.95
Phytagel (0.225%)	4.7 ± 0.41	13.75 ± 0.47	-	-

Data were recorded after 21 days and each treatment consisted of four replicates.

Fig. 7. Comparison of effect of 'Compound A' on shoot proliferation in Chlorophytum borivilianum in MS medium supplemented with BA (5 µM) (SMB) and basal MS medium (SM).

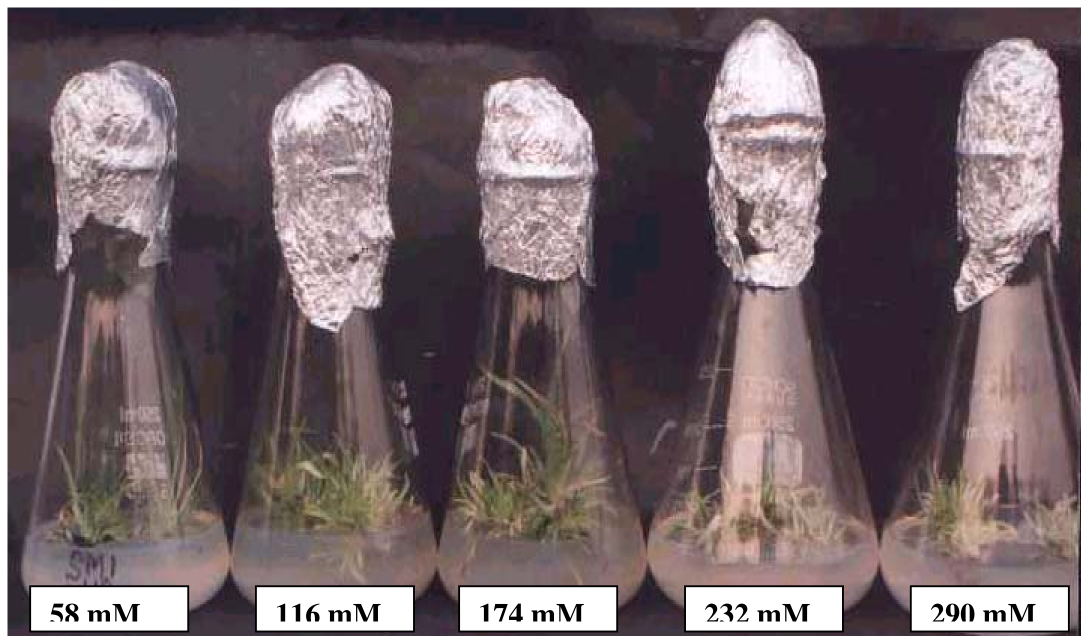

Fig. 8. Effect of different sucrose concentration (58 – 290 mM) on rooting of safed musli inoculated on MS medium supplemented with IBA (5µM).

Rooting of Microshoots

Effect of IBA, sucrose concentrations (58 – 290 mM) and heat shock (at 50 °C for 1 h) was studied on rooting of shoots on MS medium. It was observed that maximum percent (95 %) shoots rooted following heat shock on MS medium containing 5.0 µM IBA and 290 mM sucrose. On basal medium also the highest rooting frequency of 72 % was observed after the heat shock to the cultures on medium contain 290 mM sucrose (Table 5; Fig. 8 and 9).

Table-5. Effect of heat shock and different sucrose concentrations on rooting of shoots of *Chlorophytum borivilianum* inoculated in MS medium(SM) and MS medium supplemented with IBA (5µM) (SMI) after three weeks of culture.

Medium composition	Number of roots per explant (Mean ± SE) n=3				% shoots rooted			
Diff. Sucrose conc. (µM)	without heat shock		with heat shock		without heat shock		with heat shock	
	SM	SMI	SM	SMI	SM	SMI	SM	SMI
58	2.24 ± 0.58	3.33 ± 0.34	2.54 ± 0.32	3.33 ± 0.34	35	40	40	46
116	2.96 ± 0.65	3.33 ± 0.34	3.40 ± 0.57	3.66 ± 0.32	36	45	43	60
174	3.66 ± 0.32	4.33 ± 0.32	4.20 ± 0.30	5.66 ± 0.32	53	70	65	75
232	4.32 ± 0.32	6.66 ± 0.34	5.66 ±0.32	7.0 ± 0.57	60	74	70	82
290	4.82 ± 0.57	5.0 ± 0.57	6.02 ± 0.34	7.66 ± 0.32	65	80	72	95

Data were recorded after 21 days of subculture. In each flask 16-15 clumps (each with 3 – 5 shoots) were inoculated in triplicates. SM is basal MS medium and SMI is MS medium supplemented with IBA (5µM). Heat shock given after 7 days of inoculation.

Fig. 9. Effect of heat shock on rooting of safed musli inoculated in MS medium supplemented with IBA (5µM) and 116 mM sucrose.

Fig. 10. Hardening of tissue culture raised plants in shade house.

In case of SMI medium, roots were more thick and elongated, while the roots on SM medium were thin and less elongated. In both the cases colour of roots was greenish yellow, unbranched and normal in appearance. In SMI medium rooting was better at higher concentration of sucrose and heat shock further enhanced rooting significantly at higher sucrose concentrations (232 mM and 290 mM). All, increase in sucrose concentration, heat shock and IBA were found to be beneficial for rooting of microshoots.

Hardening of plantlets

The water holding capacity of soil is an important parameter, therefore, in order to find out the suitable soil mixture, plants were transferred to polybags having different soil mixtures. The experiment was carried out directly in shade house thus avoiding the interviening stage of polyhopuse (Fig. 10.). It was observed that vermicompost and Farm yard manure had significantly beneficial effect on survival rate as well as subsequent growth of the plants.

Table 6. Effect of different soil mixes on survival of plantlets of *Chlorophytum borivilianum* during hardening.

Different soil mixes	Shade House*	
	Number of Plants transplanted	% of survival
A	15	90
B	15	95
C	15	99
D	15	90
E	15	50

* Plantlets were directly placed in shade house.

A – FYM (Farm Yard Manure) : soil 1:1 ; B -Vermicompost : soil 1:1;

C -Vermicompost, perlite, FYM : soil 1:1; D -Perlite : soil 1:1 ; E- soil

After 21 days of culture of microshoots on rooting medium, which resulted in the sufficient rooting, plantlets were transplanted to plastic pots containing garden soil and vermicompost (1:1). Plantlets that were transferred to the plastic pots in polyhouse showed low percentage of survival (24%) as compared to those which were directly transferred to the shade house which showed about 90% survival (Table 7; Fig 10). In shade house plants started to elongate and leaves also start to thicken. In shade house plants were watered twice a day i.e. morning and evening.

Table-7.Survival rate of plantlets of Safed musli at different stages of Hardening.

Stage of transplantation	Number of plants transplanted	Percentage of survival (%)
Poly House (1st stage)	50	24
Shade house** (1st stage)	30	90

**Plantlets directly transferred in shade house (avoiding poly house stage).

Clonal fidelity of micropropagated plants

PCR results using 10 mer random primer (RAPD) and 18 mer (iSSR) primer indicates the similar banding pattern in regenerated plants and comparable to mother plants (Fig 11 and 12). Those results indicate the true to type nature of regenerated plant. Further analyses using more such primers is required to arrive at the firm conclusion on the clonal fidelity of these plants.

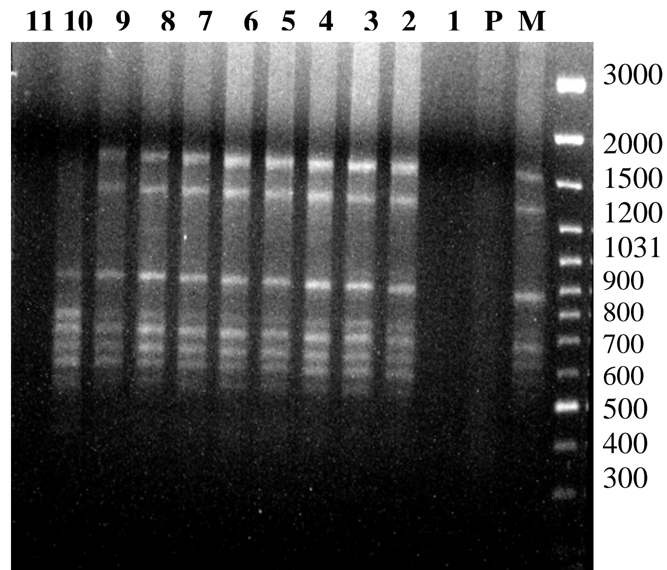

Fig. 11. RAPD gel run of *Chlorophytum borivilianum*

P – Mother plant; M – 100 bp DNA Ladder Plus ;
1 to 11 – Tissue culture raised plants

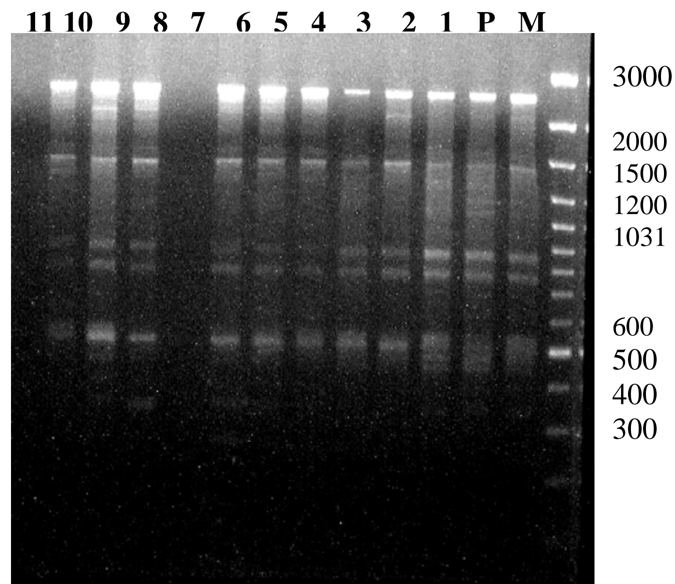

Fig. 12. iSSR gel run of *Chlorophytum borivilianum*

P – Mother plant; M – 100 bp DNA Ladder Plus ;
1 to 11 – Tissue culture raised plants

DISSCUSION

For shoot proliferation, growth regulators especially cytokinins (Lane, 1979; Stolz, 1979; Bhojwani, 1980; Garland and Stolz, 1981) are one of the most important factors affecting the response. A range of cytokinins (Kinetin, BA, 2-ip and zeatin) has been used in micropropagation work (Bhojwani and Razdan, 1992). In white clover (Bhojwani, 1981), hybrid willow (Bhojwani, 1980) and chickpea (Barna and Wakhlu, 1994). BA was the most effective cytokinin for the shoot tip, meristem and bud culture. At higher levels, cytokinins tends to induce adventitious bud formation (McComb, 1978; Zimmerman and Broome, 1980). In the present study also cultures were multiplied using a range of BA concentrations, however 5.0 μM BA was found to be optimum (Table 1).

In the present study, it was seen that heat shock (at 50 °C after 7 days of inoculation) gave better shoot proliferation in combination with high sucrose concentration (232 and 290 mM) in comparison to the explants that were not given any heat shock (Table 2). In MS medium supplemented with BA (5 μM), containing different concentration of sucrose, on an average each explant gave rise to 6 -7 shoots (Table 2). Hundred percent cultures showed shoot proliferation on this medium. Beneficial effect of heat shock was also seen on shoot growth on medium containing 174 mM sucrose. It was noticed that heat shock did not had any significant effect on shoot proliferation in medium having low sucrose concentration specifically 58 and 116 mM (Table 2).

Beneficial effect of high sucrose concentrations on different events of morhogenesis like shoot multiplication (De Bruyn and Ferreira, 1992), rooting of microshoots (Rahman *et al.,* 1992; Romano *et al.,* 1995) and somatic embryogenesis (Loiseau *et al.,* 1995) has been reported earlier. However, temperature dependent response of different sucrose concentrations, as demonstrated in the present investigation, has been reported in *Gladiolus hybridus* (Kumar *et al., 1999, 2002).*

Researchers are always in search of certain novel compounds that can have wide range of applications, so, in the present study effect of a bioactive compound ('Compound A') was studied on the growth and proliferation of cultures. For this

different concentrations of Compound A (0.0 – 5.0 mg/l) were used in basal MS and MS medium supplemented with 5 μM BA. It was observed that Compound A (1mg/l) showed enhanced shoot proliferation in *Chlorophytum borivilianum.* It was seen that with the increase in concentration of the compound A there was decrease in both shoot number and shoot length (Table 3).

The effect of two gelling agents (agar and phytagel) was compared in the present study. It was found that the phytagel has beneficial effect on shoot proliferation and growth. Earlier, beneficial effect of phytagel on shoot proliferation and growth has been reported (Kumar *et al*., 2002, 2003). Although agar is most frequently used gelling agent in tissue culture, it has been reported to differ from batch to batch (Debergh, 1983) and subsequently show variation in response due to interaction with media components (Romberger and Tabor, 1971), impurities (Nairn *et al*., 1995) and gelling strength (Debergh, 1983). However, in the present study agar was found to be beneficial for rooting of shoots, these results are in line with the earlier findings of Kumar *et al*. (2003).

Rooting response of microshoots is reported to be controlled by growth regulators in the medium (Bhojwani and Razdan, 1992), basal salt composition (Garland and Stoltz,1981; Zimmerman and Broome,1981), genotype (Rines and McCoy, 1981) as well as cultural conditions (Murashige, 1977). For most of the species auxin is required to induce rooting. NAA and IBA are most commonly used for root induction (Bhojwani and Razdan, 1992). By the use of IBA many plants such as *Lycoperscicon esculemtum (*Sibi, 1982), *Hedychium roxburgii* (Tripathi and Bitaillion, 1985), carnation (Werker and Leshem, 1987) gave *in vitro* rooting.

For the purpose induction of roots PGR-free(SM) and IBA supplemented medium(SMI) were used in the present study. But rooting was observed better in SMI medium (Table 5). In the present study higher concentrations of sucrose was found to be beneficial for root induction. Beneficial effect of high concentrations of sucrose on morphogenesis has been reported earlier (Kmar *et al*., 1999, 2002). Higher concentration of exogenous sucrose have been shown to increase the endogenous levels of reducing sugars (Romano *et al.,* 1995) which are further known to react non-enzymatically with nuclear proteins and cause modifications in

about 10% of the proteins (Bojanovic *et al.,* 1970). It is therefore possible that in the present study these beneficial properties of sugars are involved in shifting the morphogenic pathway of the tissue. Heat shock treatment to the shoot cultures also promoted rooting on medium containing high sucrose concentrations in Gladiolus (Kumar *et al*., 1999: Elisson, 1978). These heat shock induced developmental events may result from the production of stree/heat shock proteins. Earlier developmental role of heat shock proteins has been speculated by Schnall *et al.* (1991); Vierling (1991). Certain loci molecular weight HSP are known to be expressed during specific stage of somatic embryogenesis in white spruce (Dong and Dunstan, 1996).

Auxins are known to induce rooting in the microshoots/shoots (Blakesly and Chaldewtt, 1993; Bonza *et al*., 1994). In the present study, IBA was found to promote rooting in basal medium containing different concentrations of sucrose and heat shock further promoted the rooting in microshoots. This heat shock promoted rooting in auxin containing medium seems to be novel observation.

Hardening of tissue culture plants is the most crucial step in micropropagation. The plants produced are very soft to face ambient environmental conditions. (Bhojwani and Razdan, 1992) during acclimatization. Under these conditions the leaves of plants develop cuticle and its photosynthetic system starts functioning. The most crucial stage is during first 10 days in polyhouse.

The water holding capacity of soil is an important parameter, therefore, during hardening experiments involving various soil mixtures were performed. It was observed that FYM and vermicompost containins mixtures showed better results (Table 6). It may be due to the high organic content present in these mixtures that supported the plant growth. The role of organic matter present in soil in hardening of the *Chlorophytum* had been specified by Oudhia *et al*. (2001).

Rooted plantlets that were transferred from culture bottles to plastic bags containing mixture of 1:1 ratio of soil: vermicompost for their hardening prior to their final transfer to the soil, showed low percentage of survival (24%) in polyhouse and high percentage (90%) in shade house. The growth and elongation of the plants were

less in poly house whereas in shade house growth of the plants was better and they also start to elongate in shade house. The leaves also start to thicken in shade house (Table 7). Usually tissue-culture raised plants are hardened first in polyhouse and then in shadehouse but according to our observations, Safed musli showed significantly higher survival rate when directly kept in shade house. This can be very useful from commercial point of view because it also cuts down the cost during acclimatization of plants.

The hardened tissue-culture raised plant may posses deviant phenotypes due to some somaclonal variations. Therefore molecular markers have been used to determine the level of clonal fidelity (True-to-type plants) among *in vitro* propagated plants. Various protein based (protein patterns and isoenzymes) and DNA based markers can be applied to test the clonal fidelity. In the present work we used RAPD and iSSR markers to find out variants if any, amongst the regenerated plants. Earlier RAPD has been used for genetic analysis in micropropagated plants of *Populus deltoides* Marsh (Rani *et al*., 1995).

PCR results using RAPD and iSSR primer indicates the similar banding pattern in regenerated plants as in mother plants. But the work is still not sufficient enough. So further analyses using more such primers is required to arrive at the firm conclusion on the clonal fidelity of *Chlorophytum borivilianum*.

CONCLUSION

Chlorophytum borivilianum is a medicinal plant of considerable importance. It is widely used in drug industry and its demand is increasing day by day. The tuberous roots of Safed musli are the only propagule which can either be sold in the market for economic gains or saved for commercial cultivation year after year.

This has created a severe shortage of planting material for large scale cultivation. To fill the gap of demand and supply, and to provide genetically uniform planting material from a known source, micropropagation is the best alternative. Therefore, efforts are required to develop efficient micropropagation protocol for safed musli.

The objectives of the present study was to standardize conditions for establishment of axenic culture from elite germplasm, shoot proliferation, rooting of micro shoots, hardening and transfer of plants to soil. For the identification of any possible somaclones, in addition to their comparison with in terms of morphology, experiments were planned to carry out genetic analysis using DNA based markers. For this purpose we isolated DNA from both mother plant as well as plants regenerated through tissue culture.

The conclusions Drawn from this study are,

1. Surface sterilization with $HgCl_2$ (0.1% for 5-minutes) with 70% alcohol dip was best for the surface sterilization of the explants.
2. For the initiation of the culture, MS medium with BA 2.5 μM with NAA 0.5 μM was used.
3. Best shoot proliferation after heat shock was achieved on MS medium 10% sucrose and supplemented with BA (5.0 μM).
4. For shoot proliferation, medium with phytagel as gelling agent was found to be better than medium gelled with agar.
5. ‘Compound A’ (1 mg/l) enhanced shoot proliferation in the present study showing beneficial effect on shoot proliferation.
6. In the present study, IBA (5.0 μM) was found to promote rooting in basal medium containing different concentrations of sucrose (particularly 290 mM)

and heat shock further promoted the rooting in microshoots. Higher sucrose concentrations were also found to be beneficial for rooting.

7. 95% shoots showed rooting response on PGR -free medium.
8. Agar as gelling agent was beneficial for rooting of microshoots as compared to phytagel.
9. *In vitro* raised plantlets showed 24% survival if transferred to polyhouse conditions and about 90% survival when directly transferred to shade house avoiding poly house stage.
10. FYM and vermicompost showed beneficial effect on hardening of *In vitro* raised plantlets.
11. Based on preliminary study using RAPD and iSSR markers, regenerated plants were found to be similar to the mother plant.

ANNEXURE 1

Composition of basal MS medium

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

MICRO SALTS	
$MnSO_4.H_2O$	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	37.24

ADDITIVES	mg/l
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	30000

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