Propagation of *Chlorophytum borivilianum Sant . et Fernand in vitro* for conservation

Priyanka Kumari

COLLEGE OF BIOTECHNOLOGY

BIRSA AGRICULTURAL UNIVERSITY

KANKE, RANCHI- 834006

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BIRSA AGRICULTURAL UNIVERSITY

KANKE, RANCHI-834006 (JHARKHAND)

Dr. (Mrs.) Madhuparna Banerjee
Sr. Scientist Cum Assoc. Professor
(Plant Tissue Culture)

College of Biotechnology
Ph.:0651-2450789 (O)

CERTIFICATE

This is to certify that thesis entitled **Propagation of *Chlorophytum borivilianum Sant. et Fernand in vitro* for conservation** submitted in partial fulfillment of the requirements for the degree of **Master of Science in Biotechnology** of the faculty of post-graduates studies, Birsa Agricultural University, Kanke, Ranchi (Jharkhand) is the record of bonafide research carried out by **Ms. Priyanka Kumari** under my supervision and guidance. No part of thesis has been submitted for any other degree or diploma.

It is further certified that such help or information received during the course of this investigation and preparation of the thesis have been duly acknowledged.

ENDORSED

Dr. Z.A. Haider
Associate Dean
College of biotechnology
B.A.U., Ranchi- 834006

Dr.(Mrs.) Madhuparna Banerjee
Major Advisor

BIRSA AGRICULTURAL UNIVERSITY

KANKE, RANCHI-834006 (JHARKHAND)

CERTIFICATE

(By the Advisory Committee)

We, the undersigned, members of the advisory committee of **Ms. Priyanka Kumari**, a candidate for the **Degree of Master of Science in Biotechnology** have gone through the manuscript of the thesis and agree that the thesis entitled **"Propagation of *Chlorophytum borivilianum Sant. et Fernand in vitro* for conservation"** may be submitted by **Ms. Priyanka Kumari**, in partial fulfillment of the requirement for the degree of Master in Science in Biotechnology.

Dr. (Mrs.) Madhuparna Banerjee
Chairperson
Advisory Committee

Members of Advisory Committee

ENDORSED

1. *Dr. Anita Pande*

2. *Dr. Himanshu Dubey*

Dr. Z.A. Haider
Associate Dean
College of Biotechnology
B.A.U., Ranchi-834006

3. *Dr. Niraj Kumar*

BIRSA AGRICULTURAL UNIVERSITY

KANKE, RANCHI-834006 (JHARKHAND)

CERTIFICATE

Certificate of approval by the Chairman of the Advisory Committee and the External Examiner

This is to certify that the thesis entitled **"Propagation of *Chlorophytum borivilianum Sant. et Fernand in vitro* for conservation"** Submitted by **Ms. Priyanka Kumari**, in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology of the Faculty of Post-Graduate Studies, Birsa Agricultural University, Kanke, Ranchi was examined and approved on ……,2014.

……

Name and Signature of External Examiner

Dr. (Mrs.) Madhuparna Banerjee
Chairperson
Advisory Committee

Members of Advisory Committee

Associate Dean
College of Biotechnology
B.A.U., Ranchi-834006

1. *Dr. Anita Pande*
2. *Dr. Himanshu Dubey*
3. *Dr. Niraj Kumar*

DRI-cum-Dean
Post Graduate Studies
B.A.U., Ranchi-834006

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Date:

Place: Kanke, Ranchi ***Priyanka Kumari***

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LIST OF ABBERVIATION

Sl. No.	Abbreviations	Full form
1.	$AdSO_4$	Adenine Sulphate
2.	BA	6- Benzyl adenine
3.	BAP	Benzyl amino purine
4.	2, 4-D	2,4-Dichlorophenoxy acetic acid
5.	$HgCl_2$	Mercuric chloride
6.	IAA	Indole-3-acetic acid
7.	IBA	Indole-3-butyric acid
8.	KIN	6-furfuryl amino purine
9.	µg	Microgram
10.	µg/l	Microgram per litre
11.	µM	Micro molar
12.	mg/l	Milligram per litre
13.	g	gram
14.	NAA	Naphathalene acetic acid
15.	NaOH	Sodium hydroxide
16.	GA3	Gibberelic acid
17.	SA	Salicylic acid
18.	ANN	Artificial Neural Network
19.	MS	Murashige and Skoog media
20.	B5	Gamborg *et al*. Media
21.	CSIR	Council of Scientific and Industrial Research

ABSTRACT

An efficient protocol for rapid *in vitro* propagation of valuable medicinal plant *Chlorophytum borivilianum* by using shoot tip as explants were done. This plant is used in the traditional medicinal system for enhancing male potency, curative for physical weakness, natal and post natal problems, remedy for diabetes and arthritis. It is an aphrodisiacs agent used for preparation of general sex tonic, and immunity-improving drug. The *in vitro* plantlets production system was investigated on Murashige and Skoog medium with different concentrations and combinations of different hormones such as BAP alone and in combination with $AdSO_4$, with and without IAA and KIN for shoot multiplication. Shoot multiplication was induced on shoot tip. Multiplication was best (88.83%) in shoot tip explants when grown on MS medium supplemented with BAP (6.0 mg/l). Rooting on excised shootlets was highest (91.31%) in MS medium supplemented with 2.0 mg/l IBA along with 2.0 mg/l IAA. Rooted plantlets were transferred to the portray for hardening. The potting mix used was sterile coco peat. After plantation the portrays were kept under poly tunnel to retain almost 100% humidity. After 15 days, the new leaves and roots came out and the plantlets were transferred to poly bags containing soil: sand: FYM in 1:1:1 proportion and kept under the shade in green house for secondary hardening. The survival percentage in green house was found 95.83% . Transferred safed musli plantlets established better under net house condition compare to direct exposure into the field. Micro propagation seems to be the best method compared with other methods of multiplication. In Safed musli *in vitro* method increased the rate of multiplication while combating the problem of long tuber dormancy to facilitate for growing more than one crop per year.

Chapter -1

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 has medicinal properties like Aloe, Jatropa, Satavari, *Piper methystium*, Ginkgo etc. One of such important medicinal plant is *Chlorophytum borivilianum* Sant. et Fernand. It is Commonly known as Safed musli 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 is 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. *Chlorophytum borivilianum* which diploid in nature with chromosome number 2n=16 (Kumar and Subramanium, 1986).

Indian forests are rich in 'Safed Musli' its demand is increasing rapidly in the Indian as well as international drug markets. According to a report in 2005-06, the demand for dry Safed musli is to the tune of 35,000 tonnes per annum, while the supply stands at 5,000 tonnes per year Amongst the, *Chlorophytum borivilianum* produces the highest yield and highest saponin content (Shariff and Chennaveeraiah, 1972). Other important 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 rain. Its root tubers are fleshy, fascicled and directly originates from the stem disc devoid of any fibrous structure. 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 cm 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. Roots pale brown to white colour with characteristic odor and are tasteless in nature. Root tubers distinguished it from other species of *Chlorophytum* genus. The shape of tubers were cylindrical, the thickness being on the average 0.9 cm and the length 8cm.The number of tubers varies from plant to plant and on an average 5-30 tubers/ plant are observed and slightly tapering towards lower side look like pestle. It grows to a maximum height of 1.5 ft. Tubers can grow up to a depth of 10.

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).

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 dugged 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.They are then packaged in polythene bags and transported for marketing. Although Indian forests 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, 2001). 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.

Uses and Benefits of Safed Musli

- Safed musli is a very popular aphrodisiac agent, with no side effects. It is often prescribed for enhancing male potency and overcoming signs of fatigue. It is particularly used for individuals with low sperm count and low libido.
- It is also regarded as an energy booster in asthmatic conditions. The roots of the herb are also used to strengthen the general immune system of the body.
- The tuber roots of the plant have been used since ancient times, to prepare nutritive tonic for sexual weakness and is used in Ayurvedic medicines even today.
- Safed musli proves useful as a nutritive tonic for both the mother and the fetus, during pregnancy and is also used to replenish the body fluids during the post-partum stage.
- The herb improves the quantity and flow of breast milk, in feeding mothers.
- It has got health promotional benefits. It is used to control and prevent obesity and its side effects.The activities of vitamin C and antioxidant enzymes are also enhanced by the use of this plant species.
- Research has indicated that the plant species is used for curing diabetes and arthritic conditions. Safed musli is also beneficial in the treatment of natal and postnatal problems.
- Its regular use causes increase in the level of High Density Lipoprotein (HDL or good cholesterol) and decrease in the plasma and hepatic lipid profiles.
- Apart from rejuvenating the reproductive system, the herb prevents premature ejaculation and is also used in chronic leucorrhoea . 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".

The major constraint in the cultivation of safed musli is long tuber dormancy to the tune of 6-7 months. Due to long dormancy period, only one crop per year can be grown. Other constraints include shortage of quality planting material, bulky nature of tuber and lower tuber multiplication rate. 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 options. 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.*, 2003, Dave *et al.*, 2003) where in some of the parameters have been worked out. Micropropagation is an alternative and feasible means in the propagation of most of the commercially important plants. Moreover the technique is not season bound and requires only a limited quantity of initial explant and space for multiplication through the phenomenon of 'morphogenesis' based on the totipotency concept. Further studies are required to investigate the various factors which influence large scale multiplication and subsequent acclimatization. Micropropagation has the potential for large-scale propagation of elite trees.

Considering this background, the present investigation was carried out with objective.

Optimization of protocol for micropropagation to conserve as well as propagate the plant *in vitro*.

Chapter -2

REVIEW OF LITERATURE

Development of the plant tissue culture is historically linked to the discovery of cell and subsequent propounding of the cell theory. The concept of 'totipotency' which is an inherent part of the cell theory of Schleiden (1838) and Schawn (1839) is the basis of plant tissue culture. Later, Haberlandt during 1902 developed the concept of *in vitro* culture by culturing the cells of carrot stamen hairs, thus he has been regarded as the father of tissue culture. Then the discovery of auxins and cytokinins played an important role in plant tissue culture. (Skoog and Miller, 1957) revealed the hormonal control of shoot and root regeneration from tobacco callus and established the basis for manipulating organ initiation and provided the principle on which micropropagation based. The formation of nutrient media *i.e* (Murashige and Skoog, 1962) medium is the most used medium for culturing large number of plants.

2.1 Botany

Santapau and Fernandes (1955) described *Chlorophytum borivilianum* as small perennial herb with full of radical leaves appearing over the ground with the advent rains. It perennates by fleshy roots/root tubers. These are fascicled, sessile, cylindrical, 1-8 in number, brown to black skinned and white after peeling; the tubers are 3-10 cm long at maturity. Leaves are radical, 6-13 in number, spirally imbricate at the base, sessile, linear ovate, acute apex and slightly narrowed at the base. The leaves are spreading horizontally and its lower surface is rough, margins are wavy with parallel venation. It has solitary scape, 15-20 cm long, terminal, unbranched. It bears flowers over upper ¾ of it's length. The flowers are white, bracteate, pedicellate, usually arranged in alternate clusters, each cluster consisting of 3 flowers. The flower cluster are closer on the upper part of the scape, the bracts are linear papery, purplish, 1-1.5 cm long, the pedicel look whitish, jointed and kneed at the joint, 6-10 mm long, Perianth six, linear acute, 3-5 nerved, stamens six, as long as perianth, its filaments are glabrous, The anthers are yellow, linear and dehisces by longitudinal splits, style is slightly longer than the stamens, swollen at the apex, ovary is 3 lobed, angels obtuse, green globose and sessile. The fruit is loculicidal capsule, green to yellow, triquetrous, almost equal in length and width, seeds are onion like black in appearance with an angular. (Rochford and Grover, 1961) reported that the name *Chloroplytum* is derived from greek words, Chloros

meaning green and phyton meaning plant and belongs to the family Liliaceae. (Shah, 1978) described following key for identification of two economically important species of safed musli found in Gujarat. Plants are with tubers upto 30 cm long (a) *C. tuberosum*b (b) *C. borivilianum.* As described by (Naik and Nirgude, 1981) the flowers of *Chlorophytum tuberosum* are without septal glands. There are 7-9 veins on the petals. This feature is employed in the taxonomic delineation of the species. (Vaikos and Rai, 1990) studied floral anatomy of few species of *Chlorophytum* and abstracts on two species from their observations are reproduced *C. tuberosum*. Flowers are without septal glands. *C. laxum*: There are no ovarian grooves. It bears floral nectarines. The storage roots of *C. borivilianum* are the major sources of steroidal and triterpenoidal saponins, sugars, polysaccharides and amino acids. The roots are also a rich source of over 25 alkaloids, proteins, vitamins, steroids, phenolics, gallotannins, mucilage and resins (Thakur *et al.,* 2008) The plant supports an annual growth habit and has a very short life cycle (90 -100 days) (Maiti and Geetha, 2005). *Chlorophytum borivilianum* (Liliaceae), commonly known as safed musli has also been listed as endangered species (Nayar and Shastry, 1988) valued for its fasciculate storage roots .

Baldwin and Sepeese (1951) have worked on cytogeographical aspects of *C. laxum* (2n=14), and studied somatic chromosomes and the meiotic behaviour in these species based on several collections made from different localities. (Kumar and Rao, 1958) studied meiosis in *C. tuberosum* and recorded its chromosome number as n=8. (Kumar and Subramanium, 1986) reported chromosome number 2n=16 in *C. borivilianum.* (Dutta and Mitra, 1968) gave an analysis of the karyotypes of *C. tuberosum* (2=16) and *C. laxum* (2n=16) and noted two pairs of chromosome with terminal satellites in both the species. (Pahuja and Kumar, 1969) compared several taxa collected from different geographic regions and recorded chromosome number of *C. arundinaceum* (2n=42) from Bihar, *C. tuberosum* (2n=16) from Pune.

2.2 Distribution

The safed musli is widely distributed in India, mainly in southern Rajastan, Western Madhya Pradesh and Northen Gujarat. But the continued exploration of its roots for gainful trade has dwindled its frequency of distribution and the quantity brought to market is continuously going down. (Hooker, 1854) have listed more than 300 species in the genus *Chlorophytum* and suggested its probable center of origin and diversification to lie in tropical and subtropical Africa, where over 85% of the species are found.

(Vartak, 1959) reported occurance of *C. borivilianum* from Sinhagad near Mahi and Aravali hills. (Haines ,1961) reported occurance of *C. tuberosum* from santhal parganas (Rajmahal hills) and Ranchi. (Dalal *et al.,* 1987) noticed that most of the *C. tuberosum* plants were found on eastern slope and there were hardly few plants over its western sides during plant collection in Gujarat state.

2.3 Dormancy

Dalal *et al.,* (1987) reporded that *Chlorophytum borivilianum* take seven to eight months of dormancy in the fleshy roots of *Chlorophytum borivilianum*. (Trivedi and Yadav, 1989) also reported seed dormancy period of *Chlorophytum borivilianum* is 9½ to 10 months and germination at indore was 28-62% after 37 days of sowing in the petridishes. (Sheta and Goswami, 1989) recorded 7-8 months of dormancy in the fleshy roots of *Chlorophytum borivilianum*. (Jat and Bordia, 1990) reported that about 13% of seed germination in safed musli. (Rizvi *et al.,* 2007) reported that the natural regeneration of this herb is through tuberous roots that have become scarce in nature due to poor seed germination percentage (11 - 24%), low viability and long dormancy period.

2.4 Medicinal uses

Kirtikar and Basu (1975) reported that it is also known as a curative for diabetes, arthritis and to improve the immune system The use of plants for treatment of various ailments dates back to over 5000 years. Due to these medicinal properties, there has been indiscriminate collection of the roots from the natural habitats, which resulted in the decline in natural populations. 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, published (in Portugese) from Goa in 1565 is chronologically the first printed book on Indian plant drugs, which contained the most classical information on the valuable plants of Malabar hills. These are reported to have aphrodisiac properties and form an important ingredient of herbal tonics prescribed in the Ayurvedic systems of medicine. Now-a-days world-wide shift towards herbal

preparations over synthetic pharmaceuticals has realized the importance of focused research in medicinal plants reported by (Kumar *et al.*, 2004). Therefore, it was realized by (Kothari and Singh, 2003; Maiti and Geetha, 2005) 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. Increasing global demand and high market value phytochemical analysis in *C. borivilianum* has been mainly restricted to isolation, purification and characterization of saponins and polysachharides prposed by (Acharya *et al.*, 2008, 2009; Narasimhan *et al.*, 2006).

2.5 Tissue Culture

Adventitious shoots may be induced by NAA or NAA plus BAP on the meristematic regions of scale, leaf and stem of lily (Hussey, 1977). Bulblets induced from 5 mm lily segments, cultured on Murashige and Skoog's mineral salts medium, bulblets developed well in leaf explants excised before flowering and mainly in those excised within 15 mm of the leaf base (Niimi and Onozawa, 1979). (Leshem *et al.*, 1982) established *in vitro* propagation of *Lilium longiflorum* by using bulb scales is the most suitable method for its multiplication in different species. (Alderson and Rice, 1983) obtained reliable regeneration of adventitious shoots on explants of immature floral stems from tulip dry bulbs to be influenced by the stage of development of the bulb, the thickness of explants and origin of the explants within the floral stem. (Deforssard, 1985) advised that the microbial status of shoot cultures should be monitored after every 3-6 passages. Under normal circumstances, contaminated cultures should be destroyed by autoclaving and contaminated culture vessels should not be opened before autoclaving. (Matsuo *et al.*, 1987) reported that in *Lilium longiflorum*, the outer and middle scales developed many large sized bulbs where as inner bulb scales produced smaller bulblets with low sprouting. (Torres, 1989) was of the opinion that the selection of the ideal explant and thorough disinfection important for establishing *in vitro* cultures in safed musli. Sprouts and tips from tubers during the first month of storage proved to be good explants to start cultures of Gloriosa (Custers and Bergervoet, 1993). (Purohit *et al.*, 1994) described an *in vitro* clonal multiplication of the endangered species *C. borivilianum* on MS medium supplemented with 22.2 μM benzyladenine using young shoot bases as explants. (Lee *et al.*, 1994) used larger bulblets from inner segments of the bulb scales in *Lilium elegans*. (Jeong *et al.*, 1996) abtained in *Lilium*, the bulblet

development maximum from the outer scales of the bulb and many large sized bulblets could be produced from the bottom segments of bulb scales as compared to the upper ones in Lilium elegans. (Kozai *et al.,* 1997) proposed that commercial micropropagation is limited by the cost of plant production. Several species of *Chlorophytum*, collectively known as "safed musli", are endangered because of over exploitation for their tuberous roots, used as an ingredient in tonics and aphrodisiacs. (Suri *et al.,* 1999) developed a method for the rapid multiplication of *C. borivillianum* using stem discs as explants. Plant tissue culture system offers a tool for a large scale production of genetically similar plants (Wawrosch *et al.,* 1999) and is used widely for the commercial propagation of a large number of plant species, including many medicinal plants. (Rout *et al.,* 2000) propagated *C. borivilianum* by using tissue culture technique. The basal explants excised from the outer most layers of the bulbs are most suitable for *in vitro* bulblet production in two Asiatic Lilium hybrids, Arignon and Vada (Singh *et al.,* 2003). The four explants of *C. borivilianum i.e.,* seedling, roots, stem disc and leaf (Basal half) tried on nine media including seven MS media with different combinations and concentrations of the hormones and vitamins for callus development as well as for the shoot and root regeneration. The best explant found to be the leaf base, which at par with stem disc. The performance of the root explant the poorest (Gaikwad *et al.,* 2003). An improved method developed by (Pudake and Dhumale, 2003) for the large scale multiplication of *C.borivilianum* through shoot base and stem disc culture. Callus cultures of *Chlorophytum* established by (Joshi *et al.,* 2003) using young shoot bases as explants on MS medium containing various cytokinins and auxins either individually or in combination. Attempts have been made to propagate *Chlorophytum borivilianum* (safed musli) through tissue culture (Dave *et al.,* 2003, 2004; Purohit *et al.,* 2001). Shoot base and immature unopened floral bud along inflorescence axis of safed musli used as explants for clonal micropropagation (Sharma and Mohan, 2006). Micropropagation technology is advantageous due to production of high quality disease free, true to type plant independent to seasonal and other environmental condition in a comparatively smaller space (Gurel, 2009). Micropropagation is to be the effective method compared with other methods of multiplication. This protocol used to generate cost-effective protocol for large scale *in vitro* cultivation of safed musl (Jakkulwar *et al.,* 2012). The propagation of this species through seeds is restricted due to poor viability and early death of young seedlings under natural environmental conditions (Parashurama *et al.,*

2013). *In vitro* culture is an alternative method of conservation and propagation of this species.

Niimi and Onozawa (1979) used basal mixture for *in vitro* bulblet formation from leaf segments of lilies, especially *Lilium rubellum* baker and consisted of MS mineral salts medium with growth regulators and 0.7 per cent agar. Bulb scales of *Lilium longiflorum* were planted in 25 x 100 mm test tubes on Linsmaior and Skoog (LS) (1965) medium containing growth hormones and solidified with 0.8 per cent Difco-Bacto agar (Leshem *et al.,* 1982). Bulb scales from *in vitro* cultured lily bulblets divided into halves were used as explants. The induction phase (Sub culture I) performed on LS medium supplemented with growth regulators and 7.5 g/l agar (Mensuali *et al.,* 1990). Cultures of Gloriosa performed on Murashige and Skoog (1962) basal medium supplemented with growth hormones and 0.65 per cent (w/v) bacteriological agar (Custer and Bergervoet, 1993)

The first step in any successful tissue culture programme is the selection of suitable explants followed by complete disinfecting. Disinfecting the surface generally involves sterilization with one or more disinfectants followed by thorough washing. Washing the explants under running tap water for 30 minutes to 2 hours (Torres, 1989). Nodal segments of *safed musli* collected by (Sharma *et al.,* 1991) which maintained *ex situ* and washed with teepol detergenet for 15 minutes at slow speed on a magnetic stirrer and later washed thoroughly under running water for 2 hours. (Shen and Sharma, 1991) treated seeds of safed musli with 0.1% $HgCl_2$ for 7-10 minutes after soaking over night in water and washing with 5% teepol detergent solution for 10 minutes. (Razdan, 1993) reported that Successful control of contamination largely depends upon the operator's technique while transferring the sterilized explants into the sterile culture vial containing nutrient medium under aseptic conditions. During this process dust, hair, hands and clothes are the potential sources of contamination, against which it is essential to take precautions. Role of 70% ethyl alcohol and 0.1% $HgCl_2$ as disinfecting solution is very significant. (Nair *et al*., 1994) reported that addition of 100 ppm each of ampicillin and bavistin to the medium was found suitable for checking bacteria and fungal contaminations. (Yanagihava *et al.,* 1996) recommended that young shoot segments of *Chlorophytum borivilianum,* washed with tap water, surface sterilized with 2% sodium hypochlorite solutions containing 0.1% tween-20 for 10 minutes, then with 70% ethanol for 30 seconds and finally washed twice thoroughly with sterilized water before inoculation on MS medium. (Suri *et al*., 1999) pretreated the stem disc explants of safed

musli with mixture of systemic fungicide and antibiotics for 24 hour prior to sterilization effectively reduced contamination. (Gupta, 2006) washed explants thoroughly in running tap water for 30 minutes, cleaned with a solution of liquid detergent Tween 20 for 10 minutes and kept in 1% (w/v) solution of bavistin for 1 hour and rinse in 1% (v/v) solution of savlon and 2-3 washings with sterile water. Further explants surface disinfected with 0.1% (w/v) of mercuric chloride for 5 minutes then thoroughly washed 3-4 times with sterile water. (Haque *et al.*, 2009) collected dormant roots of *Chlorophytum borivilianum*, root with sprouted crown shoot buds sprayed with gentamycin (500 mg/l), 0.5% Fluconazole, 0.1% Bavistin and 0.1% Endosulphan at interval of 24 hours for seven days, surface sterilized with 1% sodium hypochlorite and 1□2 drops Tween 2 (Kemat *et al.*, 2010) collected young shoot bud of *C. borivilianum* and washed thoroughly under running tap water for 30 min, then immersed in 5 g/l (w/v) fungicide for 2 h followed by washing in tap water and rinsed three times with sterilized double-distilled water. Explants were treated with sodium hypochlorite 50% (v/v) in addition with 2 - 3 drops of Tween 20 for 20 min followed by surface disinfecting with 0.1% (w/v) aqueous mercuric chloride solution for 15 min, and finally rinsed three times with sterilized double-distilled water. (Rani and kumar, 2011) used stem disc, shoot bud, root disc, inflorescence and seeds of *Chlorophytum borivilianum* as explants. These explants washed and pretreated in a mixture solution of 0.1% streptomycin and 0.1% bavistin for 30 minutes. The pretreated explants surface sterilized with 0.2% $HgCl_2$ solution for 5 to 10 minutes. Surface sterilization carried out by (Garima and Shruthi, 2012) with 0.1 per cent $HgCl_2$ for 10 minutes, explant treated with bavistin (1% w/v) and 2-3 drops of Tween 20 for 5-20 min followed by immersion in (0.1% w/v) mercuric chloride for 7 min and washed with autoclaved distilled water for 3-4 times. (Rout *et al.*, 2013) collected the mature seeds of *E. scaber* before inoculation the seeds soaked in 1000 mg /l gibberellic acid (GA3) for 24 hours. Seeds washed in running tap water for 10 minutes and then in 5% teepol for 7 minutes, seeds kept in 70% alcohol for 30 seconds. After that the seeds treated with 0.1% $HgCl_2$ for 5 minutes and washed 3-4 times with sterile distilled water to remove traces of $HgCl_2$. (Nakhate *et al.*, 2014) washed the selected explant thoroughly under running tap water for 30 min and with Tween–20 for 10 min. Further surface disinfected with 70% alcohol for 30sec after this explants treated by 0.1% (w/v) aqueous solution of mercuric chloride for 5 minutes and again rinsed with double distilled water.

2.5.1 Shoot Induction

The advances made in tissue culture technology are directly related to the mineral content and overall composition of the culture media. Initially, tissue culturists used Knop's mineral solution (Gautheret, 1942; Nobecourt, 1937) and white's medium supplemented with various trace elements. Later Murushige and Skoog (1962) proposed a solution for tobacco tissues with concentrations of salts 25 times higher than in Knop's solution. This medium allowed five to seven times more active growth than other media. (McComb, 1978) proposed that at higher levels, cytokinins tend to induce adventitious bud formation. (Lane 1979, Bhojwani 1980) found for shoot proliferation, growth regulators especially cytokinins one of the most important factors affecting the shoot response. Stimulating axillary branching by *in vitro* culture of nodal explants is the common techniques of micro propagation proposed by (Lawrence, 1981). Scale from *in vitro* cultured bulblets of lily divided into halves were used as explants. The induction phase (Sub culture I) was performed on MS medium supplemented with 30 g/l sucrose and growth regulators medium R0 (0.1 mg/l NAA); medium R1 (0.1 mg/l NAA+ 1 mg/l BA); medium R10 (0.1 mg/l NAA + 10 mg/l BA (Mensuali *et al*., 1990). (Sharma *et al*., 1991) reported that *in vitro* clonal multiplication was best achieved on MS medium supplemented with kinetin (2.0 mg/l) and IAA (1.0 mg/l) when nodal stem segments of *Coleus forskohlii* were used. (Sen and Sharma, 1991) assessed shoot multiplication *in vitro* within 20-25 days of culture from shoot tip explants obtained from 30 days old aseptically germinated seedlings of *Coleus forskohlii*, using 2.0 mg/l of BA. (Sen *et al*., 1992) also initiated cultures from shoot tip explants of *Coleus forskohlii* on non supplemented MS basal medium as well as on MS medium supplemented with BAP (0.5- 2.5 mg/l). The best effect was observed with 2.0 mg/l BAP. In the absence of BAP however, growth of leaves, roots and elongation of shoots were much pronounced. A range of cytokinins (Kinetin, BAP) has been used in micropropagation work (Bhojwani and Razdan 1992). Beneficial effect of high sucrose concentrations on different events of morhogenesis like shoot multiplication, rooting of microshoots (Rahman *et al.,* 1992 and somatic embryogenesis (Loiseau *et al.,* 1995). (Kumar *et al.,* 1999; Elisson, 1978) reported heat shock treatment to the shoot cultures also promoted rooting on medium containing high sucrose concentrations in Gladiolus. (Custers and Bergervoet, 1993) used Sprouts and tips from tubers during the first month of storage proved to be good explants to start cultures of Gloriossa, addition of low level of 6-benzyladenine (BA) in

the medium (1.0 mg/l) improved *in vitro* plant growth, where as high level of BA (10 mg/l) caused proliferation of multiple shoots from the rhizome meristems. By applying alternatively the high and low BA levels, a method of continued micro propagation achieved, which resulted in 4-7 fold multiplication of qualitatively good plantlets every 18 weeks. *In vitro* clonal multiplication of Safed musli has been achieved by (Purohit *et al.*,1994) on Murashige and Skoog 1962 (MS) medium supplemented with 22.2 µM N6-Benzyladenine (BAP) using young shoot bases as explants. which resulted in a mean number of shoots (11.0) after 21 days of inoculation. Somatic embryogenesis in callus culture of *Chlorophytum borivilianum* first observed in immature zygotic explants and intact ovules . (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-D) which 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 those embryos. On plant growth regulator free basal MS medium, about 20% of these embryos were successfully converted into plantlets. Cytokinins shown to be the most critical growth regulators for shoot proliferation of many medicinal plants (Chen *et al.,* 1995, Rout *et al.,* 2000, Martin *et al.,* 2005, Raghu *et al.,* 2006). (Mohamad, 1997) developed a protocol for the micro propagation of crinum lily plant from inflorescence. Explants cultured on MS medium alone or supplemented with 4.4, 8.8, 13.3 µM BA and 0.5 µM NAA and incubated for 4 weeks. Maximum shoot proliferation found on medium (MS medium supplemented with 4.4 µM BA and 0.05 µM NAA). (Tefera, 1998) abtained shoot proliferation through enhanced axillary bud release in shoot tip and nodal segments. The maximum number of shoots (17.33) obtained at 6 weeks of culture. The use of basal MS media encouraged the production of only single shoot. (Nhut, 1998) used shoot tips from stems of dormant bulbs were cultured on one half strength of MS medium. Stems from plantlets derived from the shoot tips were cut into nodal segments, which were then cultured on MS medium supplemented with 2.3 µM BA. *Gloriosa superba* was micropropagated using apical buds on MS medium supplemented with 1-8 mg/l BAP and 1-2 mg/l IAA. Shoot multiplication and growth was best with BAP concentration at 4.0 mg/l (Agarwal *et al*., 1999). An efficient method developed by (Suri *et al.,* 1999) for the rapid multiplication of *Chlorophytum borivilianum*. Explants produced large number of shoots (15/explant) during first passage on B5 medium supplemented with 0.18 mM Kn and 22 µM BAP. (Dapkuniene *et al*., 2000) used bulblets of the lily as explants for culture in MS medium

supplemented with 5.0 mg/l benzyladenine and 0.1 mg/l NAA. (Xiao Ying *et al*., 2000) studied growth and callus induction on immature lily embryos. The results indicated that the 35 days old immature embryo grew well on MS medium containing 1.0 mg/l IAA, 1.0 mg/l GA3 and 6-8 per cent sucrose, attaining maturity after 30 days. Light green callus formed on immature embryos cultured on MS medium containing 0.1 mg/l NAA and 1 mg/l BA. The survival rate of the regenerated, transplanted plantlets was more than 90 per cent. An efficient protocol established by (Sivakumar and Krishnamurthy, 2000) for micro propagation of *Gloriossa superba* from shot tips and non-dormant apical buds. (Raun *et al.*,2001) developed the *in vitro* propagation of explants of Lilium cultured on MS medium containing different concentrations of 2,4-D, benzyl adenine, gibberellic acid, NAA and kinetin. (Pudake and Dhumale, 2003) given improved method for the large scale multiplication of *Chlorophytum borivilianum* through shoot base and stem disc culture. *In vitro* multiplication achieved on Murashige and Skoog medium supplemented with 2 mg/l benzyl adenine. (Dave *et al.,* 2004) found multiplication rates of 2.5 to 3 fold per 3 weeks through micropropagation. (Thakare *et al.*, 2004) attempted *in vitro* induction and characteristics of callus of *Chlorophytum tuberosum.* The explant consisting of leaf, tubers, and hypocotyl of *in vitro* germinated seed of *C. tuberosum* used. Initiation of callus was highest during 68 days in leaves on MS media supplemented with 5 mg 2,4-D/l and 1mg kinetin/l. The maximum callus induction (91.6%) observed ,the proliferated calli transferred for shoot regeneration. Combination of 2.0 mg/1 BAP + 1.0 mg/l (NAA) most effective for shoot regeneration from callus (13.7 shoot/callus). (Wawrosch *et al.,* 2005) observed that the unusual growth pattern may be attributed to prolonged growth in liquid medium under slow agitation and continuous agitation obstructs apical dominance which ultimately leads to increased adventitious branching. (Velayutham *et al.,* 2006) demonstrated that the highest number of shoots obtained on MS medium with 4.0 M BAP + 1.0 M IAA for *Chlorophytum borivilianum.* (Davood and Behzad, 2008) found after 21 days of inoculation results well documented in safed musli medium supplemented with 0.5 mg/l BAP + 0.5 mg/l NAA. (Thakur *et al.,* 2008) carried out extraction of saponins and sapogenins by chromatography and quantification of individual saponin aglycones and sugars performed by (Jin *et al.,* 2004 and Xin *et al.*, 2003). Extraction, chromatography and quantification of amino acids performed by (Hess and Sherma 2004). (Haque *et al.,* 2009) inoculated shoot buds on semisolid modified MS medium supplemented with

different combinations of Kn (0.0, 1.0, 1.5, 2.5, 3.0, 3.5, 4.0,4.5 mg/l) and BAP (0.01 with 1.5, 2.0, 2.5, 3.0, 3.5, 4.0,4.5 mg/l) or BAP or Kn with IAA,IBA and NAA for shoot proliferation. (Scheidt *et al.,* 2009) proposed that liquid culture to be ideal in micropropagation and automation and reduced the cost of manul labour (Roels *et al.,* 2006) and for reducinplant production cost many plant propagated in mass on liquid culture medium using bioreactor. (Haque *et al.,* 2009) found the maximum number of shoots obtained in MS medium supplemented with Kn (3.0 mg/l) and BAP (2.0 mg/l), but shoots small when the concentration of kinetin raised. MS medium containing low concentration of auxin along with cytokinine increases the rate of shoot multiplication. 73 shoots/culture produced at a combination of Kn (3.0 mg/l) and BAP (2.0mg/l) and 8 shoots / culture produced at a combination of Kn (6.0 mg/l) and BAP (2.0 mg/l). (Usha *et al.,* 2010) inoculated shoot tips on the culture medium Murashige and Skoog (1962) supplemented with 3% (w/v) sucrose and different concentrations of 6-benzylaminopurine (BA; 0.0, 2.22, 4.44, 6.66, 8.87, 11.09 and 13.3 μM) or kinetin (0.0, 2.32, 4.65, 6.97, 9.29, 11.6 and 13.9 μM) plus 1-naphthaleneacetic acid (NAA; 1.34 and 2.69 μM) or indole-3-acetic acid (IAA, 1.42 and 2.85 μM) tested for shoot multiplication. (Kemat *et al.,* 2010) described an efficient protocol for shoot proliferation, MS medium supplemented with various concentrations of Cytokine (BAP 0 – 1.0 mg/l). Three shoot clumps (each clump of 2-3 shoots) inoculated per culture flask in triplicates. In another experiment MS media supplemented with 30 gm/l agar + 0.5 mg/l BAP. For callus induction, MS medium supplemented with various concentration combination of Cytokine (BAP 0-1 mg/l) and Auxine (NAA 0-1 mg/l). (Rizvi *et al.,* 2012) studied on effect of variation in culture conditions on shoot biomass production performed separately for each variable. Sucrose content varied from 0 (control) to 9%, pH of culture medium ranged from 3.86 to 7.86, inoculum density varying from 0.09-0.90 g/culture flask and different volumes (10-60 ml) of the culture medium/culture flask tested. The *in vitro* cultures of *C. borivilianum* grown in 250 ml Erlenmeyer flasks containing liquid MS growth medium supplemented with 22.2 μM BAP. All the experimental studies performed on the same medium composition. (Galal, 2012) studied on physiological phenomena influenced by SA include seed germination, stomatal regulation photosynthesis and stress tolerance in plants. The promotory role of salicylic acid in callus growth, shoot development, rooting and hardening of *in vitro* derived plantlets has been observed in Ziziphus spina- Christi. (Basu and Jha 2013) described an alternative protocol for the development of a stable fast growing, non transformed root culture system in *C. borivilianum* and the

simultaneous quantitative analysis of total steroidal saponins, amino acids and sugars. To proliferate the callus, explants taken and subcultured into full strength MS basal medium supplemented with different concentrations of either 2,4-D (0.25, 0.5, 1.0, 1.5 or 2.0 mg/l) or BAP (0.25, 0.5, 1.0, 1.5 or 2.0 mg /l) alone and also in combination with Kin (0.25, 0.5, 1.0, 1.5 or 2.0 mg /l for massive callus induction (Rout *et al.*, 2013). (Rizvi *et al.*, 2013) represented an Artificial Neural Network (ANN) based computational scheming of chemical and biological parameters at flask level for mass multiplication of plants through micropropagation, using bioreactors of large volumes. The optimal culture environment at small-scale for maximum shoot biomass yield in *Chlorophytum borivilianum*, a medicinal plant predicted by using Elman back propagation neural network (Elman-BPNN) approach, in terms of sugar content, pH of the culture medium, inoculum density (no. of shoot base explants/culture flask), and volume of growth medium/culture flask. New shoots started to produced shoots on MS medium + BAP 0.5 mg/l concentrations. The callus induction in *C. borivilianum* at 0.50 mg/l BAP and 0.25 mg/l NAA observed and maximum cell mass with brown coloured callus developed (Nakhate *et al.*, 2014). (Rizvi and Kukreja, 2014) used a bench top stirred bioreactor for propagation of *C borivilianum* with liquid medium by multiple shoot culture, one week old shoot along with shoot base 1.5cm inoculated in liquid medium shake flask containing 22.2 µM BAP. An inoculums 120 explants/2.5 l Ms media supplemented with BAP found optimal for shoot regeneration. After 4 weeks of culture 4.4 fold increase in biomass.

2.5.2 ROOTING

(Hussey, 1977) transferred shoots to media with low concentrations or no cytokinin spontaneously formed the roots. Root induction in single shoots occurred in the presence of IAA and IBA in the media. (Sharma *et al.*, 1991) found IAA at 1.0 mg/l proved the best, with root initiation being observed within a week after subculture. Rooting observed highest in half the strength of MS medium supplemented with low concentration of IBA. (Rehman *et al.*, 1992) abtained the maximum of (52.40) roots obtained in 1.0 mg/l IBA with an average length of 6.60 cm. (Bhojwani and Razdan, 1992, genotype (Rines and McCoy, 1981) reported rooting response of microshoots as well as cultural conditions (Murashige, 1977) to be controlled by growth regulators in the medium. 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). Hardening of

tissue culture plants is the most crucial step in micropropagation. The plants produced are very soft to face ambient environmental conditions 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 poly house. After 21 days of culture on rooting media, the plantlets shifted to polythene bags filled with various soil mixes for hardening prior to final transfer to natural conditions (Bhojwani and Razdan, 1992). (Purohit *et al.,* 1994) developed an *in vitro* multiplication of the species *Chlorophytum borivilianum* on MS medium supplemented with 22.2 µM BA using shoot bases as explants. All shoots rooted when transferred to MS medium containing ¾ strength organic and inorganic constituents and 9.8 µM IBA. The percentage of shoots that formed roots and the number of roots per shoot varied significantly with different concentrations of IBA, IAA, or IBA+ IAA. Optimal rooting (94.1%) with no intervening callus observed within 10-14 days of transfer to medium containing 4.90 µM IBA+ 2.85 µM IAA with 2% of sucrose (Chen *et al*. 1995; Rout *et al*., 2000; Martin *et al*., 2005; Raghu *et al*., 2006). The excised shoots transferred to half strength MS basal semisolid medium supplemented with different concentrations and combinations of Indole-3-butyric acid (IBA; 0.0, 0.49, 1.23, 2.46 and 4.90 µM) or IAA (0.0, 0.57, 1.42, 2.85 and 5.71 µM) and 2% sucrose for root initiation. This is in concordance with the results of (Sahoo and Chand 1998) and (Chandramu *et al.,* 2003) and agreement with the findings of (Rani and Nair 2006). (Saurabh *et al.,* 2005) reported that good rooting response observed when individual regenerated shoots inoculated onto MS with 1.48M IBA, 3.90M ancymidol and 3% sucrose. Sterilized soil minimized the cost of transplantation as documented by (Anand *et al*., 1997). (Nhut, 1998) reported multiple shoots derived from micropropagation of *Lilium longiflorum* rooted on half of the strength of MS medium containing 1.1 µM NAA. (Tefera, 1998) reported that although IAA at 1.0 mg/l effective in inducing roots, normal rooting observed in hormone free medium. Rooting of regenerated shoots of *Chlorohytum borivilianum* best in B5 medium containing 0.49 µM IBA. Plantlets formed *de novo* tuberous roots (3/plant) from the stem region in a medium containing IBA 0.49 µM, glucose (10 g/l) and sucrose 80 g/l (Suri *et al.*,1999) within 10-14 days of transfer to medium containing 4.90µM IBA+2.85µM IAA with 2% sucrose. (Purwito *et al.,* 2001) found *in vitro* produced shoots from bulbs of calla lily rooted in one-half strength MS medium supplemented with 1mg/l IBA and the highest survival in the field. In *Chlophytum borivillianum* plantlets rooted by subculturing them into the medium containing kinetin or BAP (5.0 mg/l) and IBA (4.0 mg/l). Then plantlets

successfully transferred to pots containing a mixture of soil, sand and farmyard manure (2:1:1) (Jadhav and Hegde, 2001). Regenerated shoots of lily transferred to bulb induction medium containing 60 g sucrose, 4.4 µM BA and 0.05 µM NAA. These shoots rooted and transferred to soil (Mohamad, 2002). (Pudake and Dhumale, 2003) reported among the different combinations involving auxins (IBA/NAA) tested for rooting, IBA (3.0mg/l) relatively better. Upto 90% plantlets established in pots by hardening treatment where the plants first transferred to sterile sand and kept in mist chamber under high humidity. (Ganorkar *et al.,* 2004) recorded highest tuber per plant and tuber yield of safed musli with the application of 75 kg N per ha. However it on par with application of 50 kg N per ha application of 75 kg per ha increased the fresh and dry root yield of safed musli over all the nitrogen levels, except 50 kg N per ha. (Wankhade *et al.,* 2004 and Kothari and Singh, 2003). Application of 75 kg N per ha recorded the highest uptake of nitrogen over 50 kg and 25 kg per ha at all the growth stages of safed musli (Ganorkar *et al.,* 2004). (Haque *et al.*,2009) reported four week old healthy excised shoots on transfer to 1/2 strength MS medium containing 3% sucrose (w/v) supplemented with 1.0mg/l IBA formed vigorous fibrous and thick strong roots during the culture of 4 weeks. Maximum roots / shoots observed in 1/2 strength MS medium containing 1.0 mg/l IBA. (Velcheva *et al.,* 2005; Debiasi *et al.,* 2007) reported IBA is more efficient than NAA for root proliferation in safed musli. (Rout and Sahoo, 2013) proposed the elongated shoots rooted in half-strength MS medium supplemented with different concentrations of auxins (NAA , IAA and IBA). NAA more suitable for root induction (6.2 shoots/callus) when compared to IAA and IBA (3.5 and 3.7, respectively). The in vitro regenerated plantlets successfully transferred to the greenhouse for acclimatization. The survival of the plantlets under ex vitro condition was recorded as 77% . Roots of *Chlorophytum borivilianum* regenerated in MS medium containing 3.0 mg/l NAA gave more number of roots. The root proliferation observed on full strength of MS medium after inoculation with the increasing concentration (1.0 to 3.0 mg/l) for indole acetic acid (IAA) at 1.0mg/l and naphthalene acetic acid (NAA) at 3.0 mg/l. (Nakhate *et al.,* 2014) transferred the regenerated shoots to MS medium containing various auxins IAA (0– 1.0mg/l) and heat shock also analyzed on rooting. In another 250 ml flask 10 – 15 shoot clumps inoculated in each flask in triplicates and root development recorded after 21 days. (Nakhate *et al.,* 2014) reported that root induction in the regenerated shoots grown in IAA with different concentrations the beneficial effect observed in 0.25 mg/l IAA medium, showing mean number 3.06 units of roots initiated. Plantlets transplanted to small plastic cups for

hardening pots containing sand, soil, vermicompost (1:1:1). The transplanted plantlets kept under shades and then transferred to normal environmental condition through this process of acclimatization almost 76% survivals of the tissue cultured plantlets achieved. (Rizvi and Kukreja, 2014) reported shoot regenerated in bioreactor rooted *ex vitro* on 3-4 strength of liquid medium supplemented with 9.8 µM IBA Plantlets with 100% rootting of microshoot hardened and established in glasshouse with 85% survival rate.

Chapter–3

MATERIALS AND METHODS

3.1 MICROPROPAGATION

To meet the objective of the work, experiments were conducted in plant tissue culture laboratory, College of Biotechnology, Birsa Agricultural University, Ranchi during year 2013-2014. A thorough insight of the materials used and the methodology applied during the course of the experimentation is as follows:

3.1.1 Materials:-

3.1.1.1 Selection of mother plants:

Healthy and mature plants of *Chlorophytum borivilianum* growing in mother nursery of College of Biotechnology, B.A.U., Ranchi were used as source of explants.

3.1.1.2 Collection of explants: Shoot tips were used as explants.

3.1.2 Methods:-

3.1.2.1 Preparation of culture media

3.1.2.1.1 Composition of MS basal media:

For *in vitro* regeneration in *Chlorophytum borivilianum* explants, 34.41gm/l of dehydrated medium (Hi media) dissolved in double distilled water and supplemented with various combinations of plant growth regulators (cytokinins and auxins).

Table 3.1: Chemical Constituents of MS basal media

	Amount(mg/l)
Macro nutrients	
Solution I	
KNO_3	1900.0
NH_4NO_3	1650.0
$MgSO_4.7H_20$	370.0
$KH_2.PO_4$	170.0
Solution II	
$CaCl_2.2H_2O$	440.0
Solution III	
$ZnSO_4.7H_2O$	8.6

H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Solution IV	
$MnSO_4.4H_2O$	22.3
Solution V	
KI	0.83
Solution VI	
Na_2EDTA	32.75
$FeSO_4.7H_2O$	27.50
Solution VII	
Nicotinic acid	0.5
Pyridoxin- HCl	0.5
Thiamine-HCl	0.1
Solution VIII	
Myoinositol	100.0
Solution 1X	
Glycine	2.0

Hormonal regime

For Bud breaking and multiplication

1. MS basal media
2. MS basal media + KIN (1.0, 3.0, 5.0 mg/l)
3. MS basal + BAP (2.0, 4.0 ,6.0 mg/l)
4. MS basal + BAP (6.0 mg/l) + $AdSO_4$ (25.0 ,50.0 , 75.0, 100.0 mg/l)
5. MS basal + BAP (2.0 mg/l) + $AdSO_4$ (50.0 mg/l)
6. MS basal + BAP(6.0 mg/l) + $AdSO_4$ (100.0 mg/l) + IAA (0.2 mg/l)
7. MS basal + BAP (2.0 mg/l) + KIN (1.0 mg/l)

For Root Induction

8. MS basal + IBA (1.0 , 2.0 , 3.0 , 4.0 mg/l)
9. MS basal + IBA (2.0 , 3.0 mg/l) + IAA(2.0 mg/ l)
10. MS basal + IBA (2.0 mg/l) + Charcoal (2.0 gm/l)

11. MS basal + IBA (2.0 mg/l) + IAA (2.0 mg/ l) + Charcoal (2.0 gm/l)

The pH of the prepared media was adjusted between 5.6- 5.8 (using 1N HCl and 1N NaOH as per need). As a gelling agent 0.75% (w/v) agar was added to the media. To dissolve agar properly, media was boiled. The ready media was then poured into 300 ml capacity of sterilized glass bottle (50 ml/ bottle) and capped tightly with autoclavable polypropylene caps.

3.1.2.2 Sterilization

3.1.2.2.1 Sterilization of glassware and media

In plant tissue culture, sterilization is an important step to prevent any microbial contamination (fungal, bacterial etc). Double distilled water, glassware, cotton wools, brown papers and other instruments were autoclaved at 121.6^0 C temperature and 15 lbs/sq. inch pressure for one hour and sterilized glass bottles containing media were autoclaved for 15 minutes at the same temperature and pressure.

3.1.2.2.2 Sterilization of explants

After thorough washing under tap water for 30 minutes, all explants were treated with 0.1% bavistin, a systemic fungicide. The shoot tips were treated for 30 minutes. Then the explants were washed thoroughly with distilled water (3-4 times). The explants were then surface sterilized with 0.05%, 0.1% 0.15% and 0.20 % (W/V) $HgCl_2$ for, 10, 15 and 20 minutes under laminar air flow. After that, explants were washed 3-4 times with sterile distilled water to remove each and every trace of mercuric chloride. The effect of different concentrations of $HgCl_2$ viz. 0.05%, 0.1%, 0.15% and 0.20% with respect to treatment durations was noted and survival percentage was recorded.

3.1.2.3 Establishment of culture

Surface sterilized explants were inoculated aseptically under laminar air hood on MS media supplemented with different combinations and concentrations of plant growth hormones to induce bud breaking. Percentage of explants showing bud breaking on media was recorded after 4 and 6 weeks.

3.1.2.4 Maintenance of culture

After inoculation, the culture bottles were kept in the culture room at $25^o \pm 2^o$ C temperature with photoperiod regime of 16 hours light (at 3000 lux approx) and 8 hrs of dark period. The relative humidity was adjusted to approximately 60 %.

3.1.2.5 Subculture

The shoot tip of *Chlorophytum borivilianum* were transferred into new bottle of same media to overcome excessive phenolic exudation and subcultured at every 10- 15 days. The number of shoots multiplied on different media supplemented with different concentrations was recorded at different time interval (after 4 and 6 weeks)

3.1.2.6 Root induction

For *in vitro* root initiation, the regenerated shootlets were excised and transferred to MS basal media in different concentrations and combinations of hormonal regime and result was recorded after 3 and 5 weeks.

3.1.2.7 Hardening

The cultured plantlets were taken out from the media and were thoroughly washed with tap water to remove adhering media from them. For primary hardening, the plantlets were transferred to portray containing coco peat and vermicompost (1:1). The portrays were then kept in poly tunnel for 15 days. After that the plantlets were transferred to poly bags containing soil: sand: FYM (1:1:1) for secondary hardening and kept under the shade net.

3.1.2.8 Observations

The cultures were examined periodically and the responses were recorded on the basis of visual observations. The effects of different treatments in respect to time were noted and data have been represented as mean percentage and mean number.

RESULTS AND DISCUSSION

4.1 Micropropagation of *Chlorophytum borivilianum*

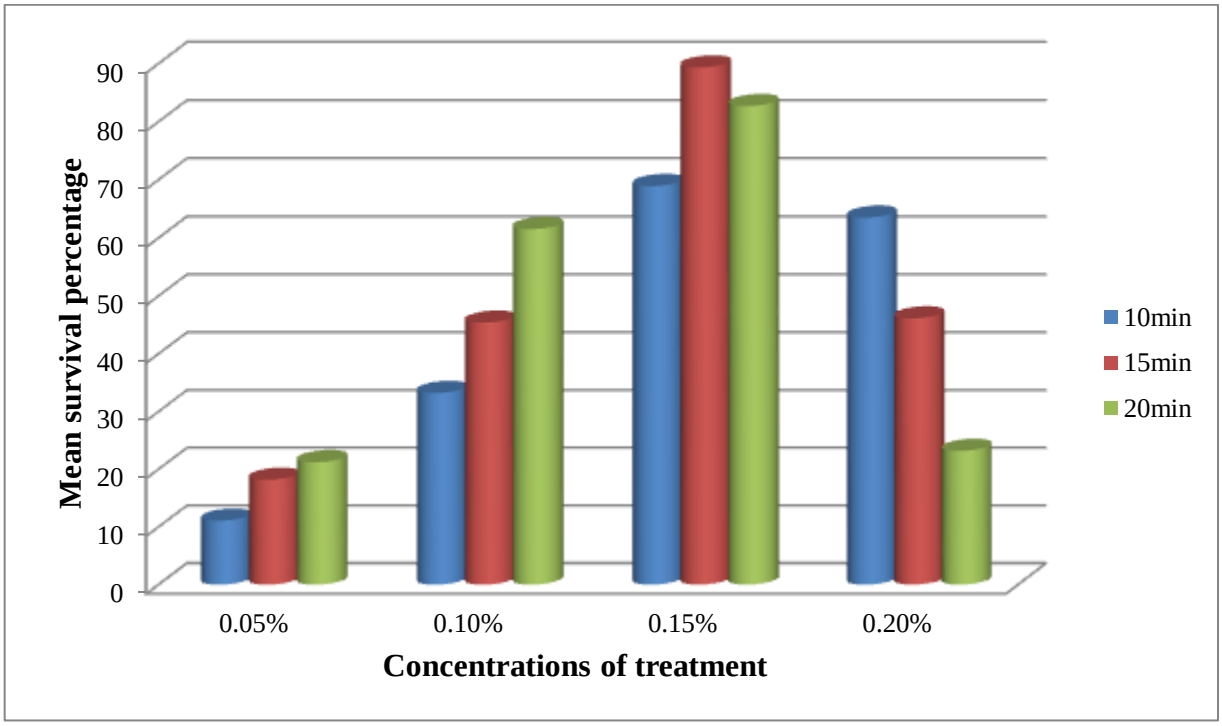
4.1.1 Standardization of surface of sterilization of the explants

In the present work mercuric chloride was used as sterilant. Simiral use of mercuric chloride have also been reported by several authors (Haque *et al*., 2009, Usha *et al*., 2010, Rani and kumar, 2011, Garima and Shruthi, 2012, Rout and Sahoo, 2013) who used to sterilize various explants of *Chlorophytum borivilianum.* To optimize surface sterilization shoot tips of *Chlorophytum borivilianum* were treated with various concentrations (0.05% , 0.10% , 0.15% ,0.20%) and durations (10min ,15min, 20min) of $HgCl_2$. Maximum survival (percentage 89.33) of shoot tip was observed when treated with 0.15% for 15min (table 4.1 and graph4.1). The increase of $HgCl_2$ concentrations as well as duration of treatment reduced the survival of explants.

Table 4.1: Efficacy of $HgCl_2$ treatment on mean survival percentage of Shoot tips of *Chlorophytum borivilianum*.

$HgCl_2$ concentrations (%)	**Mean survival Percentage**		
	10 Minutes	**15 Minutes**	**20 Minutes**
0.05	11.09	18.18	21.23
0.10	33.32	45.40	61.53
0.15	68.92	**89.33**	82.70
0.20	63.49	46.15	23.18

Graph-4.1: Efficacy of $HgCl_2$ treatment on mean survival percentage of Shoot tips of *Chlorophytum borivilianum* .



4.1.2 Bud breaking

For bud breaking, sterilized explants were aseptically inoculated on MS media supplemented with different concentrations and combinations of hormonal regime. In contrast to the finding of (Garima and Shruthi, 2012) , who reported sprouting of buds after 18 days, the shoot initiation was observed after 12 days of culture in the present study. Amongst 13 different combinations as well as concentrations of phytohormones, highest bud breaking 85.18% was observed on MS media supplemented with 6.0 mg/l BAP after 4 weeks and 88.83% after 6 weeks of inoculation of shoot tip explants (table 4.2 and graph 4.2). Bud breaking percentage was found 82.88% after 4 weeks and 85.37% after 6 weeks when inoculated in MS supplemented with 6.0 mg/l BAP in combination with 100.0 mg/l $AdSO_4$ and 0.2 mg/l IAA. BAP in combinations with $AdSO_4$ in 5 different concentrations were also tried of which 6.0 mg/l BAP in combination 100.0 mg/l $AdSO_4$ was found best showing 81.81% after 4 weeks and 85.23% of bud breaking after 6 weeks (table 4.3, and graph 4.3). Three different concentrations of KIN were also used for initiation out of which 5.0 mg/l KIN was found best showing 68.33% and 74.48% bud breaking after 4 and 6 weeks respectively (table 4.4 and graph 4.4) . While BAP in combinations with KIN concentration was not found effective in bud breaking, it was 71.48% after 4 weeks and 75.08% after 6 weeks

when inoculated in 2.0 mg/l BAP in combination 1.0 mg/l KIN (table 4.2 and graph 4.2) So, BAP alone was found more effective for bud breaking than BAP along with adenine sulphate, IAA and Kinetin. During fresh culture establishment maximum phenolic exudation was observed immediately after each inoculation. The effect of exudates was found toxic to the plants. Rapid subculture could not found effective to prevent phenolic exudation. Bud breaking was declined when cultured for long period in the same media due to accumulation of phenolic compound leading to browning of culture media. A comperative account of effect of all combinations of hormones used was shown in table 4.5 and graph 4.5.

Table-4.2: Effect of various concentrations of BAP alone and in combinations with KIN on mean bud breaking percentage on shoot tip explants

Concentrations	Mean percentage of bud breaking	
	After 4 weeks	After 6 weeks
2.0 mg/l BAP	70.07±0.06	74.33±0.04
4.0 mg/l BAP	74.88±0.08	77.37±0.11
6.0 mg/l BAP	**85.18±0.04**	**88.83±0.01**
2.0 mg/l BAP+1.0 mg/l KIN	71.48±0.14	75.08±0.08

Graph-4.2: Effect of various concentration of BAP alone and in combinations with KIN on mean bud breaking percentage on shoot tip explants

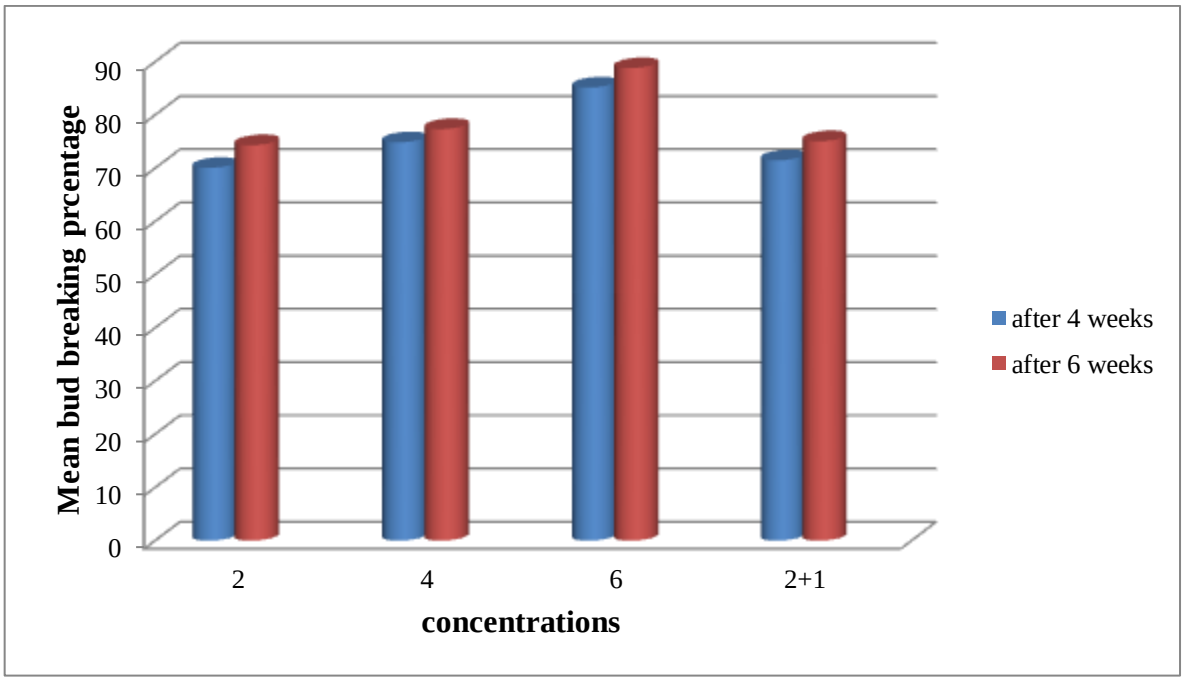


Table-4.3: Effect of various concentrations and combinations of BAP and $AdSO_4$ with and without IAA on mean bud breaking percentage on shoot tip explants

Concentrations	Mean percentage of bud breaking	
	After 4 weeks	After 6 weeks
2.0 mg/l BAP+50.0 mg/l $AdSO_4$	72.68±0.16	75.25±0.10
6.0 mg/l BAP +25.0 mg/l $AdSO_4$	73.19±0.10	76.18±0.09
6.0 mg/l BAP +50.0 mg/l $AdSO_4$	79.95±0.22	84.21±0.66
6.0 mg/l BAP +75.0 mg/l $AdSO_4$	80.92±0.18	84.23±0.12
6.0 mg/l BAP +100.0 mg/l $AdSO_4$	81.81±0.09	85.02±0.05
6.0 mg/ BAP l+100.0 mg/l $AdSO_4$+ 0.2 mg/l IAA	**82.88±0.06**	**85.37±0.05**

Graph-4.3: Effect of various concentrations and combinations of BAP and $AdSO_4$ with and without IAA on mean bud breaking percentage on shoot tip explants

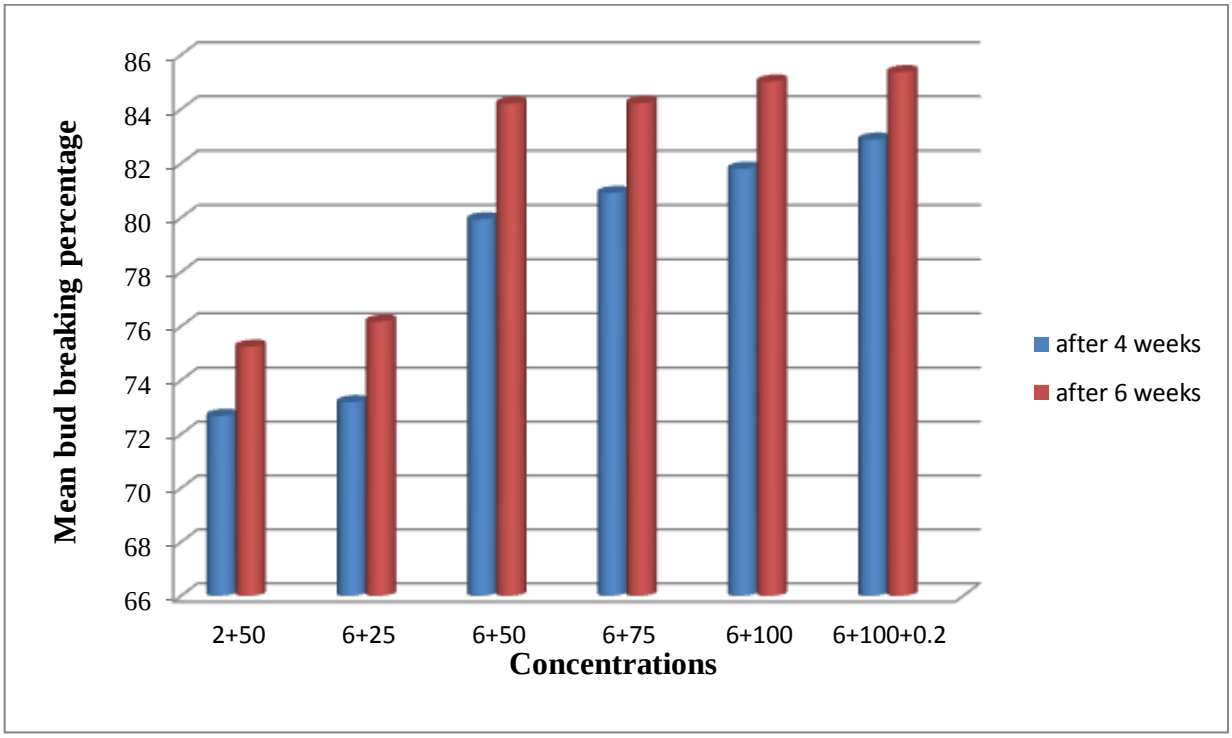


Table-4.4: Effect of different concentrations of KIN on mean bud breaking percentage on shoot tip explants

Concentrations	Mean percentage of bud breaking	
	After 4 weeks	**After 6 weeks**
1.0 mg/l KIN	55.23±0.11	59.25±0.06
3.0 mg/l KIN	67.23±0.15	72.11±0.13
5.0 mg/l KIN	**68.33±0.13**	**72.48±0.16**

Graph-4.4: Effect of different concentration of KIN on mean bud breaking percentage on shoot tip explants

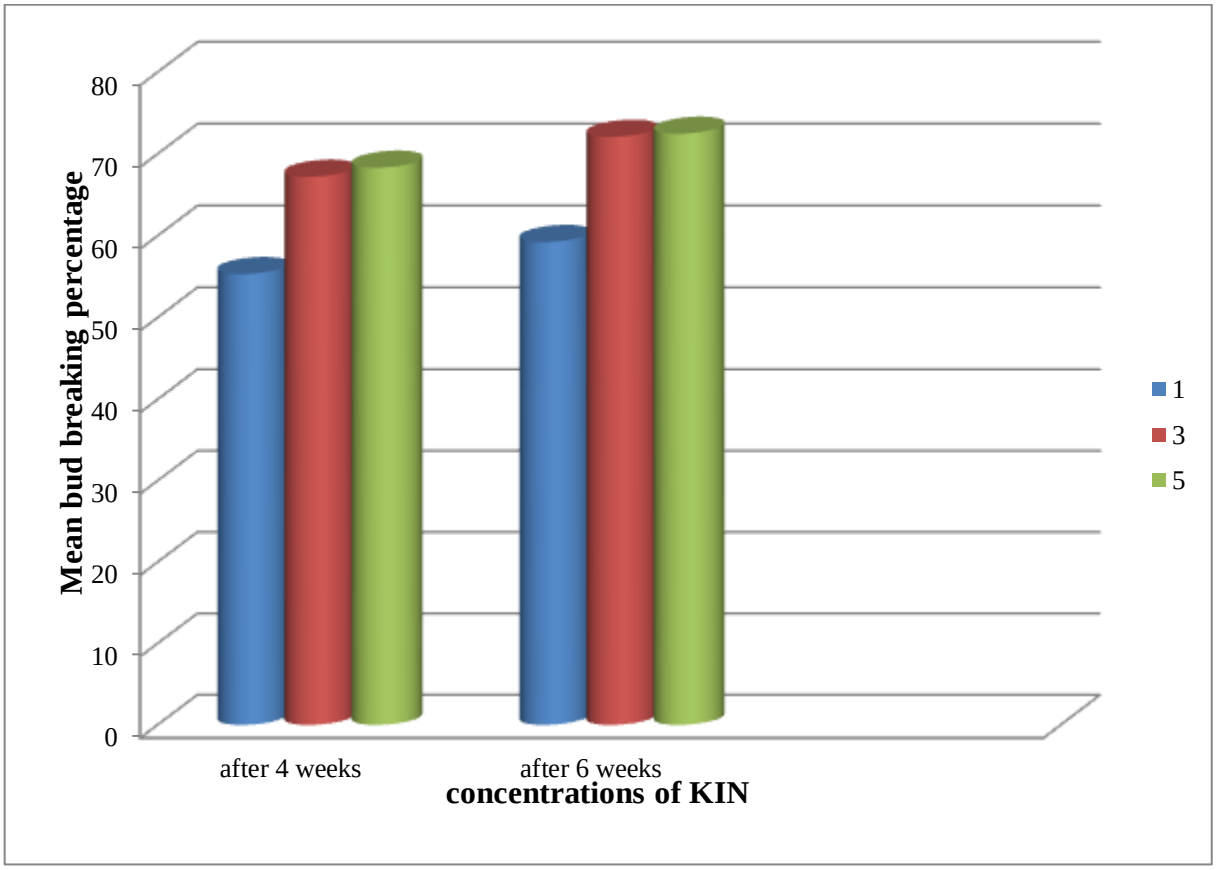
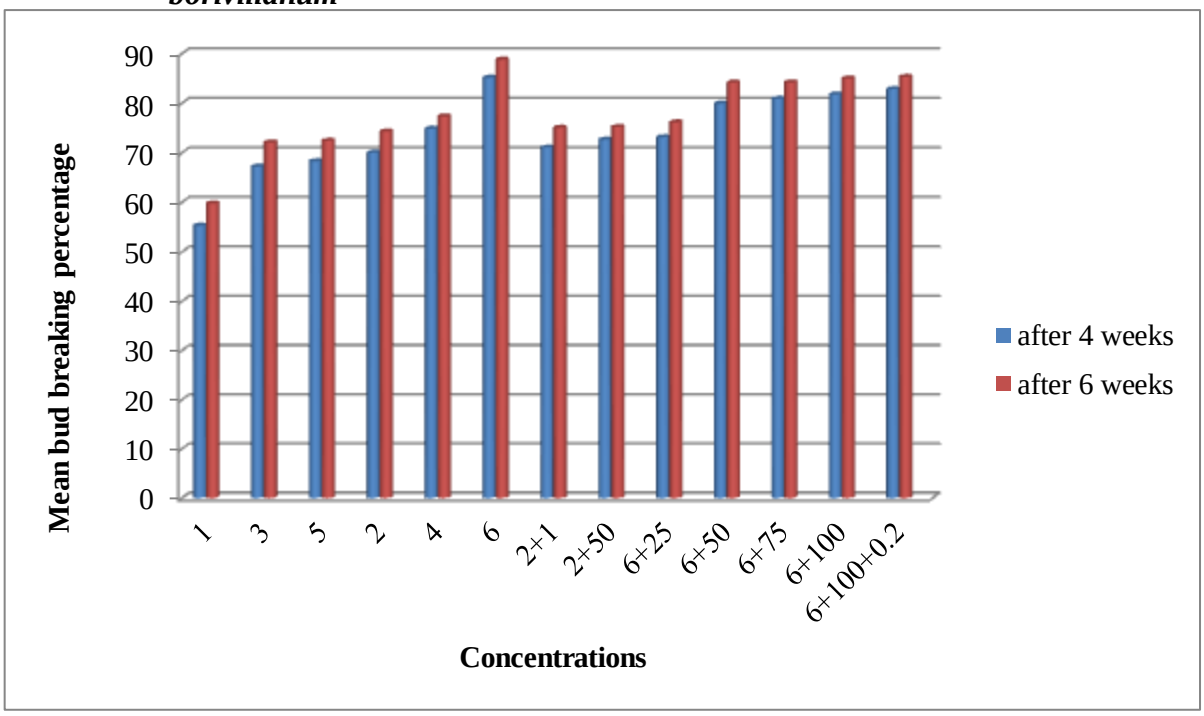


Table-4.5: Comparative account of effect of different concentrations and combinations of hormones on bud breaking of *Chlorophytum borivilianum*

Concentrations	Mean percentage of bud breaking	
	After 4 weeks	After 6 weeks
1.0 mg/l KIN	55.33±0.11	59.83±0.06
3.0 mg/l KIN	67.23±0.15	72.11±0.13
5.0 mg/l KIN	68.33±0.13	72.48±0.16
2.0 mg/l BAP	70.07±0.06	74.33±0.04
4.0 mg/l BAP	74.88±0.08	77.37±0.11
6.0 mg/l BAP	**85.18±0.04**	**88.83±0.01**
2.0 mg/l BAP +1.0 mg/l KIN	71.09±0.14	75.08±0.08
2.0 mg/l BAP +50.0 mg/l $AdSO_4$	72.68±0.16	75.25±0.16
6.0 mg/l BAP +25.0 mg/l $AdSO_4$	73.19±0.10	76.18±0.09
6.0 mg/l BAP +50.0 mg/l $AdSO_4$	79.95±0.22	84.21±0.66
6.0 mg/l BAP +75.0 mg/l $AdSO_4$	80.92±0.18	84.23±0.12
6.0 mg/l BAP +100.0 mg/l $AdSO_4$	81.81±0.09	85.02±0.05
6.0 mg/l BAP +100.0 mg/l $AdSO_4$ +0.2 mg/l IAA	82.88±0.06	85.37±0.05

Graph -4.5: Comparative account of effect of different concentrations and combinations of hormones on bud breaking of *Chlorophytum borivilianum*



4.1.3 Shoot multiplication

In present study shoot multiplication was found to be induced in same media which were found responsive for bud breaking. The number of shoot multiplied on MS media supplemented with different concentrations of hormones was recorded (table 4.6, 4.7, 4.8,). Highest number of shoots per explants 8.84 was observed after 6 weeks of inoculation on MS media supplemented with 6.0 mg/l BAP and minimum number of shoots was 4.75 on MS media supplemented with 1.0 mg/l KIN after 6 weeks of inoculation. In three different concentrations of BAP , 6.0 mg/l BAP was found best i.e 6.88 and 8.84 after 4 weeks and 6 weeks of inoculation respectively(table 4.6 , graph 4.6 and fig 4.2 and 4.3) and minimum number of shoot per explant 4.28 after 4 weeks and 4.68 after 6 weeks when inoculated in 2.0 mg/l BAP (table 4.6 , graph 4.6 and Fig 4.6 and 4.7). Media containing BAP along with $AdSO_4$ in different concentrations was also found effective for induction of multiplication. In media supplemented with 6.0 mg/l BAP and 100.0 mg/l $AdSO_4$, maximum mean number of shoot per explants was 6.06 after 4 weeks and 7.53 after 6 weeks of inoculation(table 4.7 , graph 4.7 and Fig4.10 and 4.11). while in 6.0 mg/l BAP +25.0 mg/l $AdSO_4$, the minimum mean number of shoots per explant was observed 5.05 after 4 weeks and 6.12 after 6 weeks of inoculation (table 4.7, graph 4.7 and fig 4.16 and 4.17). Second highest number of shoots per explants in BAP plus $AdSO_4$ and IAA was 6.10 in 6.0 mg/l of BAP and 100.0 mg/l $AdSO_4$ and 0.2 mg/l IAA after 4 weeks and 7.82 after 6 weeks of inoculation (table 4.7, graph 4.7, fig4.8 and 4.9). However the rate of multiplication in KIN containing media was found comperatively lower. Maximum number of shoot was found in media with 5.0 mg/l KIN i.e 4.32 after 4 weeks and 4.32 after 6 weeks(table 4.8, graph4.8, fig4.26 and 4.27) and minimum number of shoot was observed in 1.0 mg/l KIN 2.45 after 4 weeks and 2.75 after 6 weeks was observed (table 4.8 , graph 4.8 and fig 4.22 and 4.23). BAP and KIN combinations was found not that effective in shoot multiplication it was 4.45 after 4 weeks and 4.98 after 6 weeks when inoculated in 2.0 mg/BAP and 1.0 mg/l KIN (table 4.6, graph 4.6 and fig4.20 and 4.21). Moreover, BAP alone was found significantly effective to enhance bud breaking percentage as well as multiplication rate. Purohit *et al*., 1994 Haque *et al*., 2009 reported that maximum shoot prolifiration occured in BAP containing media However, present study deals with refinement of existing protocol of *Chlorophytum borivilianum* and as compared with the findings of (Rizvi *et al*.,2013) and(Purohit *et al*., 1994) the bud breaking percentage as well as rate of multiplication was found to increase significantly after addition of adenine sulphate. Use of $AdSO_4$ in

Chlorophytum borivilianum has not been reported earlier. A comperative account of effect of all combinations of hormones used for shoot multiplication was shown in table 4.9 and graph 4.9.

Table-4.6: Effect of different concentrations of BAP alone and with KIN on mean number of shoots per explant of *Chlorophytum borivilianum*

Concentrations	Mean no. of shoots/ explant	
	After 4 weeks	After 6 weeks
2.0 mg/l BAP	4.48±0.37	4.78 ±0.08
4.0 mg/l BAP	5.15±0.40	6.23±1.30
6.0 mg/l BAP	**6.88±0.38**	**8.84±1.42**
2.0 mg/l BAP +1.0 mg/l KIN	4.45±0.30	4.98±0.33

Graph-4.6: Effect of different concentrations of BAP alone and with KIN on mean number of shoots per explant of *Chlorophytum borivilianum*

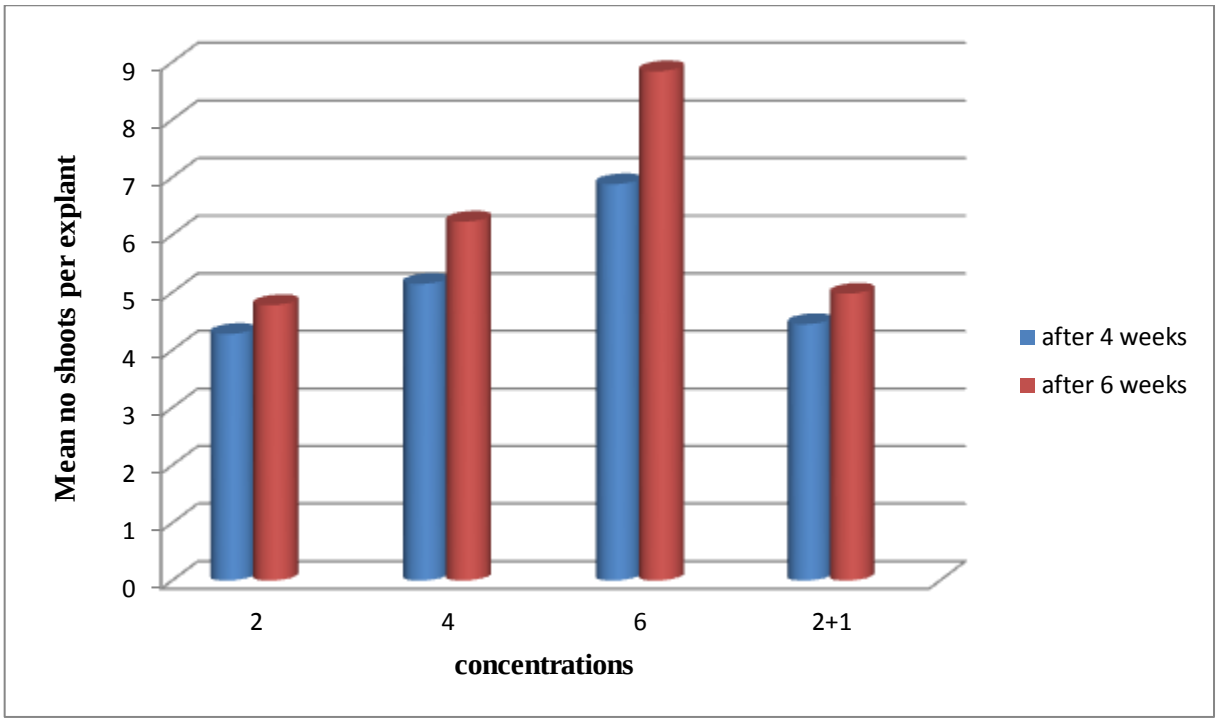


Table-4.7: Effect of different concentrations and combinations of BAP along with $AdSO_4$ and IAA on mean number of shoots per explant of *Chlorophytum borivilianum*

Concentrations	Mean no. Of shoots/ explant	
	After 4 weeks	**After 6 weeks**
2.0 mg/l BAP+50.0 mg/l $AdSO_4$	4.68±0.15	5.02±0.98
6.0 mg/l BAP +25.0 mg/l $AdSO_4$	5.05±0.85	6.12±0.23
6.0 mg/l BAP +50.0 mg/l $AdSO_4$	5.94±0.34	7.28±0.56
6.0 mg/l BAP +75.0 mg/l $AdSO_4$	6.02±0.50	7.35±1.17
6.0 mg/l BAP +100.0 mg/l $AdSO_4$	6.06±0.32	7.53±0.40
6.0 mg/l BAP +100.0 mg/l $AdSO_4$ + 0.2 mg/l IAA	**6.10±0.54**	**7.82±0.73**

Graph-4.7: Effect of different concentrations and combinations of BAP along with $AdSO_4$ and IAA on mean number of shoots per explant of *Chlorophytum borivilianum*

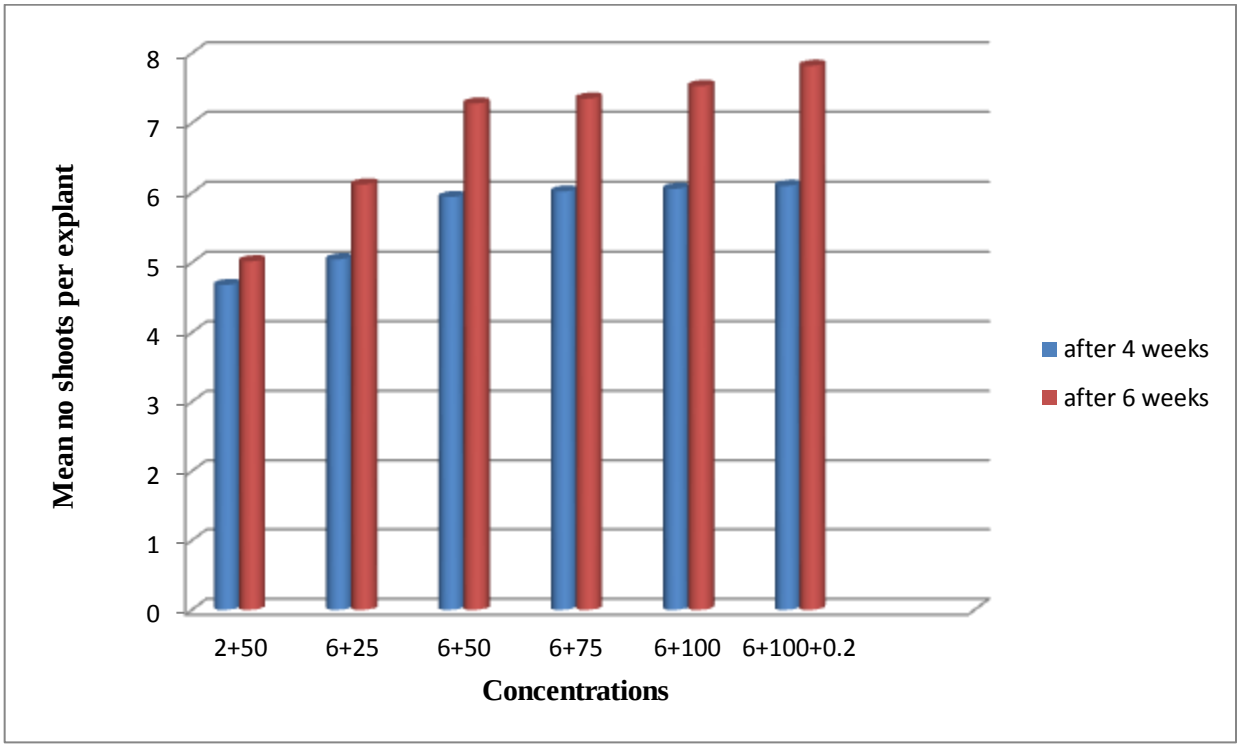


Table-4.8: Effect of different concentrations of KIN alone on mean number of shoots per explant of *Chlorophytum borivilianum*

Concentrations	Mean no. Of shoots/ explant	
	After 4 weeks	**After 6 weeks**
1.0 mg/l KIN	2.45±0.34	2.75±0.55
3.0 mg/l KIN	3.33±0.46	4.27±0.91
5.0 mg/l KIN	**3.71±0.22**	**4.32±0.90**

Graph-4.8: Effect of different concentrations of KIN alone on mean number of shoots per explant of *Chlorophytum borivilianum*

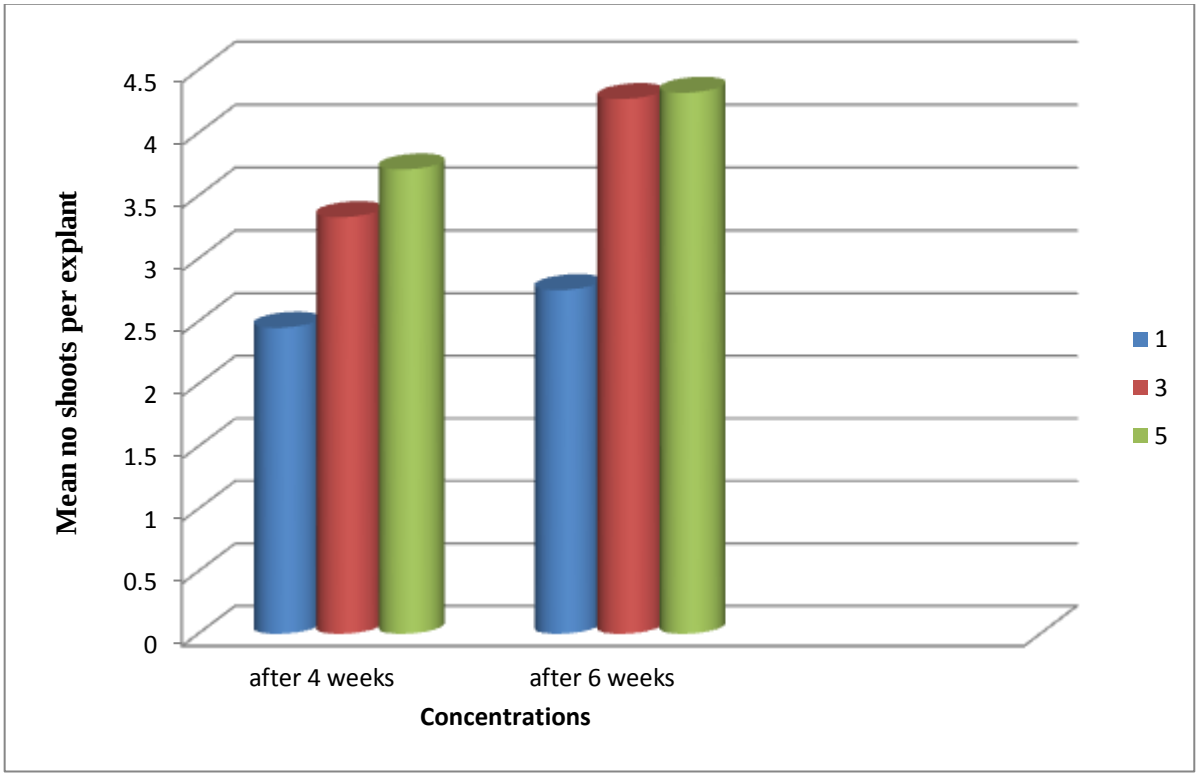
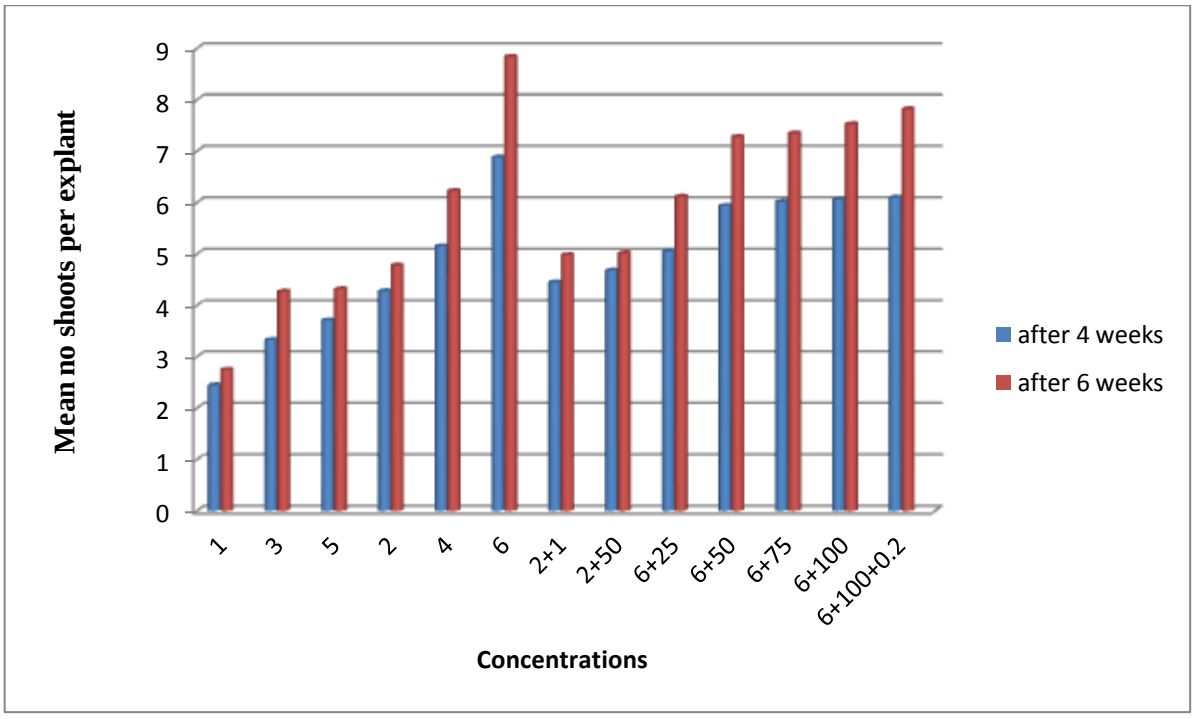


Table-4.9: Comparative account of effect of different concentrations and combinations of hormones on mean number of shoots per explant of *Chlorophytum borivilianum*

Concentrations	Mean no. Of shoots/ explant	
	After 4 weeks	After 6 weeks
1.0 mg/l KIN	2.45±0.34	2.75±0.55
3.0 mg/l KIN	3.33±0.46	4.27±0.91
5.0 mg/l KIN	3.71±0.22	4.32±0.90
2.0 mg/l BAP	4.28±0.37	4.78±0.08
4.0 mg/l BAP	5.15±0.40	6.23±1.30
6.0 mg/l BAP	**6.88±0.38**	**8.84±1.42**
2.0 mg/l BAP +1.0 mg/l KIN	4.45±0.30	4.98±0.33
2.0 mg/l BAP +50.0 mg/l $AdSO_4$	4.68±0.15	5.02±0.98
6.0 mg/l BAP +25.0 mg/l $AdSO_4$	5.05±0.85	6.12±0.23
6.0 mg/l BAP +50.0 mg/l $AdSO_4$	5.94±0.34	7.28±0.56
6.0 mg/l BAP +75.0 mg/l $AdSO_4$	6.02±0.50	7.35±1.17
6.0 mg/l BAP +100.0 mg/l $AdSO_4$	6.06±0.32	7.53±0.40
6.0 mg/l BAP +100.0 mg/l $AdSO_4$ +0.2 mg/l IAA	6.10±0.54	7.82±0.73

Graph-4.9: Comparative account of effect of different concentrations and combinations of hormones on mean number of shoots per explant of *Chlorophytum borivilianum*



4.1.4 Induction of Rooting on excised shootlets

The excised shootlets were inoculated in eight different sets of media. MS basal with IBA alone and in combination with IAA and with and without charcoal to induce roots. All the sets were found effective to induce root ,but the best rooting percentage was observed in MS media supplemented with 2.0 mg/l IBA along with 2.0 mg/l IAA i.e .81.81% after 3 weeks and 91.31% after 5 weeks (table 4.11 and graph 4.11) and second best rooting percentage was found 80.25% after 3 weeks and 86.63% after 5 weeks when inoculated in Ms media with 3.0 mg/l IBA along with 2.0 mg/l IAA (table 4.11 and graph 4.11). Out of four different concentrations of IBA, 2.0 mg/l was found best showing78.81% and 80.70% rooting after after 3 and after 5 weeks(table 4.10 and graph 4.10). MS media supplemented with 2.0 gm/l charcoal with 2.0 mg/l IBA showed 38.46% and 49.10% rooting after 3 and after 5 weeks. In comparison to this media with 2.0 mg/l IAA showed 47.05% after 3 weeks and 51.33% rooting after 5 weeks(table 4.11 and graph 4.11). So far the previous reports on *Chlorophytum borivilianum* is concerned, there is no report of using charcoal in the medium. Rehman *et al*., 2003, Bhojwani and Razdan, 1992 Saurabh *et al*., 2005 reported shoot multiplication and simultaneous rooting. A comperative account of effect of all combinations of hormones used was shown in table 4.12 and graph 4.12.

Mean number of root was observed maximum in media containing 2.0mg/l IBA along with 2.0 mg/l IAA and the result was 21.66 after 3 weeks and 37.86 after 5 weeks of inoculation(table 4.14 graph 4.14 fig 4.28 and 4.29) . In media containing 3.0 mg/l IBA with 2.0 mg/l IAA mean number of root was found 14.42 after 3 weeks and 26.81 after 5 weeks of inoculation (table 4.14, graph 4.14 fig 4.30 and 4.31) . Out of four different concentrations of IBA, 2.0 mg/l was found best showing 9.69 and 19.34 mean number of roots after 3 weeks and 5 weeks of inoculation respectively (table 4.13, graph 4.13 and fig 4.32 and 4.33). In charcoal containing media result was found insignificant. Mean number of root was found 3.37 after 3 weeks and 9.98 after 5 weeks when inoculated in 2.0 mg/l IBA with 2.0 gm/l Charcoal(fig 4.40) .while in contrast to that media with 2.0 mg/l IAA induced 4.37 after 3 weeks and11.19 roots after 5 weeks per shootlets (table 4.14 and graph 4.14 fig 4.41). However , IBA in combination with IAA was found more effective, charcoal was not found more effective in root induction on excised shootlets. A comperative account of effect of all combinations of hormones used was shown in table 4.15 and graph 4.15.

Table-4.10: Effect of IBA alone on root initiation percentage on excised shootlets

Concentrations	Percentage of root initiation	
	3 weeks	**5 weeks**
1.0 mg/l IBA	58.30	61.50
2.0 mg/l IBA	**78.80**	**80.70**
3.0 mg/l IBA	61.23	71.40
4.0 mg/l IBA	69.13	76.92

Graph-4.10: Effect of IBA alone on root initiation percentage on excised shootlets

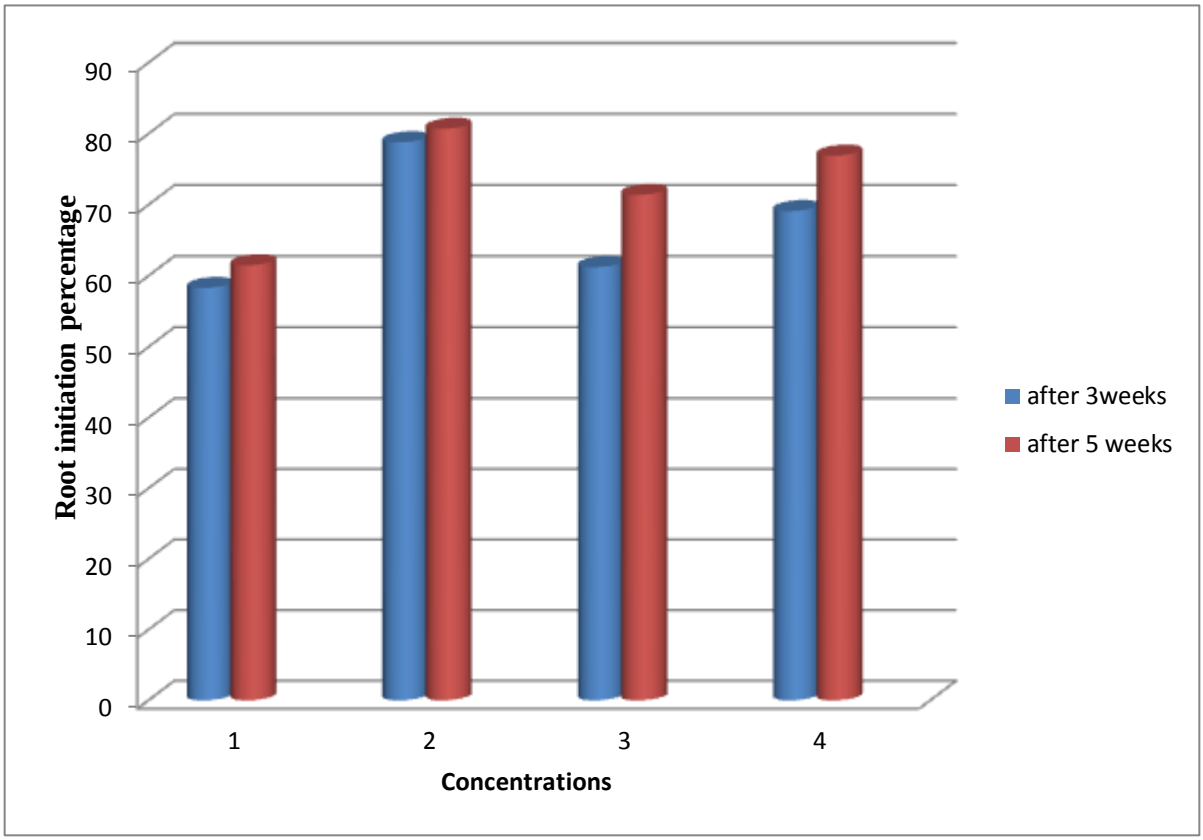


Table-4.11: Effect of IBA along with IAA and with and without Charcoal on root initiation percentage on excised shootlets

Concentrations	Percentage of root initiation	
	3 weeks	**5 weeks**
2.0 mg/l IBA+2.0 mg/l IAA	**81.81**	**91.31**
3.0 mg/l IBA +2.0 mg/l IAA	80.25	86.63
2.0 mg/l IBA +2.0 gm/l Charcoal	38.46	49.10
2.0 mg/l IBA +2.0 mg/l IAA+ 2.0 gm/l Charcoal	47.05	51.33

Graph-4.11: Effect of IBA along with IAA and with and without Charcoal on root initiation percentage on excised shootlets

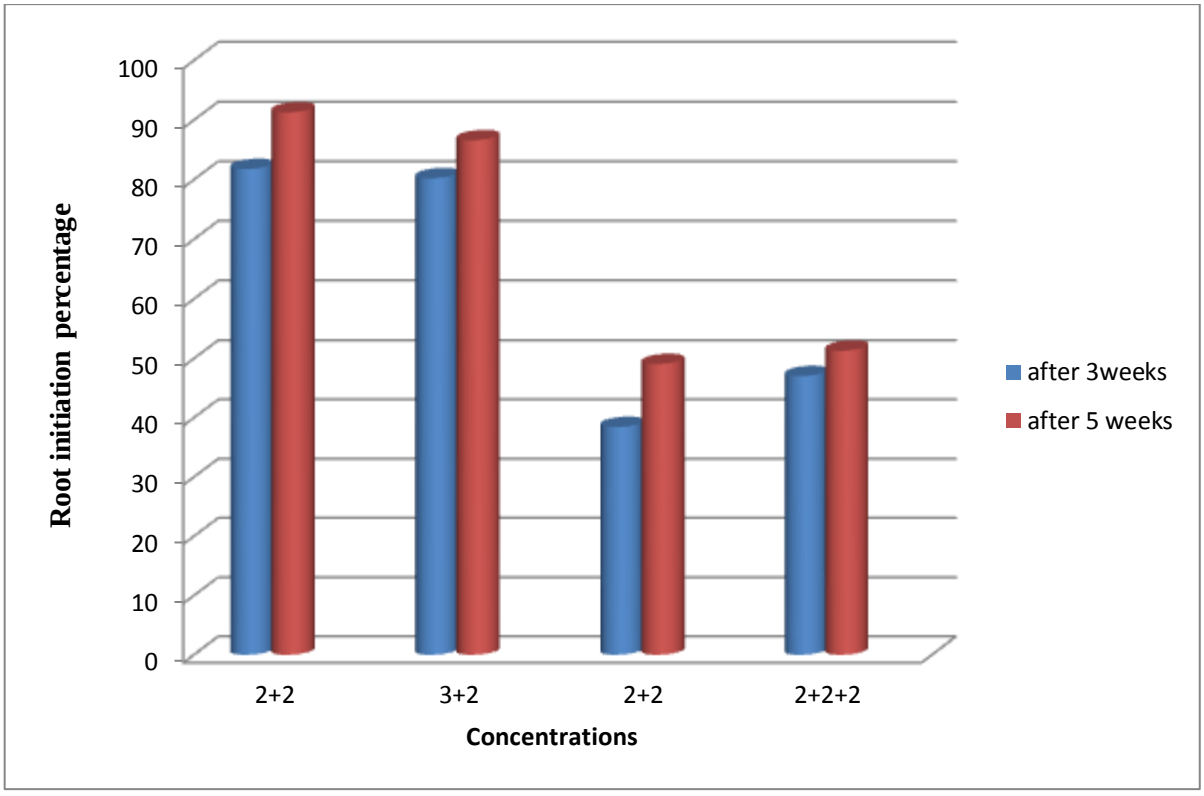


Table-4.12: Comparative account of effect of different concentrations and combinations of rooting hormones on root induction percentage

Concentrations	Percentage of root initiation	
	3 weeks	**5 weeks**
1.0 mg/l IBA	58.30	61.50
2.0 mg/l IBA	78.80	80.70
3.0 mg/l IBA	61.23	71.40
4.0 mg/l IBA	69.13	76.92
2.0 mg/l IBA+2.0 mg/l IAA	**81.81**	**91.31**
3.0 mg/l IBA +2.0 mg/l IAA	80.25	86.63
2.0 mg/l IBA +2.0 gm/l Charcoal	38.46	49.10
2.0 mg/l IBA +2.0 mg/l IAA+ 2.0 gm/l Charcoal	47.05	51.33

Table-4.12: Comparative account of effect of different concentrations and combinations of rooting hormones on root induction percentage

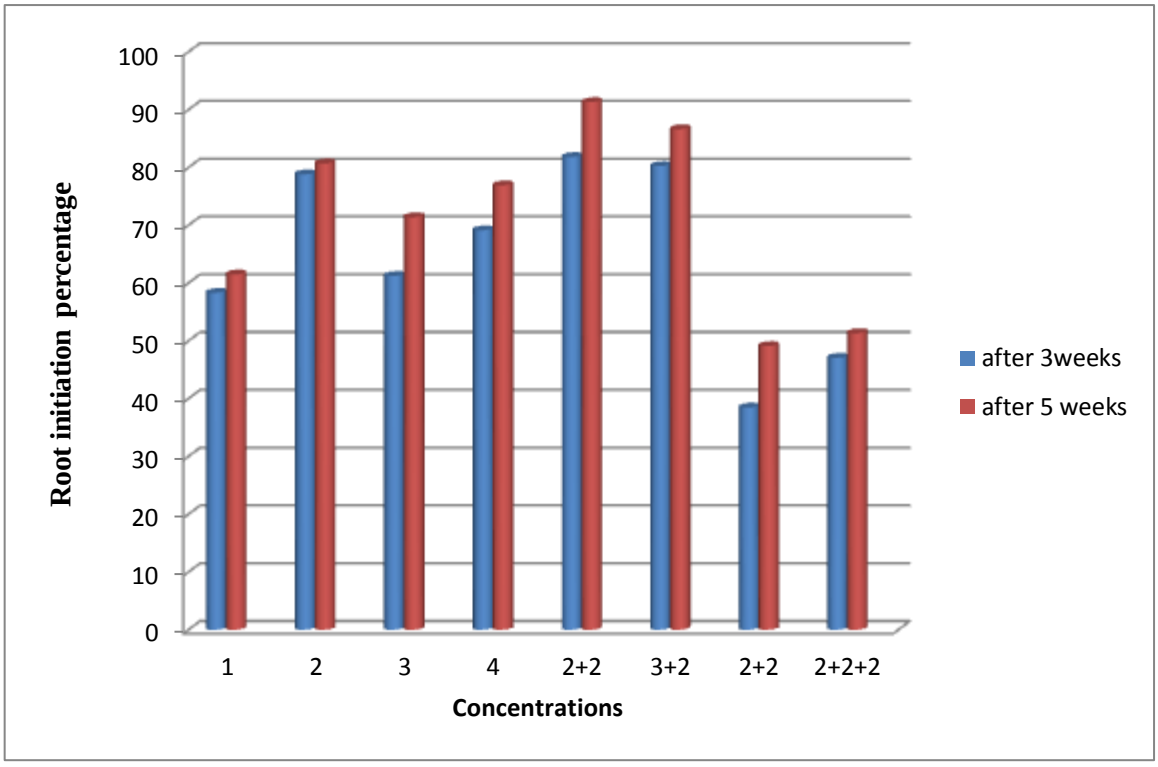


Table-4.13: Effect of IBA alone on induction of mean number of roots.

Concentrations	Mean no. of roots /shootlet	
	3 weeks	**5 weeks**
1.0 mg/l IBA	3.14	8.25
2.0 mg/l IBA	**9.69**	**19.34**
3.0 mg/l IBA	6.20	13.49
4.0 mg/l IBA	7.60	16.77

Graph 4.13: Effect of IBA alone on induction of mean number of roots.

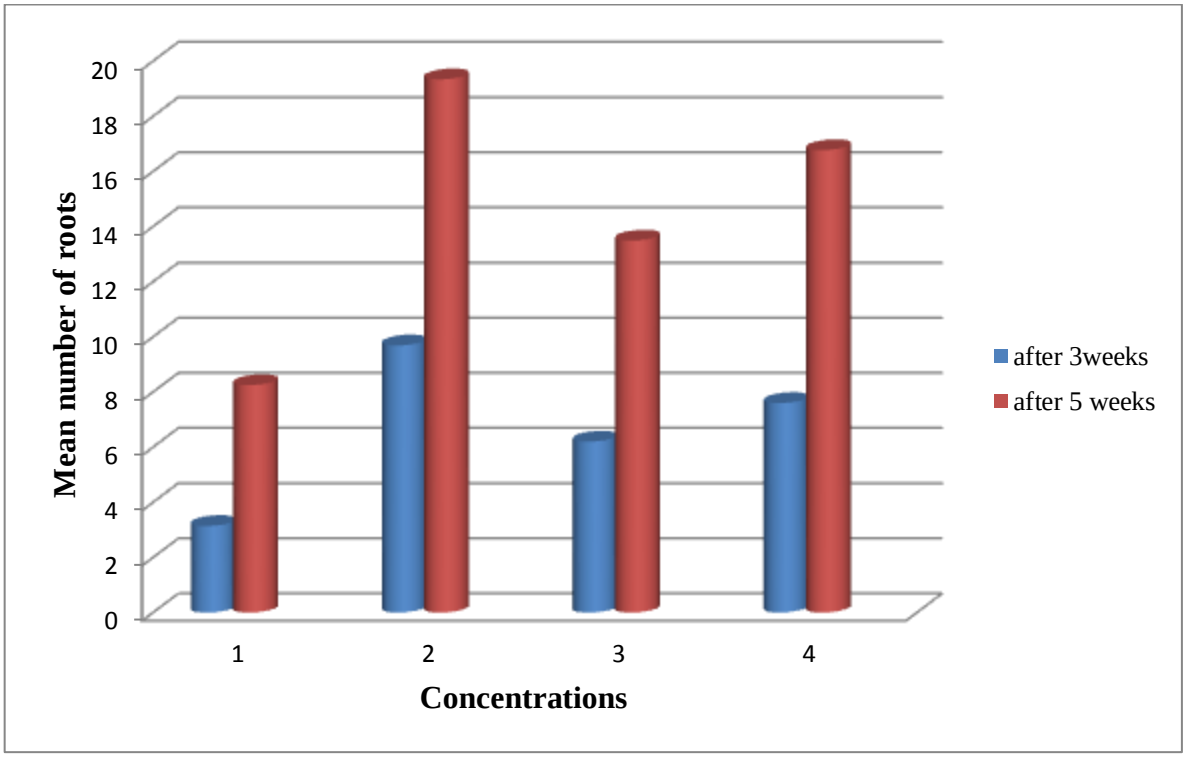


Table-4.14: Effect of IBA along with IAA and with and without Charcoal on induction of mean number of roots

Concentrations	Mean no. of roots /shootlet	
	3 weeks	**5 weeks**
2.0 mg/l IBA+2.0 mg/l IAA	**21.66**	**37.86**
3.0 mg/l IBA +2.0 mg/l IAA	14.42	26.81
2.0 mg/l IBA +2.0 gm/l Charcoal	3.75	9.98
2.0 mg/l IBA +2.0 mg/l IAA+ 2.0 gm/l Charcoal	4.37	11.19

Graph-4.14: Effect of IBA along with IAA and with and without Charcoal on induction of mean number of roots

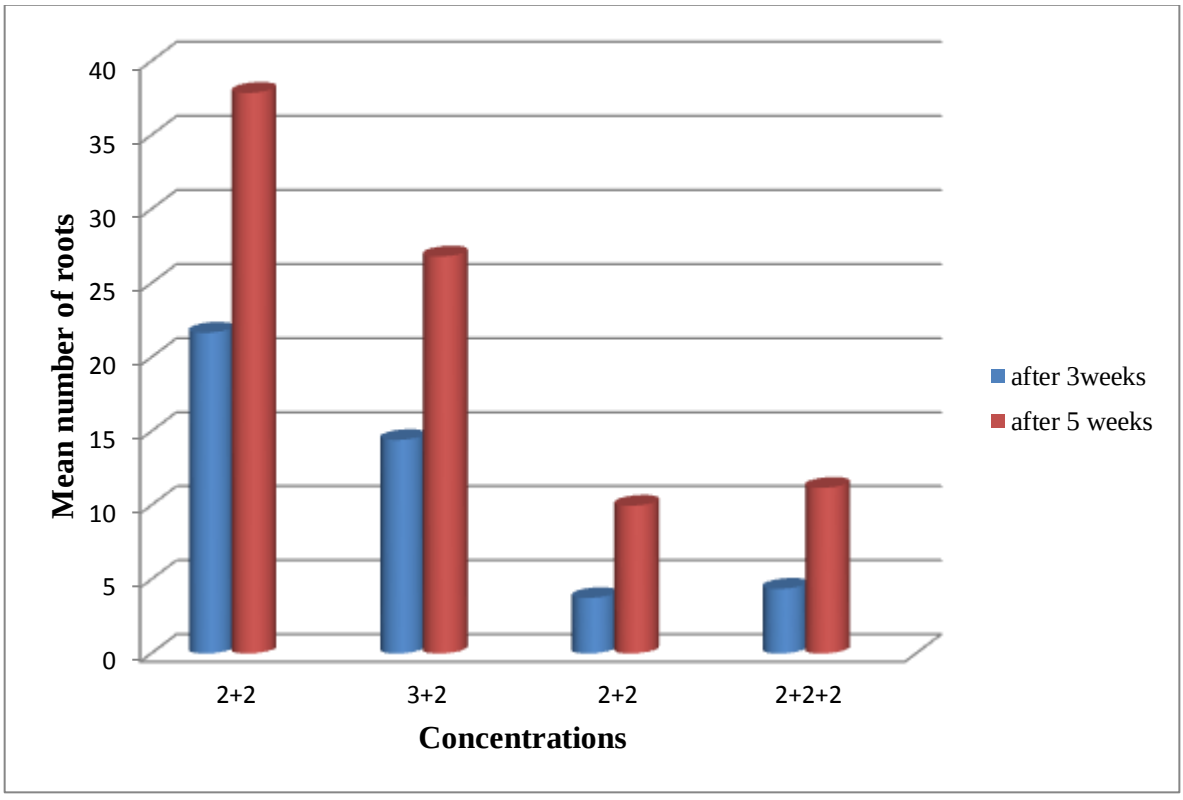
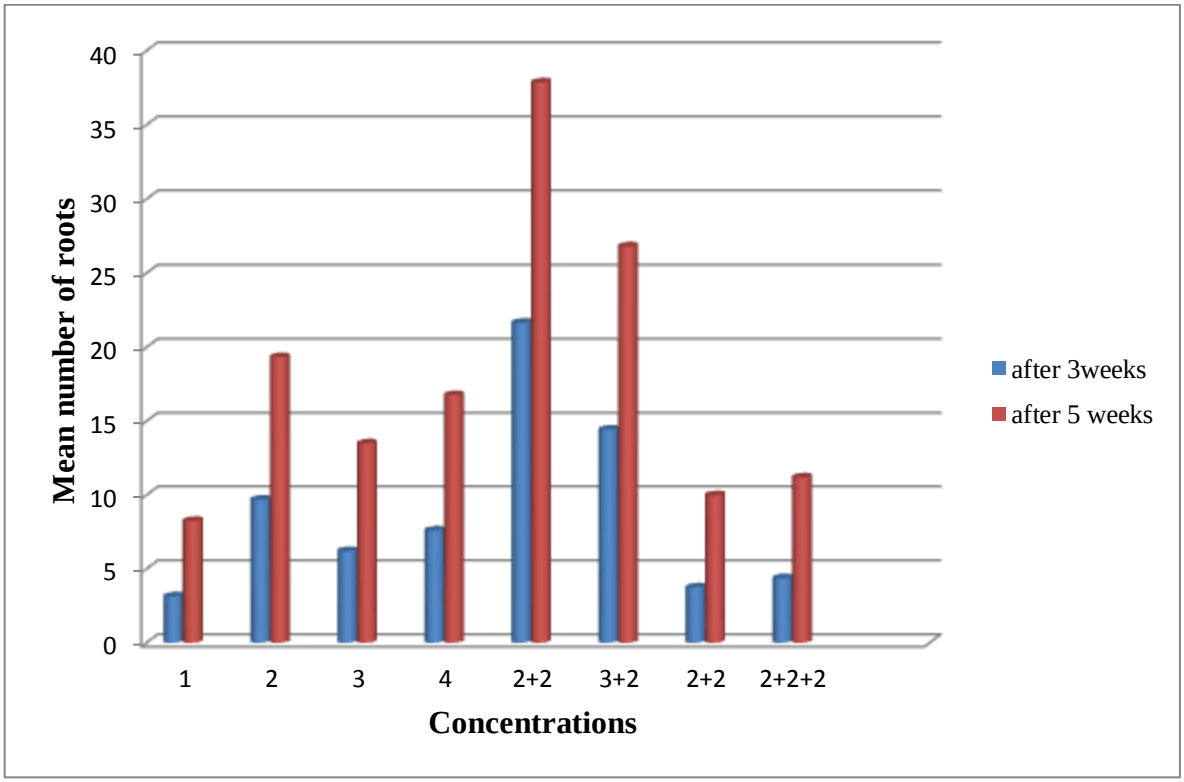


Table-4.15: Comparative account of effect of different concentrations and combination of rooting hormones on mean number roots on excised shootlet

Concentrations	Mean no. of roots /shootlet	
	3 weeks	**5 weeks**
1.0 mg/l IBA	3.14	8.25
2.0 mg/l IBA	9.69	19.34
3.0 mg/l IBA	6.20	13.49
4.0 mg/l IBA	7.60	16.77
2.0 mg/l IBA+2.0 mg/l IAA	**21.66**	**37.86**
3.0 mg/l IBA +2.0 mg/l IAA	14.42	26.81
2.0 mg/l IBA +2.0 gm/l Charcoal	3.75	9.98
2.0 mg/l IBA +2.0 mg/l IAA+ 2.0 gm/l Charcoal	4.37	11.19

Table-4.15: Comparative account of effect of different concentrations and combination of rooting hormones on mean number roots on excised shootlet



4.1.5 Hardening

It is often found tissue culture raised plants transferred directly in the natural condition without any preconditioning do not survive. Several factors are responsible for the difficultly. The *in vitro* regenerated plantlets after rooting are to be transferred from the aseptic condition under high humidity to an entirely different environment prevailing *in vivo*. The major factors affecting establishment are infection and desiccation. The rooted plantlets were washed thoroughly with double-distilled water to remove trace of medium and dipped in 1% Bavistin (systemic fungicide) solution. The rooted plantlets were then transferred to the protray for hardening. The potting mix used was sterile coco peat. After plantation the portrays were kept under poly tunnel to retain almost 100% humidity (fig 4.43). After 15 days, the new leaves and roots came out and the plantlets were transferred to poly bags containing soil: sand: FYM in 1:1:1 proportion and kept under the shade in green house for secondary hardening. In the present study plantlets transferred under net house condition showed 95.83 survival percentage. After 20 - 30 days the plants were ready for field transplantation. So, acclimatization of tissue culture raised plants is equally important as the ultimate goal is to transfer those plants to the field. These results simulates with the results of (Nhut, 1998), (Suri *et al.*, 1999), (Pudake and Dhumale, 2003).

Chapter–5

SUMMARY AND CONCLUSIONS

The present study on Tissue culture studies in safed musli (*Chlorophytum borivilianum*) was conducted at the tissue culture laboratory, College of Biotechnology, Birsa Agricultural University. Traditionally safed musli is propagated through seed tubers. Safed musli seed tubers are bulky material and involve high costs for packaging, shipping, storage and distribution. The successive multiplication of seed tubers in soil carries the risk of infection and build up of pathogens which leads to degeneration of tubers. Moreover safed musli tubers possess a long inherent dormancy period of about 6-7 months. Hence, commercial cultivation of safed musli crop is restricted to certain period (May- August) of the year *i.e.* one crop per year. For all these difficulties, *in vitro* multiplication of safed musli allows large multiplication of disease free propagules in a short duration and on a year round basis. Hence, the investigations were carried out to standardize the protocol for rapid multiplication with standardization of growth regulator concentrations and combinations for better culture establishment and to have a high shoot and root proliferation. Further acclimatization of plantlets under net house for the survival of plants was also studied. The present study reveals a viable protocol for shoot multiplication in different concentrations of phytohormones. The type and quantity of exogenous supply of hormones were standardized. Among 4 different concentrations of $HgCl_2$, shoot tip showed highest survival percentage 89.33% after treatment with $HgCl_2$ for 15 min in 0.15%. Out of 13 different phytohormones, multiplication of shoot tip was found best (88.83%) in 6.0 mg/l BAP rather than BAP in combinations with $AdSO_4$ with and without IAA and KIN . 2.0 mg/l IBA in combination with 2.0 mg/l IAA of rooting medium found best (91.31%) to induce roots on excised shootlets *in vitro* than IBA alone and Charcoal containing media. Percentage of survival of plantlets during hardening was found about (95.83%).

Chapter – 6

FUTURE PROSPECTS

The studies at molecular level may define the changes observed at different stages of development in a better way; so that the interaction among the different hormones and its effect at molecular level could be understand easily. Meristem tip culture technique would be further used for the production of virus free plants because this method did not verify whether the tissue culture plantlets were free from viruses or not. Protocol for micropropagation established may be used to conserve as well as produce this valued plant in large scale. *In vitro* multiplication of safed musli plants can be tried with other explant and culture media for early and high rate of multiplication. Callus development in leaf base and tuber explants can be tried by using various growth regulators in different concentrations and combinations. Proper hardening procedure can be tried to achieve maximum field establishment in safed musli plantlets raised through *in vitro* technique. Field performance of tissue culture raised safed musli plantlets can be tried during off season *i.e.* when safed musli tubers are in dormant condition.

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Fig 4.1 Mother Plant

Fig 4.2: Shoot multiplication shoot tip in Ms media supplemented with 6.0 mg/l BAP after 4 weeks

Fig 4.3: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP after 6 weeks

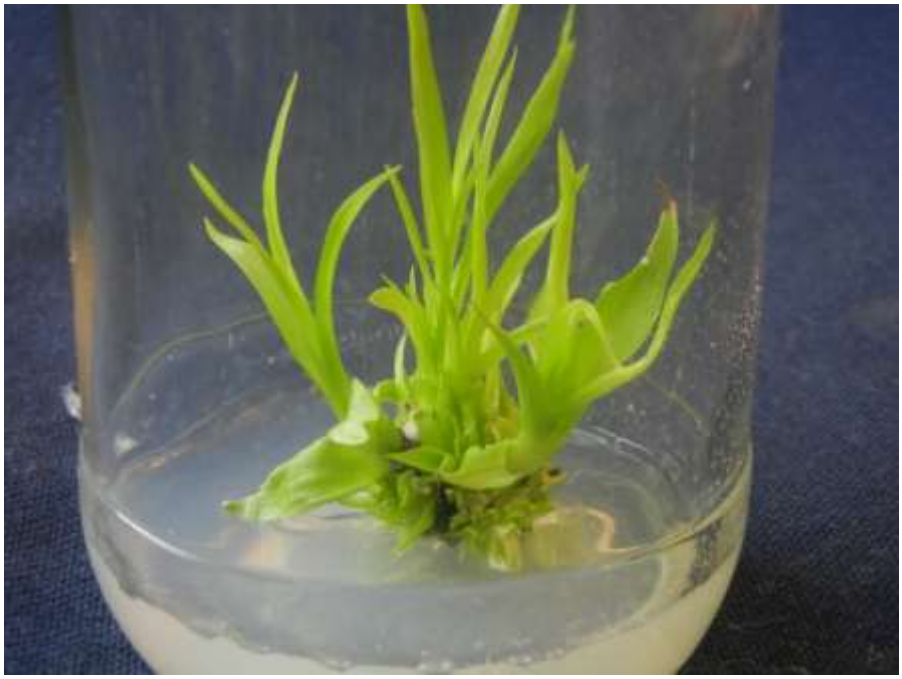

Fig 4.4 Shoot multiplication on shoot tip in Ms media supplemented with 4.0 mg/l BAP after 4 weeks

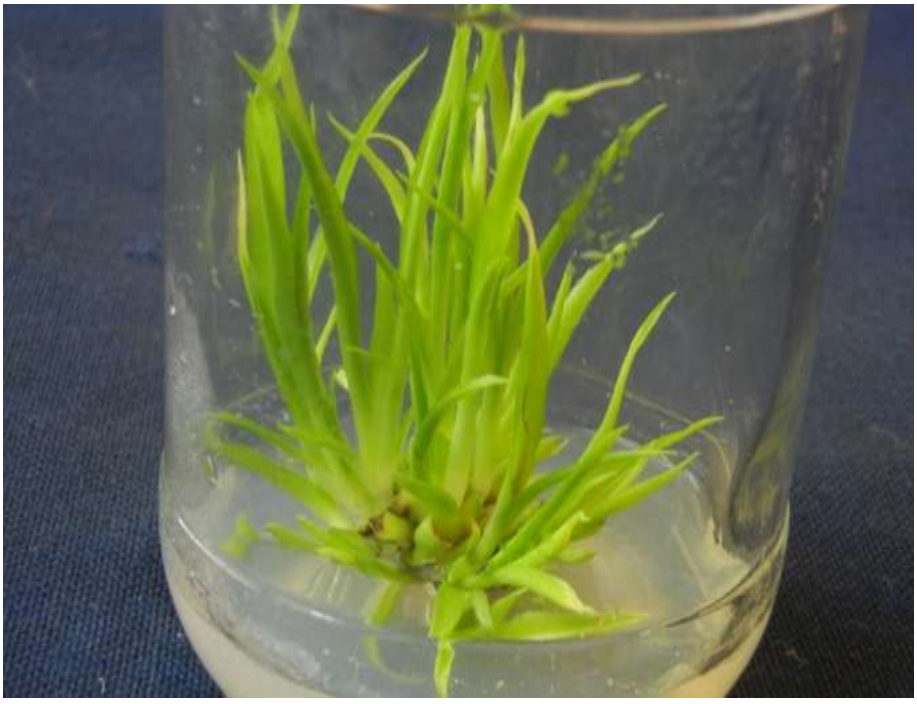

Fig 4.5: Shoot multiplication on shoot tip in Ms media supplemented with 4.0 mg/l BAP after 6 weeks

Fig 4.6: Shoot multiplication on shoot tip in Ms media supplemented with 2.0 mg/l BAP after 4 weeks

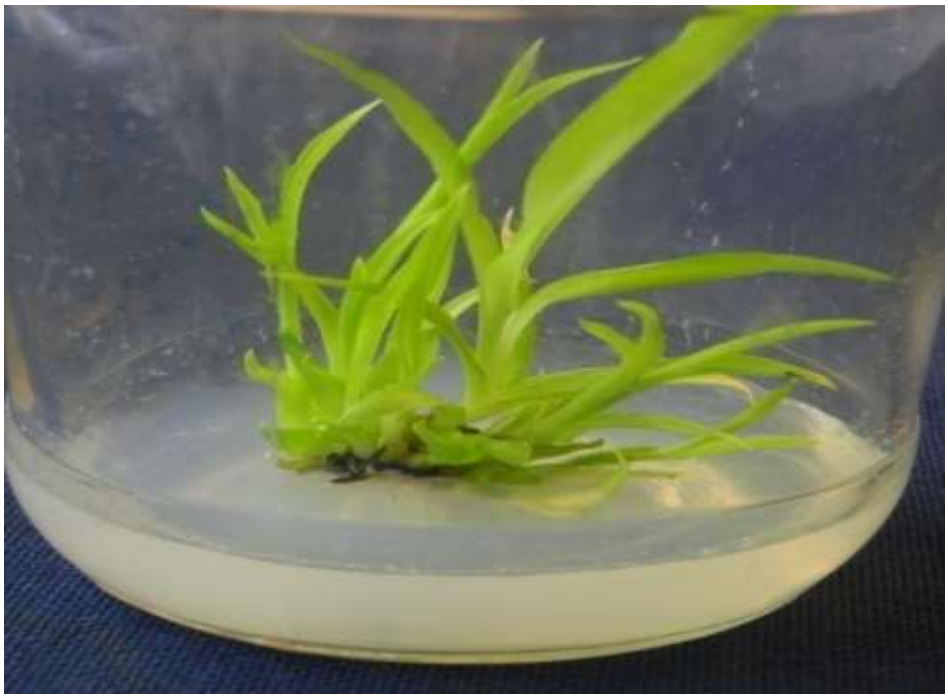

Fig 4.7: Shoot multiplication on shoot tip in Ms media supplemented with 2.0 mg/l BAP after 6 weeks

Fig 4.8: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+100.0 mg/l $AdSO_4$ + 0.2 mg/l IAA after 4 weeks

Fig 4.9: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+100.0 mg/l $AdSO_4$ + 0.2 mg/l IAA after 6weeks

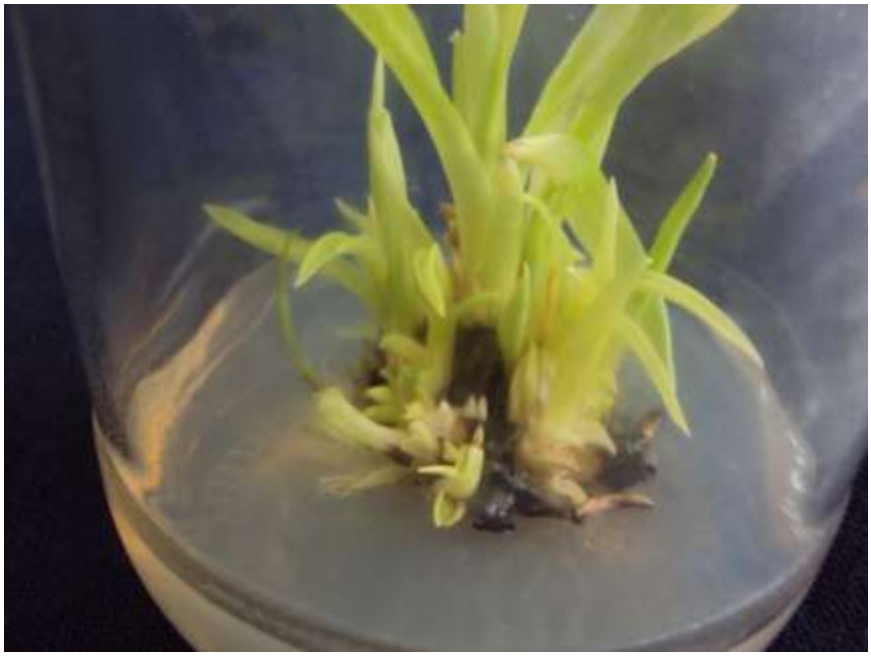

Fig 4.10: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+100.0 mg/l $AdSO_4$ after 4 weeks

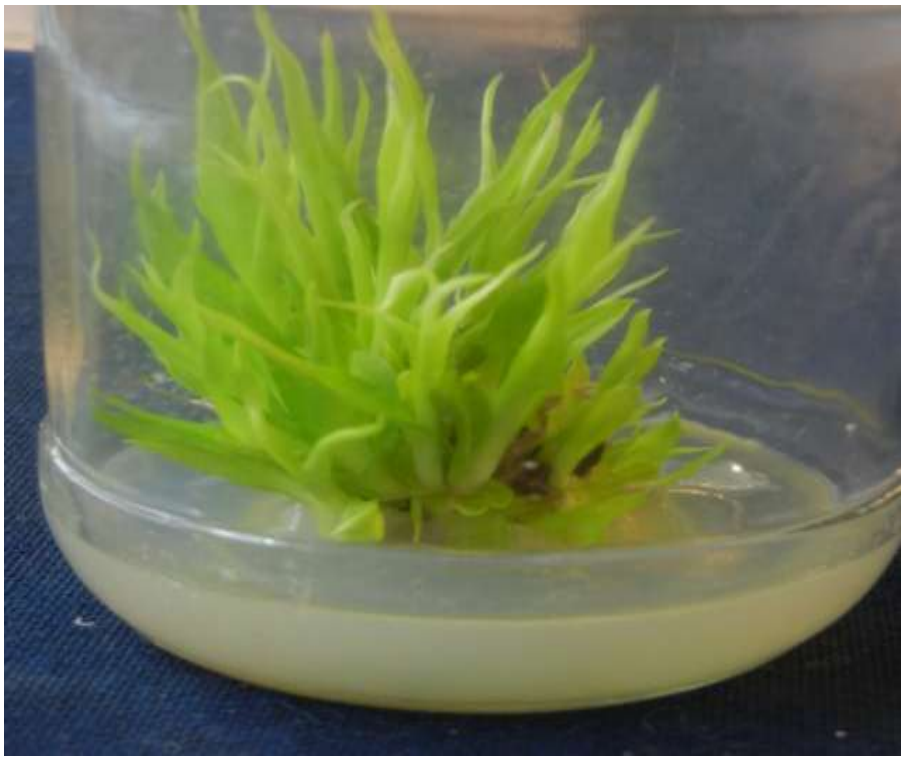

Fig 4.11: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+100.0 mg/l $AdSO_4$ after 6weeks

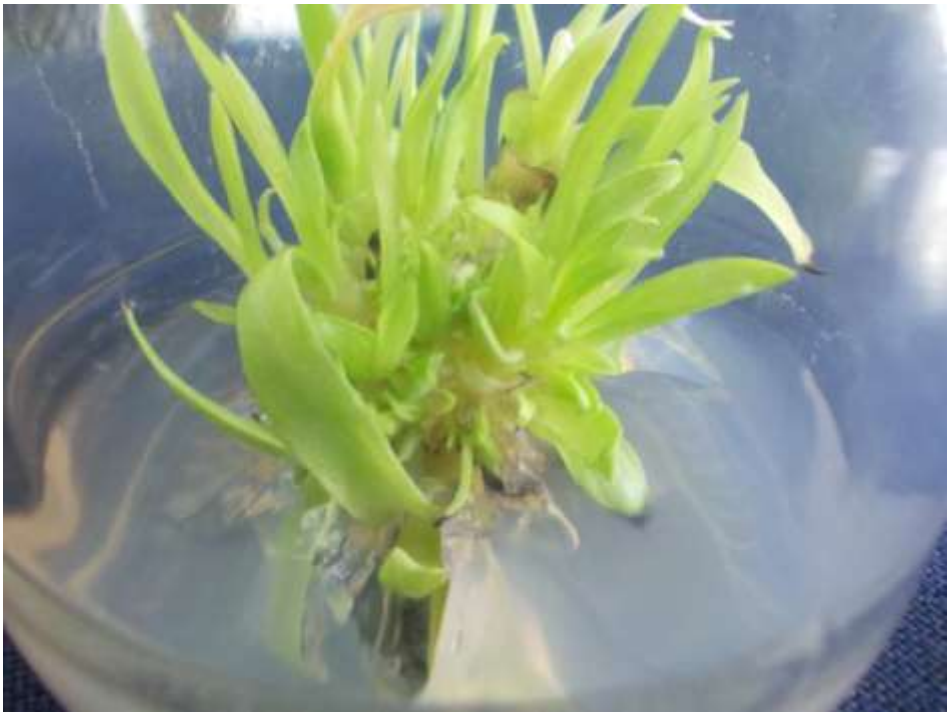

Fig 4.12: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+75.0 mg/l $AdSO_4$ after 4 weeks

Fig 4.13: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+75.0 mg/l $AdSO_4$ after 6weeks

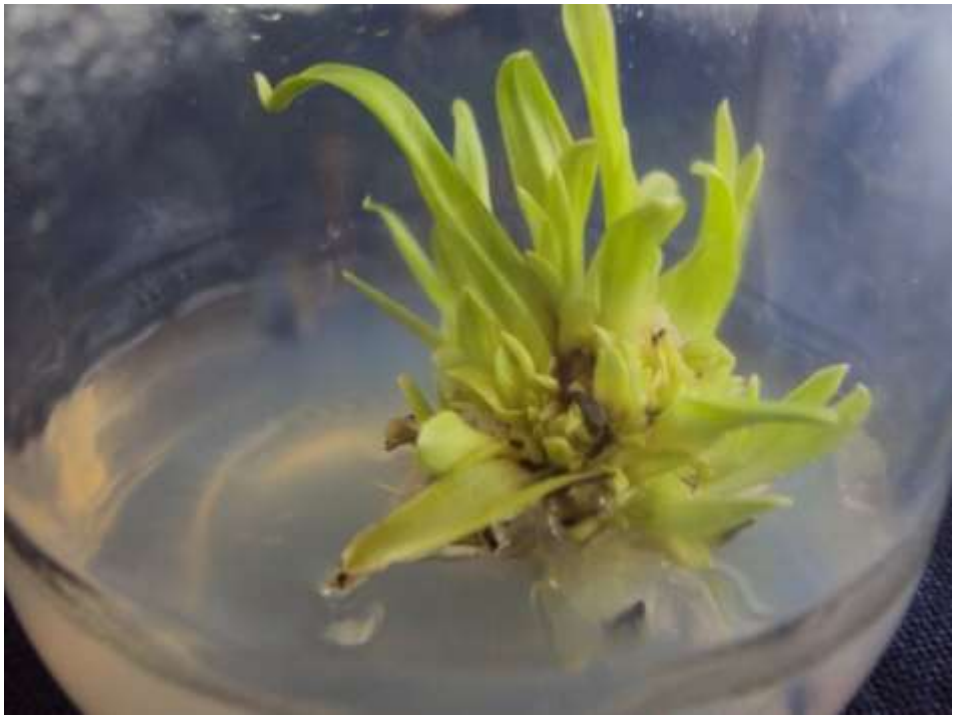

Fig 4.14: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+50.0 mg/l $AdSO_4$ after 4 weeks

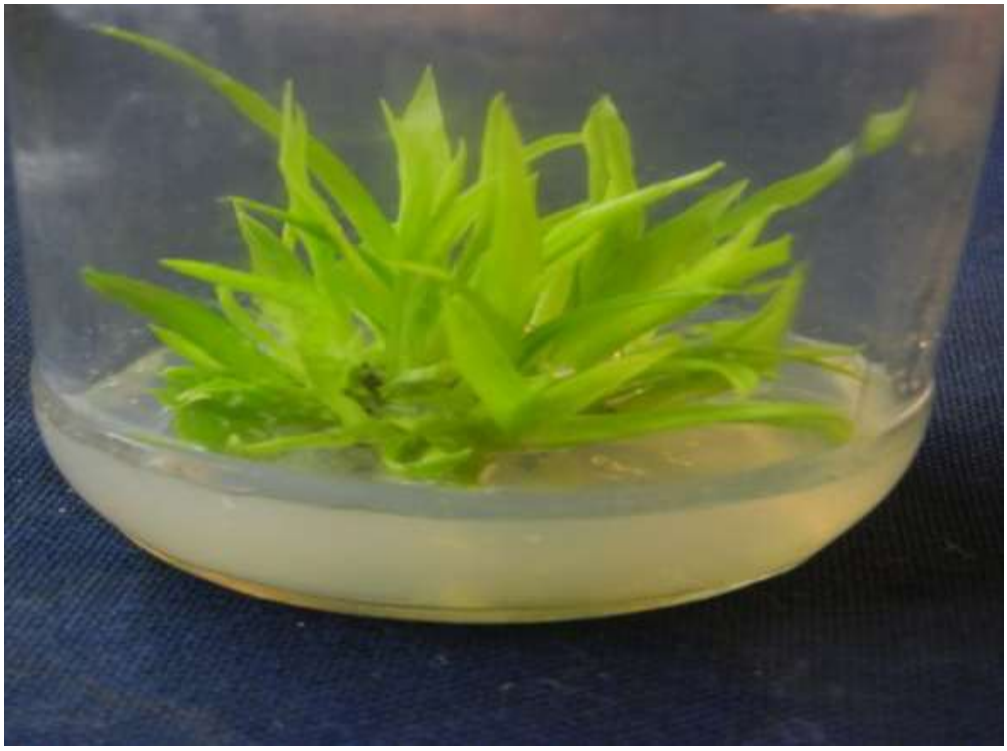

Fig 4.15: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+50.0 mg/l $AdSO_4$ after 6weeks

Fig 4.16: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+25.0 mg/l $AdSO_4$ after 4 weeks

Fig 4.17: Shoot multiplication on shoot tip in Ms media supplemented with 6.0 mg/l BAP+25.0 mg/l $AdSO_4$ after 6weeks

Fig 4.18: Shoot multiplication on shoot tip in Ms media supplemented with 2.0 mg/l BAP+50.0 mg/l $AdSO_4$ after 4 weeks

Fig 4.19: Shoot multiplication on shoot tip in Ms media supplemented with 2.0 mg/l BAP+50.0 mg/l $AdSO_4$ after 6weeks

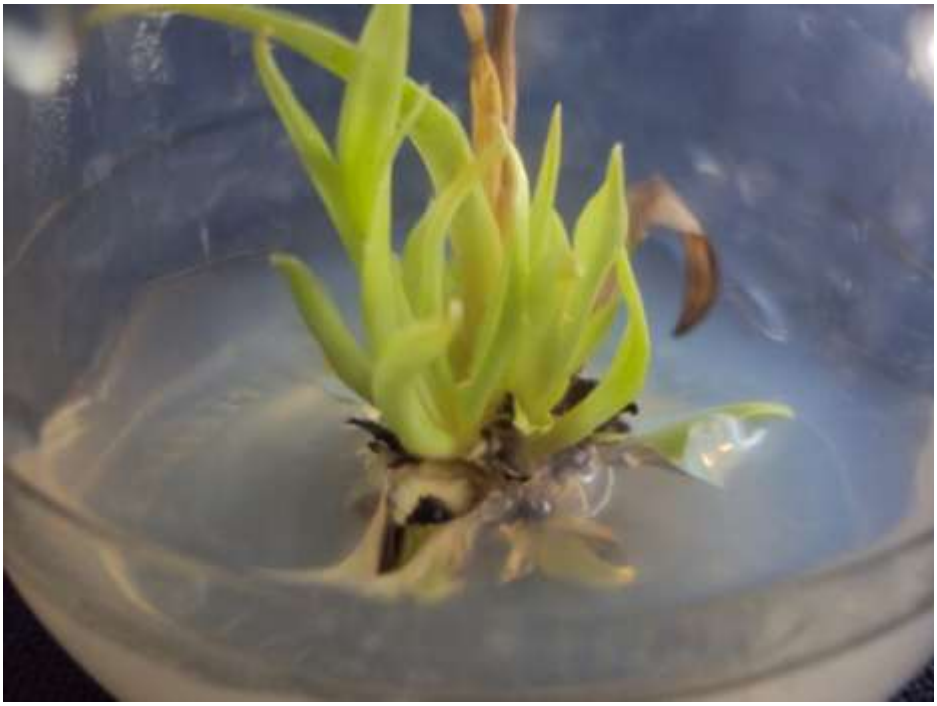

Fig 4.20: Shoot multiplication on shoot tip in Ms media supplemented with 2.0 mg/l BAP+1.0 mg/l KIN after 4 weeks

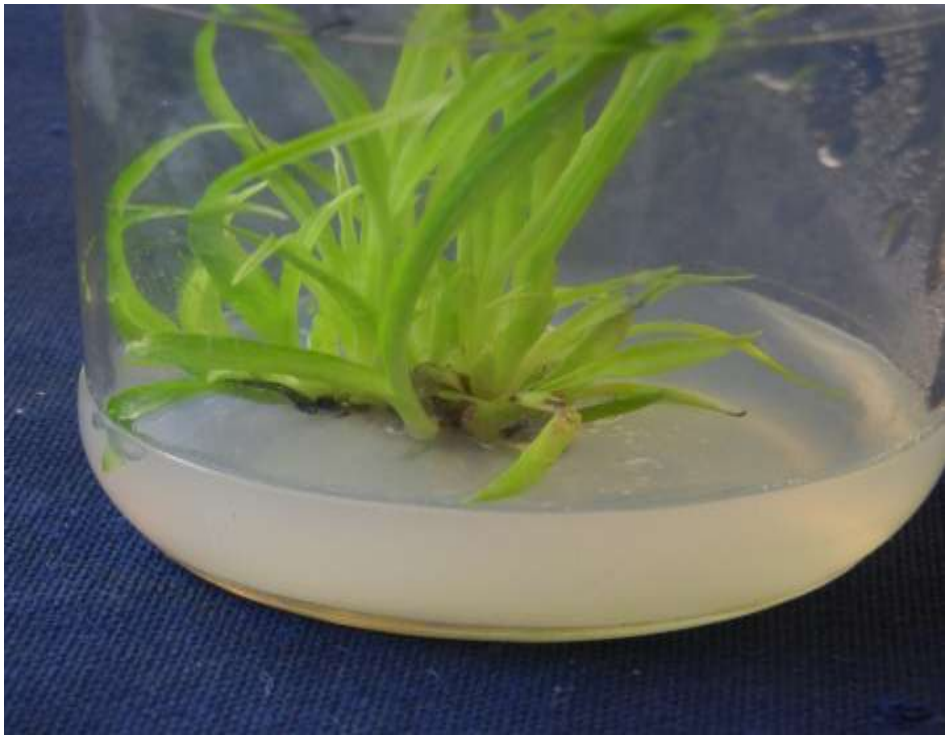

Fig 4.21: Shoot multiplication on shoot tip in Ms media supplemented with 2.0 mg/l BAP+1.0 mg/l KIN after 6weeks

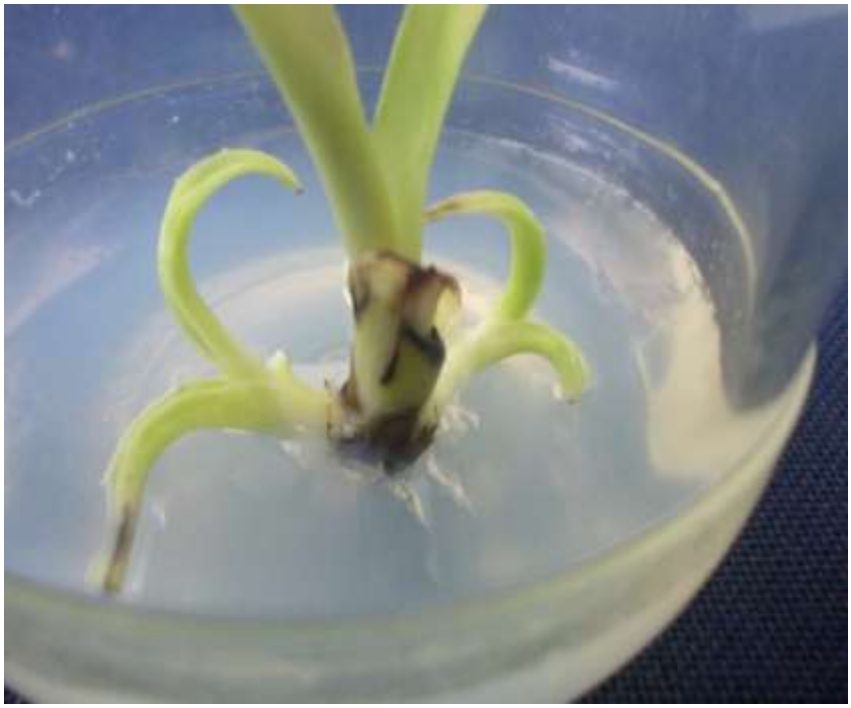

Fig 4.22: Shoot multiplication on shoot tip in Ms media supplemented with 1.0 mg/l KIN after 4weeks

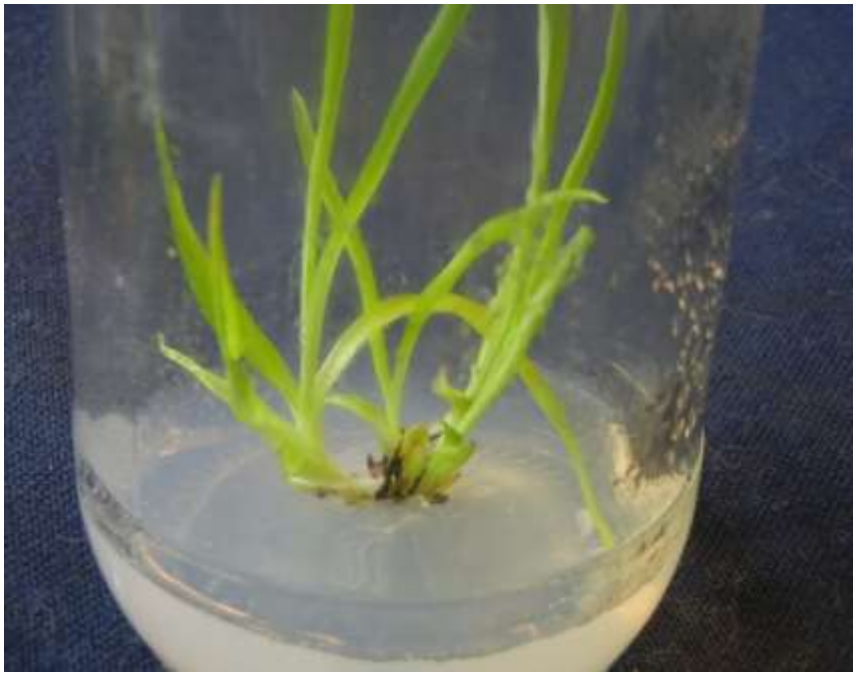

Fig 4.23: Shoot multiplication on shoot tip in Ms media supplemented with 1.0 mg/l KIN after 6 weeks

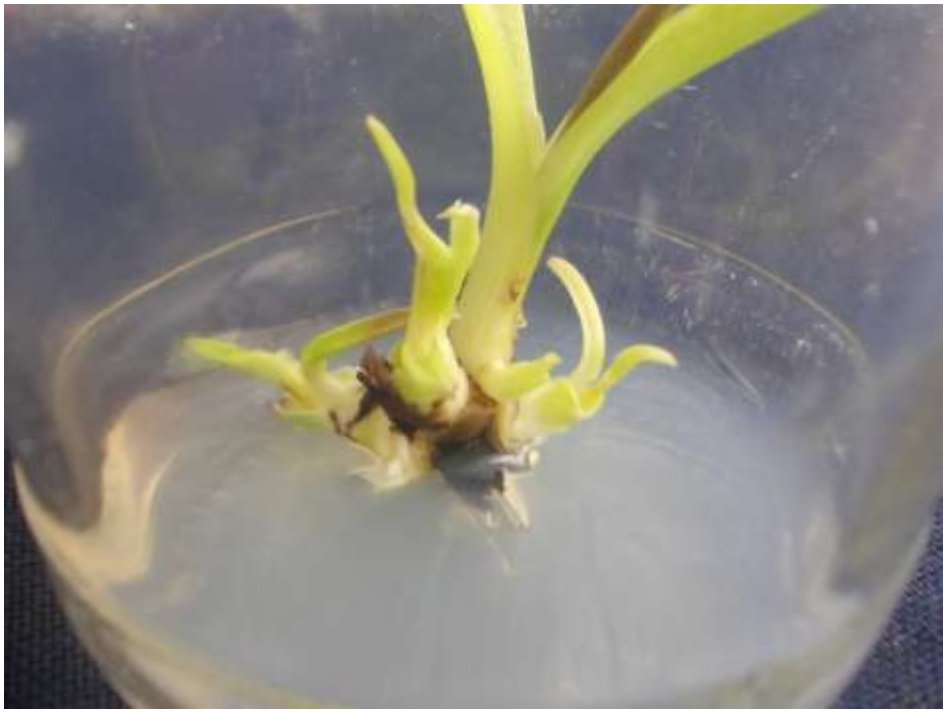

Fig 4.24: Shoot multiplication on shoot tip in Ms media supplemented with 3.0 mg/l KIN after 4weeks

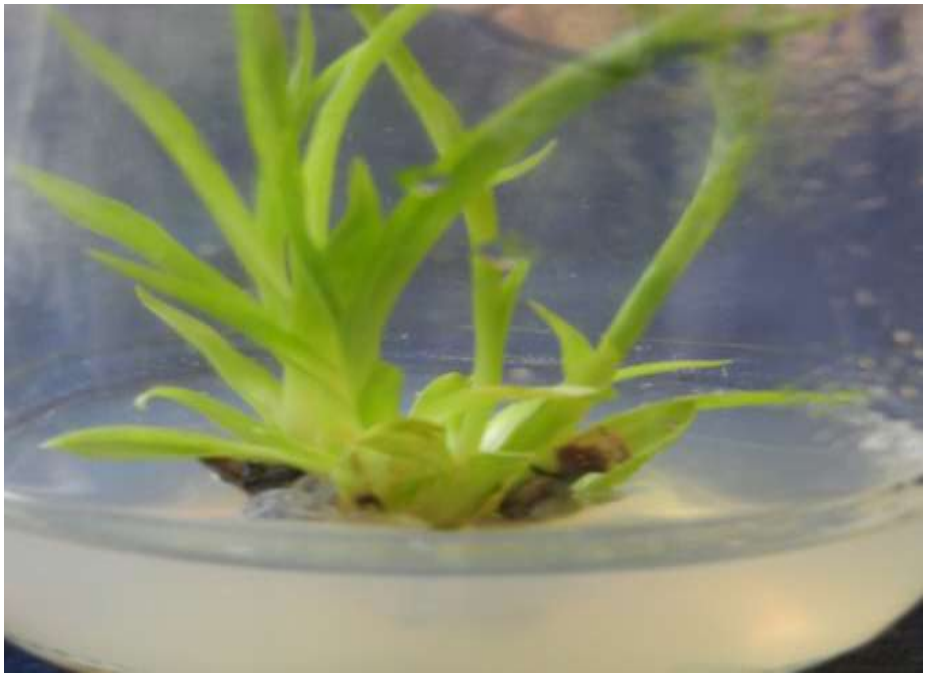

Fig 4.25: Shoot multiplication on shoot tip in Ms media supplemented with 3.0 mg/l KIN after 6 weeks

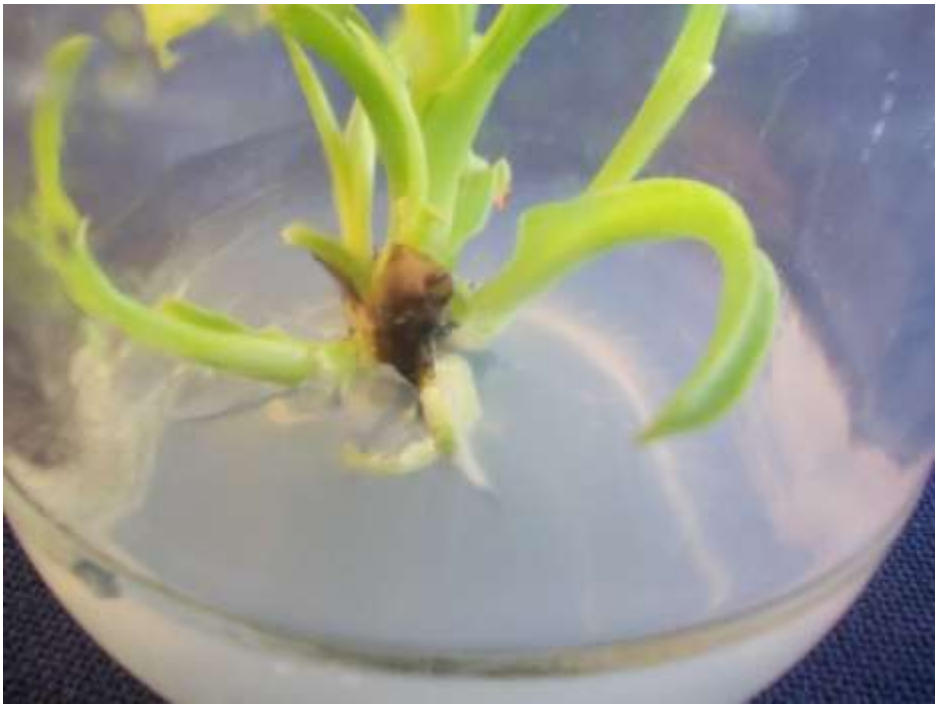

Fig 4.26: Shoot multiplication on shoot tip in Ms media supplemented with 5.0 mg/l KIN after 4 weeks

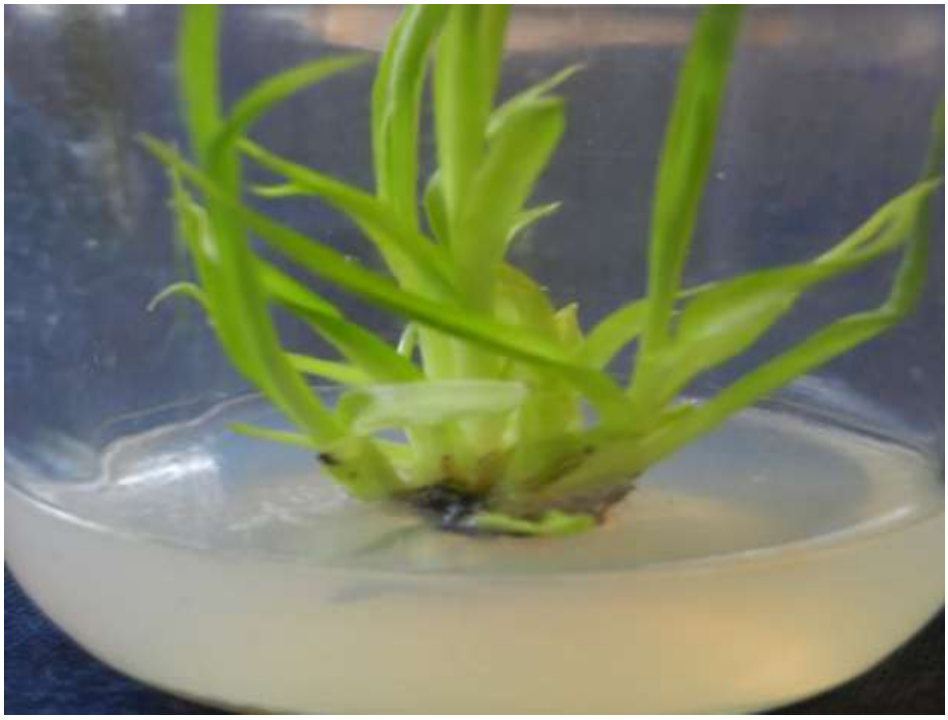

Fig 4.27: Shoot multiplication on shoot tip in Ms media supplemented with 5.0 mg/l KIN after 6 weeks

Fig 4.28: Root initiation from excised shoot in MS basal with 2.0 mg/l IBA+2.0 mg/l IAA after 3 weeks

Fig 4.29: Root initiation from excised shoot in MS basal with 2.0 mg/l IBA+2.0 mg/l IAA after 5 weeks

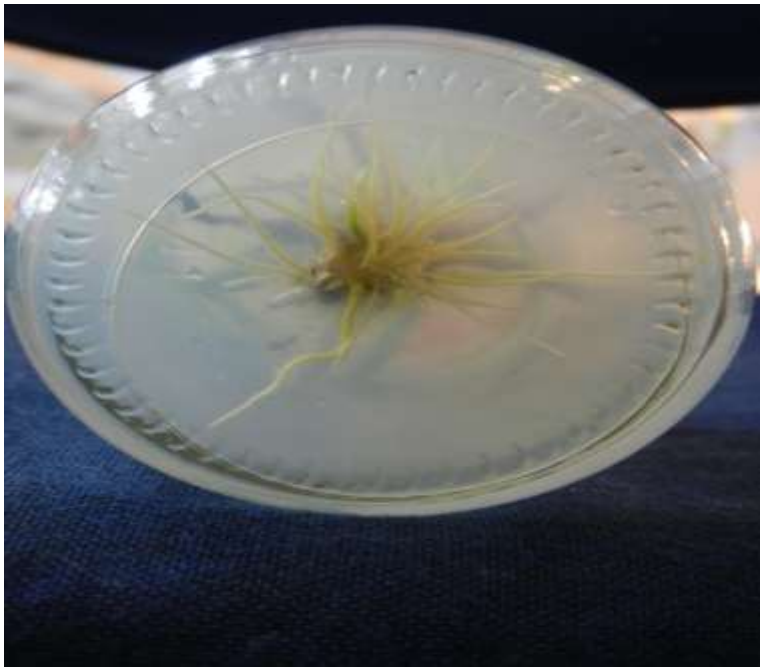

Fig 4.30: Root initiation from excised shoot in MS basal with 3.0 mg/l IBA+2.0 mg/l IAA after 3 weeks

Fig 4.31: Root initiation from excised shoot in MS basal with 3.0 mg/l IBA+2.0 mg/l IAA after 5 weeks

Fig 4.32: Root initiation from excised shoot in MS basal with 2.0 mg/l IBA after 3 weeks

Fig 4.33: Root initiation from excised shoot in MS basal with 2.0 mg/l IBA after 5 weeks

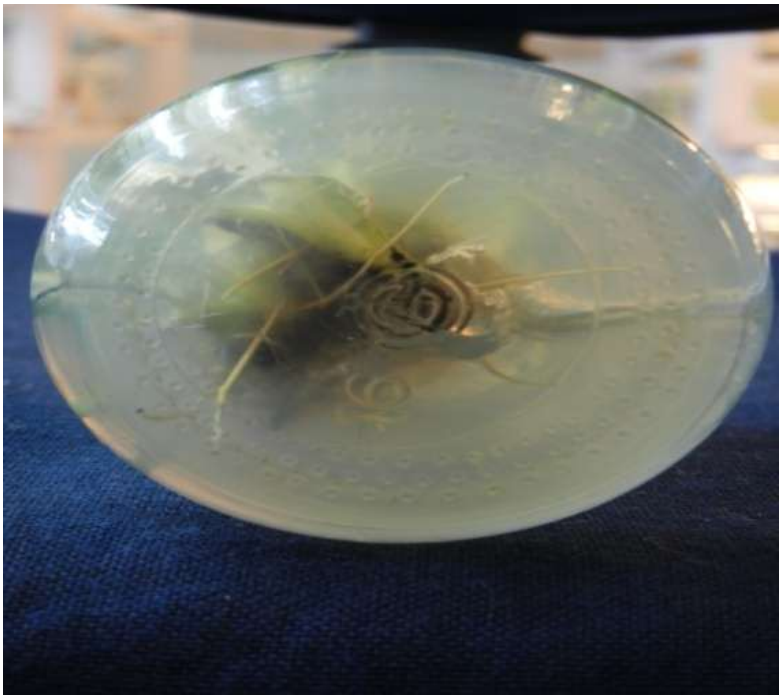

Fig 4.34: Root initiation from excised shoot in MS basal with 3.0 mg/l IBA after 3 weeks

Fig 4.35: Root initiation from excised shoot in MS basal with 3.0 mg/l IBA after 5 weeks

Fig 4.36: Root initiation from excised shoot in MS basal with 4.0 mg/l IBA after 5 weeks

Fig 4.37: Root initiation from excised shoot in MS basal with 4.0 mg/l IBA after 5 weeks

Fig 4.38: Root initiation from excised shoot in MS basal with 1.0 mg/l IBA after 3 weeks

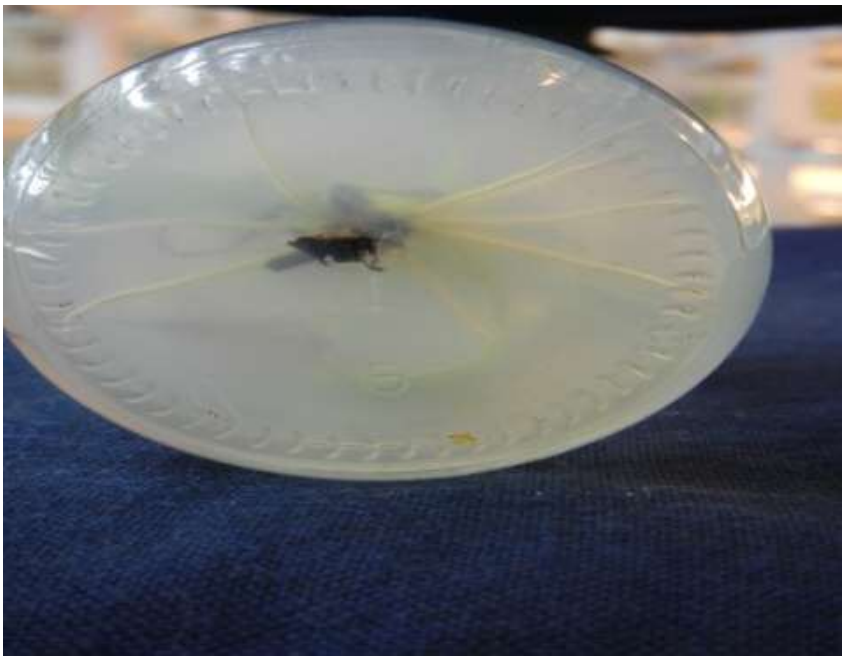

Fig 4.39: Root initiation from excised shoot in MS basal with 1.0 mg/l IBA after 5 weeks

Fig 4.40: Root initiation from excised shoot in MS basal with 2.0 mg/l IBA+2.0 gm/l Charcoal after 5 weeks

Fig 4.41: Root initiation from excised shoot in MS basal with 2.0 mg/l IBA+2.0 gm/l Charcoal after 5 weeks

Fig 4.42: Plantlets with roots obtained in different rooting media ready for primary hardening.

A: 1.0 mg/l IBA

B: 2.0 mg/l IBA

C: 3.0 mg/l IBA

D: 4.0 mg/l IBA

E: 2.0 mg/l IBA+ 2.0 mg/l IAA

F: 3.0 mg/l IBA+ 2.0 mg/l IAA

G: 2.0 mg/l IBA+ 2.0 gm/l Charcoal

H: 2.0 mg/l IBA+ 2.0 mg/l IAA 2.0 gm/l Charcoal

Fig 4.43: Rooted plantlets transferred to the portray

Fig 4.44: Hardened plant in portray under green house after 15 days

Fig 4.45: Secondary hardened plants after 21 days

Fig 4.46: Secondary hardened plants after 30 days