Optimizationof Tissue Culture Conditions of *Lallementia royleana* (Benth.) Benth. and Assessment of Somaclonal Variations

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in

Plant Biochemistry and Molecular Biology

By

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A dissertation submitted in the partial fulfilment of the requirements for the degree of Master of Philosophy in Plant Biochemistry and Molecular Biology

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DECLARATION

This is to certify that the dissertation entitled **"Optimization of Tissue Culture Conditions of *Lallementia royleana* (Benth) Benth and Assessment of Somaclonal Variations"** submitted by **Huma Arshad** is accepted in its present form by the Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan, as satisfying the dissertation requirement for the degree of *M.Phil.* in Plant Biochemistry and Molecular Biology.

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Dedicated

to

My heaven on earth

My Parents

Special thanks to

Samar Naseer

And

Riffat Batool

The most encouraging and helpful friends

I ever had

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree centigrade
µg	Microgram
µl	Microliter
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
bp	Base pair
cm	Centimeter
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
GA_3	Gibberellic acid
HCl	Hydrochloric acid
hrs	Hours
IAA	Indole-3-acetic acid
l	Litre
mg	Milligram
ml	Milliliter
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
NARC	National agricultural research centre
ng	Nanogram
NTSYS	Numerical taxonomy and multivariate analysis system
PCR	Polymerase chain reaction
pmol	Picomolar

RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNAse	Ribonuclease
rpm	Revolutions per minute
SIMQUAL	Similarity for quantitative data
SSR	Simple sequence repeat
TAE	Tris acetate EDTA
TE	Tris EDTA

ABSTRACT

Lallementia royleana (Benth.) Benth. or Tukhm-e-Balango is an important medicinal plant that is cultivated largely for its seeds which are frequently used in traditional system of medicine. In Pakistan due to over exploitation this plant has been declared endangered from the wild but it is being cultivated in some regions. Tissue culture techniques have been exploited for the micropropagation of many plants, including a wide range of threatened medicinal plants. In the present study, an efficient protocol for direct regeneration of this highly medicinal plant was optimized through different explants (leaves, nodes, internodes and petioles) by using different combinations of phytohormones (NAA, BAP, IAA, Kinetin and 2,4-D). The callogenic ability of all these explants was also checked on MS media supplemented with these phytohormones, but they gave poor callogenic response. Of all the explants used nodal explants gave best organogenic response (80 %) producing 17.75 shoots per explant, on MS media supplemented with BAP 1 mg/l. Whereas, NAA 1 mg/l gave best rooting response, producing 24 roots per explant. *In vitro* propagation often results in genetic and phenotypic variations in regenerated plants. In the present study these somaclonal variations in regenerated plants were analyzed by PCR based RAPD markers, as compared to the seed derived control plant. Random decamer RAPD primers, from OPC series (OPC1-OPC5) were used for this purpose. OPC primers revealed overall genetic variation of 36.8 % among all the eleven samples of *Lallementia royleana*. Among all the regenerated plants highest polymorphism was detected in *in vitro* regenerated shoot on 1.0 mg/l NAA that produced 18 amplified bands out of which eight bands were polymorphic. To construct similarity matrix along with corresponding dandrogram NTSYS PC version 2.0 was used based on RAPD data. The present study shows that different hormonal combinations and concentrations used for micropropagation of plants can induce genetic variation even in directly regenerated plants, and RAPD is an effective and reliable method to analyze these variations.

INTRODUCTION

Lallementia royleana (Benth.) Benth. belongs to the family Lamiaceae formerly known as Labiateae. It is commonly known as black psyllium. It is commonly known as tukh-e-balangu or tukhmalanga in Pakistan and India, also called as balangu and balangu sherazi in some parts of the world (Naghibi *et al.*, 2005).

Lamiaceae is a cosmopolitan family comprising of about 180 genera and over 3500 species. It consists of annual to perennial plants which could be herbs, subshrubs or shrubs. Members of Lamiaceae are usually aromatic and have sessile oil glands or glandular hairs. Some members such as lavender, mint, oregano, thyme, basil and rosemary are used and widely cultivated for culinary purposes. Presence of secondary metabolites such as terpenes and phenolics is well established in labiates. These are also rich source of essential oils which are used for flavouring and in perfume industry (Valdes III *et al.*, 1987). Some Labiates are cultivated for ornamental purposes as well.

The genus Lallemantia belongs to the tribe Stachyoideae-Nepeteae of family Lamiaceae. It is found in various regions of Europe and Asia and is represented by 5 species in South West and central Asia. In Pakistan it is represented by only one species i.e., *Lallementia royleana*. It morphologically differs from all of the other Labiateae members found in Pakistan as it has flattened pedicels, awns are present at the margins of the bracts and have closed fruiting calyces. It is cultivated for its mucilaginous seeds which are used medicinally (Flora of Pakistan).

1.1 Plant Morphology

Lallementia royleana is an annual herb which may be branched or unbranched at the base. It has an erect stem which is quadrangular and has a dense indumentum of short eglandular retrose hairs and is leafy. Leaves are simple, oblong to obovate in shape. It has an erect stem which is quadrangular, 5-30 cm in size, with a dense indumentum of short eglandular retrorse hairs and is leafy. Leaves are simple, 15-20 x 7-15 mm in size, oblong-obovate, crenate, cuneate, below with short glandular hairs and scattered sessile oil globules, petiole are up to 15-20 mm. Inflorescence starts

from near the base of stem. Verticillasters are present in the axils of the leaves and are numerous, 6-8 flowered, distant or contiguous. Bracts are several, linear-oblong as long as or longer than calyx, sessile or shortly petiolate, cuneate, with marginal awns 2-4 mm long. Calyx tubular, 6-7 mm, prominently veined or ribbed, with short eglandular spreading hairs and a few sessile oil globules, upper lip has 3 ovate obtuse lobes, lower lip has 2 narrower lobes which are all shortly acuminate. Teeth are clearly convergent in fruit and closing the mouth. Corolla is pale lilac, blue to whitish pink in colour which is 7-8 mm, slightly longer than calyx, upper lip is 1.5 mm long and is shorter than lower lip. Seeds are 2.5 x 1 mm in size, dark brown in colour. These are markedly triquetrous, with a small attachment scar. They are clearly mucilaginous on wetting (Flora of Pakistan).

1.2 Geographic Location

Lallementia royleana is cultivated in different regions of Europe, Western Asia, Pakistan, Turkey, Iran and India (Moghaddam, 2011). In Pakistan *Lallementia royleana* is found wild in Chitral, Malakand, Khyber, Hazara, Abbottabad, Swat, South Waziristan, Kaninguram, Kurram, Parachinar, Kohat, Hangu, Rawalpindi, Attock, Wah Gardens, Jhelum, Quetta, Zhob, Kalat, Sibi, Surab and Makran (Flora of Pakistan).

1.3 Chemical Constituents

The yellowish oil is obtained from the aerial parts of *Lallementia royleana* which has a strong and pleasant aroma. The major constituents of the oil are 9.8 % trans-carveol, 16.4 % verbenone and 8.9 % β-cubebene. However, some other identified compounds in essential oil are 1,8-cineole, limonene, β-trans-ocimene, β-cis-ocimenes, β caryophyllene and β-bourbonene (Ghannadi and Zolfaghari, 2003).

1.4 Medicinal Importance

Lallementia royleana is an important medicinal plant and has a long history in phytomedicine. It is an aromatic herb and the seeds are of great interest in folk medicine as they are mucilaginous. These are utilized in the treatment of many ailments, such as some hepatic, renal and nervous diseases. Seeds of *L. royleana* are also used in preparing general tonic, expectorant and aphrodisiac preparations (Ghannadi and Zolfaghari, 2003). They are also helpful in abscesses, inflammations

and gastrointestinal problems (Abdulrasool *et al.*, 2011). They are also considered to have cooling, sedative and diuretic properties and are used in flatulence and constipation. Seeds are soaked in water and then putting the layer on a boil can help to cure the pain or bursting of boil. Seeds of *Lallementia* have extensive swelling properties due to which these have been investigated to be used as a super disintigrant in the formulation of nimesulide orodispersible tablets (Malik *et al.*, 2011). Leaves of the plant have been reported to possess antiemetic activity. (Mohtasheemul *et al.*, 2012). Very few *Lallementia* species have been chemically studied so far.

1.5 Economic Importance

Lallementia royleana is cultivated in different regions of Europe, Western Asia, Pakistan, Turkey, Iran and India for its mucilaginous seeds. These seeds are an important ingredient of cooling drinks. These seeds are also used in traditional system of medicine to cure many ailments (Abdulrasool *et al.*, 2011).

1.6 Tissue Culture

Every plant cell has the unique potential to regenerate into a whole plant. This property of a plant cell is called totipotency and serves as the foundation of all tissue culture work. Plant tissue culture is the aseptic culture of cells, tissues and organs under physically and chemically defined *in vitro* conditions (Thorpe, 2007). Tissue culture relies on three fundamental abilities of plants namely totipotency, dedifferentiation and competency. Totipotency is the genetic potential or capacity of the plant cells to regenerate into a complete plant. Although theoretically all plant cells are totipotent but the meristematic cells express it best of all. Dedifferentiation is the capacity if mature cells to regain the meristematic activity and start the process of growth again and competency is the potential of a cell or a tissue to develop. Schwann and Scheilden put forward the cell theory in 1938 proposing the cell to be the basic unit of organisms and stating that the cells are autonomic and capable of regenerating into a complete plant. Their theory as clearly establishing the concept of totipotency provided the basis for plant cell and tissue culture. Theoretical infrastructure of plant tissue culture was provided by Gottleib Haberlandt (1902). He was the first one to cultivate isolated plant cells on an artificial medium *in vitro*. He believed that unlimited fragmentation would not be influenced by cellular proliferation. He cultivated isolated and photosynthetic cells of the leaves of *Lamium purpureum*, and

other cells differentiated functionally i.e, petiolar cells of *Eichhornia crassipes*, *Urtica*'s glandular hairs and *Pulmonoria*, and *Tradescantia*'s stamen hair cells by using Knop's (1865) salt solution (Vasil, 2008). Although he was unsuccessful in his attempts as the cells grew in size and survived for several months, accumulated starch but failed to proliferate. His predictions were falsified. Haberlandt failure to obtain proliferation in his cultures was partly due to the relatively simpler nutrients, as his culture medium mainly consisted of Knop's solution, asparagine, peptone and sucrose, and partly due to the use of highly differentiated cells. He predicted that artificial embryos can successfully be cultivated from somatic cells thus he clearly established the idea of totipotency. He proposed the use of embryonic fluids i.e., coenocytic liquid endosperm, for inducing cell divisions in somatic cells. Later on coconut milk was successfully used on a large scale in tissue culture studies for inducing cell division in vegetative cells. These serve as valid reasons for crediting Haberlandt as founder of the science of plant cell culture (Vasil, 2008).

Cultures of excised root tips and embryos were achieved by Molliard (1921), Robbins (1922) in United States and Kotte (1922) in Germany, on Knop's (1865) mineral solution. Robbins was the first one to develop a technique for the culture of isolated roots. These cultures were of limited success as on the minimal medium some growth was observed but it could not be maintained for more than few weeks (Vasil, 2008).

For the following years further progress in the field of tissue culture was hindered due to non-availability of the nutrient solution which was capable of favouring isolated plant cells and tissues growth. Culture of isolated root tips was achieved in some other studies. Some years down unlimited growth in cultured plant cells was first achieved by Philip Rodney White (1932) mainly because of the explants having meristematic cells. This resulted in successful maintenance of cultures of tomato's excised root tips and other plants for indeterminate time period. Further work was carried out on root culture on a medium which was completely defined. Initially these cultures were meant for viral studies but later on these proved to be a key instrument for physiological studies. Successful bud cultures were also achieved later on. Embryo culture of Barley was achieved in the initial years of last century. After this embryos of some nonviable seeds were rescued which were produced as a result of a cross between *Linum austriacum* and *Linum perenne*. Same

was done for some early ripening fruit species for full embryo development (Vasil, 2008). These were some of the earliest applications of *in vitro* culturing. Gautheret (1934) was the first one to obtain true plant tissue cultures from cambial cells of *Acer pseudoplatanus*. These cultures ceased to grow after 18 months. Similar work was done with explants of *Robinia pseudoacacia*, *Salix capraea* and *Ulmus campestre*. He used agar solidified medium of Knop's solution, cysteine hydrochloride and glucose (Thorpe, 2007).

Significance of indole-3-acetic acid (IAA) as a hormone that can influence division and growth of cells was also established meanwhile. Nobecourt (1939) studied the effects of auxin on growth of explants of carrot. Growth of cambial explants in the presence of IAA for a longer period of time was reported by Gautheret (1939) and White (1939). Due to improved nutrient solution and use of IAA and aseptic cultures, growth of tobacco stem and carrot root was maintained for indefinite period of time (White 1939, Nobecourt 1939 and Gautheret 1934, 1939, 1985). White (1943, 1963) developed a new nutrient solution i.e., White's medium. It included the ingredients of Uspenski and Uspenskaia's medium for algae (1925), micronutrients, nicotinic acid, pyridoxine, glycine and thiamine. Until 1960's this medium was used quite frequently for plant tissue cultures (Vasil, 2008).

1940s, 1950s and 1960s were the year of rapid development of new techniques and improvement of already existing ones (Thorpe, 2007). Albert C. Hildebrandt carried out the most systematic and detailed studies of nutrients required by cultured plant tissues. Result of these studies was the development of a nutrient medium containing high levels of mineral salts (Vasil, 2008). During that time Toshio Murashige was working on growth of cultures of tobacco pith tissues which was optimum as well as predictable. The growth increased four times by adding an aqueous extract of tobacco leaves to White's medium. These results led to the development of a completely defined and new nutrient solution which was mainly due to the inorganic constituents of the leaf extract. Ash of tobacco leaf extracts, phosphate, nitrate and potassium salts and ammonium, on addition to white's medium also generated same results. A completely defined and new nutrient medium was formulated on the basis of these results called the Murishage and Skoog or MS medium (Vasil, 2008). This medium also included a mixture of four vitamins, myo-inositol and chelated iron to make it more stable (Murashige and Skoog, 1962). The

most extensively used formulation in plant tissue culture to date is MS medium. First cytokinin was discovered from the breakdown products of sperm DNA of herring during further studies by Carlos Miller. Cytokinin was soon identified to be 6-furfurylaminopurine and was very effective in inducing cell division at very low concentrations. Due to this many other species could also be cultures indefinitely (Thorpe, 2007). Naturally occurring cytokinins were discovered later on in many tissues. Coconut water also found to contain cytokinins. It was also established by Skoog and Miller (1957) that the ratio of auxin and cytokinin determined the fate of the cells of tobacco callus. High auxin to cytokinin ratio induced rooting whereas higher levels of cytokinins favoured shooting. Proliferation of callus occurred at intermediate levels (Thorpe, 2007).

Due to the development of many *in vitro* techniques the field of tissue culture blossomed over time. It was applied to solve many problems in basic biology, forestry, agriculture and horticulture. These applications involve clonal propagation, disease elimination, plant improvement and the production of secondary metabolites. By 1985, about 100 angiosperm species were regenerated using protoplast (Thorpe, 2007). The most widely used application of the tissue culture technology is the micro propagation of plants.

Plants have been a major source of medicine from ancient times. Almost all cultures have used plants as a source of medicine (Nalawade and Tsay, 2004). Medicinal plants have an important role in the global economy as approximately 85 % of traditional medicines are prepared using different plant parts or their extracts (Vieira and Skorupa, 1995). During the last few years there has been an increased interest in the use of medicinal plants in health care due to which the demand for herbal medicines has increased and most of the pharmaceutical industries obtain the raw material from wild populations (Bodeker, 2002). In modern medicine many plants serve as source of direct therapeutic agents as well as raw material for more complex semisynthetic chemical compounds (Akerele, 1992). This practice has threatened the genetic diversity of many pharmaceutical plants due to over exploitation. Destruction of plant rich habitats due to agricultural encroachments and urbanization is another factor.

Before the discovery of cytokinin the major *in vitro* regeneration related work was centered on tobacco. After that complete flowering plants of carrot were regenerated from phloem cells. Micro propagation of medicinal plants remained neglected for a long time. During recent years there have been several reports relating to direct and indirect regeneration of medicinal plants (Chaturvedi *et al.*, 2007). A great number of medicinally important plants have been regenerated *in vitro*. These include *Allium sativum* (Ayabe and Sumi, 1998), *Aloe vera* (Cavallini *et al.*, 2003), *Ananas comosus* (Sripoaraya *et al.*, 1982), *Atropa belladonna* (Chaturvedi *et al.*, 1982), *Artemesia annua* (Gulati *et al.*, 1996), *Azadirachta indica* (Sharma *et al.*, 2002), *Carica papaya* (Agnihotri *et al.*, 2004), *Cassia fistula*, *Catharanthus roseus* (Bajaj *et al.*, 1988), *Curcuma domestica* (Balachandran *et al.*, 1990), *Digitalis lanata* (Erdei *et al.*, 1981), *Glycyrrhiza glabra* (Shah and Dalal, 1982), *Hyoscymus niger* (Cheng and Raghavan, 1985), *Mentha arvensis*, *Mentha piperita* (Rech and Pires, 1986), *Ocimum basilicum* (Ahuja *et al.*, 1982), *Panax ginseng* (Choi *et al.*, 1998), *Papaver somniferum* (Nessler, 1982), *Rauvolfia serpentina* (Chaturvedi, 1979), *Rosmarinus officinalis* (Misra and Chaturvedi, 1984), *Saussorea lappa* (Arora and Bhojwani, 1989), *Withania somnifera* (Kulkarni *et al.*, 2000) and *Zingeber officinale* (Nadgauda *et al.*, 1980) etc.

In phytopharmaceutical preparation variations have been observed in medicinal quality and content. Medicinal content varies from plant to plant and is also affected by season of collection and cultivation period (Nelawade and Tsay, 2004).

1.7 Plant Growth Regulators

In *in vitro* culturing all physical and chemical needs of a plant must be met. External environmental conditions (light, temperature etc.) must be favorable for growth. The growth medium should supply all the essential mineral ions which are required by the plant for its growth and development. In addition organic supplements such as amino acids and vitamins are also required by plants. Plant growth regulators are small molecules that define the developmental pathways of cells and are very crucial for culturing.

In a broader sense plant growth regulators can be defined as any substance that is capable of influencing plant growth and development. These could be naturally occurring or synthetic and are effective at very low concentrations. Five main classes

of plant growth regulators are used in tissue culture i.e., auxins, cytokinins, gibberellins, abcisic acid and ethylene. Went (1928) in Netherland for the first time isolated and quantified plant hormone and named it as''wuchstoff'' (hormone). He stated that no growth is possible without hormone. Later on this hormone was identified to be a naturally occurring auxin which regulates the amount, type, and direction of plant growth. Auxins are found throughout the plant kingdom. They are mainly found in growing areas i.e., meristems as well as in other areas of plants.

The most important naturally occurring hormone IAA was first isolated by Kögl (Kögl and Kostermans, 1934) in Copenhagen in crystalline form from pregnant women's urine and by Thimann (1935) in the United States from cultures of a fungus i.e., *Rhizopus suinus*. Thimann and his co-workers studied auxin and contributed a lot in understanding the structure and function of auxin. They also contributed in the development of a large collection of synthetic auxin such as 2, 4-D which is being extensively used in plant tissue culture now (Vasil, 2008).

The first cytokinin i.e., kinetin was discovered as a breakdown product of herring sperm DNA by Carlos Miller. Kinetin was found to induce cell division at very low concentrations in the presence of auxins. This class of plant growth regulators was initially named kinin but was changed later on to cytokinin due to chances of confusion with another class of chemicals found in animal systems. Up till now, identified naturally occurring cytokinin are very few in number however many synthetic compounds have been synthesized having varying levels of activities. The determination of the auxin-cytokinin ratio regulating morphogenesis of plants is considered to be an important milestone for the understanding of morphogenesis of plants and for studying regeneration and micropopagation of plants from cultured tissues (Vasil, 2008).

Gibberelins are naturally occurring and synthetic structurally related compounds which are involved in regulating cell elongation and also play role in determining plant height and fruit set. Very few gibberellins are used in tissue culture and GA_3 is one of them. Another plant hormone abscisic acid inhibits cell division. It is mostly used to promote cells to take a distinct developmental pathway such as somatic embryogenesis.

Ethylene is a naturally occurring plant growth regulator which is gaseous. It is involved in controlling fruit ripening. Its use in tissue culture is very limited however it does poses as a problem for culturing as some cell cultures produce ethylene. If it accumulates it can inhibit the growth and development of the culture (Ramage and Williams, 2002).

Prediction of the effect of plant growth regulator is difficult as there is difference in culture response among species and varieties. Auxins and cytokinins are most widely used plant growth regulators in tissue culture and there ratio determines the type of culture that is regenerated (Sugiyama, 1999).

Cultures are initiated from the sterile pieces taken from the whole plant and are known as explants. These may be pieces of organs such as leaves, stems or roots or these may be specific cell types such as endosperm or pollens. Efficiency of the culture is affected by the features of the explant used (Gamborg, 1976).

1.8 Tissue Culture of Lamiaceae

Lamiaceae is best known for the culinary herbs as it includes basil, oregano, mint, rosemary, marjoram, thyme, savory and sage. Teak (*Techtona grandis*) is one of the most important timbers of the world. It is greatly valued for its use in marine applications. *Vitex* and *Gmelia arborea* are also used as wood source. Many mint species are commonly used in perfumes and cosmetics. *Salvia divinorum* is a hallucinogen. Many species of Lamiaceae are used for therapeutic use. These members are widely used in traditional system of medicine e.g. relief of stomachache and diarrhea. Due to the presence of these economically and medicinally important plants within this family other genera have remained shadowed from scientific probing. Quiet extensive scientific work regarding tissue culture has been done on several species belonging to this family e.g. *Ocimum basilicum, Ocimum gratissimum, Ocimum tenuiflorum, Ocimum americanum, Salvia nemorosa, Salvia officinalis, Salvia canariensis, Mentha piperita, Mentha viridis* etc.

Phippen and Simon (2000) provided protocol for regeneration of *Ocimum basilicum* using leaf explants. The regenerated plants were acclimatized under greenhouse conditions afterwards. Later on, Siddique and Anis (2008) provided an improved and efficient plant regeneration system of *Ocimum basilicum* and also

successfully acclimatized regenerated plants to *ex vitro* conditions. These plants required more extended time for acclimatization than generally accepted. Lim *et al.* (2009) obtained callus of *Ocimum sanctum* by using leaf explants. A protocol for root culture of *Ocimum sanctum* was provided by Shilpa *et al.* (2010) using leaf explants. In another study, Gopi *et al.* (2006, a) developed a rapid system for regeneration of *Ocimum gratissimum* using nodal explants. These regenerated plants were successfully acclimatized. In some other reports micropropagation of *Mentha viridis* from nodal and shoot tip explants (Raja and Arockiasamy, 2008) and of *Salvia nemorosa* from shoot tips and leaves (Skala and Wyosokinska, 2004) has been reported as well.

Research work regarding *Lallementia royleana* is scanty. No scientific study regarding regeneration of *Lallementia* Genus has yet been reported. The present research work aims for the development of an efficient protocol for direct regeneration of *Lallementia royleana*.

1.9 Somaclonal Variation

Tissue culture is being used in both research and commercial applications. It not only provides a method of mass propagation, but also makes possible the production of disease-free plants, mutants, and secondary plant products. Another important use is the genetic engineering of plants. A single plant can be genetically modified and grown into a mature plant or plants having new characteristics.

Tissue culture utilizes the regenerative potential of plant cells. It involves the development of a whole plant from repetitive and rapid divisions of a limited number of plant cells under physically and chemically defined conditions. Investigations have revealed that cell or tissue cultures undergo frequent genetic changes i.e., polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplifications and mutations which are expressed at biochemical or molecular levels due to which cultures isolated from single explant, or even a single cell can show variation after repeated subculture. These tissue culture induced phenotypic and genotypic variations are collectively termed as somaclonal variations (Larkin and Scowcroft, 1981).

The occurrence of somaclonal variation is associated with point mutations, chromosomal rearrangements and recombination, DNA methylation, altered sequence

copy number, transposable elements, and seems to be influenced by the genotype, explant type, culture medium and age of the donor plants (Jain *et al.*, 1998). Chromosoml abberations are frequently found in plants regenerated from tissue culture (Jain, 2001). Translocations, duplications, deletions and inversions have all been detected. Normal cell cycle controls, which prevent cell division before the completion of DNA replication, are presumed to be disrupted by tissue culture, resulting in chromosome breakage. Chromosome breakage could be induced by altered levels of DNA methylation which is supposed to be a principal factor in tissue culture induced mutagenesis (Phillips *et al.*, 1994). Methylation changes might affect variation in several ways. These changes could result in chromatin structure alterations which may lead to late replication of heterochromatin and, therefore, to chromosome breakage, and changes in gene expression (Kaeppler, 2000).

Somaclonal variations are unpredictable in nature and can be heritable and non-heritable. Generally these result in a range of genetically stable variations in qualitative as well as quantitative traits. Somaclonal variation has advantages as well as disadvantages. The changes can occur in agronomically important traits on the other hand these may not occur for more complex agronomic traits. Changes occur at high frequency which can result in novel phenotypes which may not be achieved by conventional breeding. It can help in isolating biotic and abiotic stress tolerant lines. However the changes produced can also be insignificant as these are unpredictable in nature and may not be genetically stable due to DNA methylation and transposon elements. Selection of somaclones also requires extensive field testing (Jain, 2001).

The mutagenic nature of tissue culture has been extensively studied and reviewed. Tissue culture system itself acts as a mutagenic system because cells experience traumatic experiences from isolation, and may undergo reprogramming during plant regeneration which is different than under natural conditions. Reprogramming or restructuring of events can create a wide range of variations in newly regenerated plants (Jain, 2001).

Studies of tissue culture based variations have usually been based on phenotypic differences in regenerated plants and their progeny. Somaclonal variations have found to cause variation in leaf morphology, height and maturity of plant and disease resistance (Hammerschlag, 1992). Morphological markers have proved to be

useful in some studies but these have many limitations as they have limited diversity and are affected by the environment and growth stage of the plant (Tanksley *et al.*, 1989). However, genomic changes are the basis for the phenotypic alterations.

Biochemical markers are also a good tool for the detection of somaclonal variation as these are based on variations in protein structure. Isozymes have been used to detect somaclonal variation among regenerated apple plants which showed polymorphism (Martelli *et al.*, 1993). Isozyme analysis of regenerated date palm plants showed variations which were age dependent (Saker *et al.*, 2000). Somatic embryo-derived plants of napier grass were analyzed by Shenoy & Vasil (1992) for several isozymes however no variation was found among regenerants. Sabir *et al.* (1992) investigated the frequency of genetic change giving rise to somaclonal variation in sugerbeet on the basis of isozyme variation. No genetic variation among the isozyme pattern was detected in all genetic variants. Use of developmentally and physiological stable enzymes including alcohol dehydrogenease, malate dehydrogenase, phosphoglucomutase and phosphoisomerase for analytical studies was proposed by Bouman and De klerk (2001).

A wide variety of molecular methods are used now a days for the characterization of plant genomes (Henry, 1998). Most of them have the potential to be used for the analysis of somaclonal variation. DNA based markers have been applied widely in the analysis of somaclonal variation (Piccioni *et al.*, 1997; Henry, 1998). The Random amplified polymorphic DNA (RAPD) technique has several advantages such as the ease and rapidity of analysis, a relatively low cost, availability of a large number of primers and the requirement of a very small amount of DNA for analysis (William *et al.*, 1990). RAPD analysis using polymerase chain reaction (PCR) in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals.

DNA based markers like methylation sensitive restriction fragment length polymorphism (RFLP) (Jaligot *et al.*, 2000; and Kubis *et al.*, 2003); microsatellites (Alou *et al.*, 2004), and Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1993; and Rival *et al.*, 1998) have been frequently used in recent studies to detect the somaclonal variation (Venkatachalam *et al.*, 2007). Veilleux *et al.* (1995) used both RAPD and SSR techniques to characterize the genetic composition of anther-

derived potato plants. Wolff *et al.* (1995) used RAPD, SSR, and RFLP markers to evaluate somaclonal variation in vegetatively propagated chrysanthemum cultivars. In sugarcane RAPD polymorphisms were observed in plants regenerated from embryogenic callus, indicating infrequent gross genetic changes during tissue culture (Taylor *et al.*, 1995). RAPD marker analysis showed variation in cryopreserved embryogenic clones of *Picea glauca* (DeVerno *et al.*, 1999). Recently, Munir *et al.* (2011) assessed the somaclonal variation in *in vitro* cultured *Solanum tuberosum* plants thrugh RAPD analysis. Genetic variation was found between *in vitro* and *in vivo* grown plants of *Sylibum marianum* based on RAPD markers (Mahmood *et al.*, 2010). RAPD analysis showed distinct variation in profiles to confirm menthol tolerance and high menthol content character of the genotype that favoured the *in vitro* selection of *Mentha arvensis* clones (Dhawan *et al.*, 2003). The changes in the banding pattern obtained in basil plants regenerated *in vitro* suggested the existence of genetic variation that might affect the biochemical synthesis of phytoproducts (Rady and Nazif, 2005).

1.10 Aims of Study

Lallementia royleana is a plant of medicinal as well as economic importance but no protocol for its *in vitro* regeneration has yet been developed. Therefore, the present study was designed to achieve the following objectives:

- To develop an efficient protocol for *in vitro* culturing of *Lallementia royleana*
- To determine the effect of different hormonal concentrations on callogenesis and organogenesis
- To determine the effect of type of explant used on direct regeneration
- To determine the somaclonal variations produced as a result of different hormonal concentrations used for *in vitro* propagation of *Lallementia royleana* using RAPD markers

MATERIALS AND METHODS

In the present study experiments were carried out to optimize condition for callogenesis and organogenesis of *Lallementia royleana*. The experimental work was carried out in three steps. First, conditions for callus induction or callogenesis were optimized on Murashige and Skoog (MS) medium using different plant growth hormones i.e. Indole 3-Acetic acid (IAA), 1-Naphthaleneacetic acid (NAA), 6-Benzylaminopurine (BAP) and 2,4-Dichlorophenoxyacetic acid (2,4-D) in different concentrations. In the second step, different explants were transferred to regeneration medium for checking their organogenic ability with respect to the explant origin. Third, different genetic variations occurred during organogenesis were determined by applying RAPD markers. A brief account of material and methods used and all procedures adopted are given below.

2.1 Collection of Plant Material

The seeds of *Lallementia royleana* were obtained from National Agriculture Research Centre (NARC) Pakistan.

2.2 Sterilization of Glassware

All glass ware used in the experiments were washed thoroughly using a commercially available domestic detergent. These were then washed three times with distilled water prior to sterilization. The forceps, scissor, beakers and media were sterilized by autoclaving at 15 Ibs pressure/square inch, at 121°C for 15 minutes before use.

2.3 Sterilization of Seeds and Inoculation

Seeds were soaked in distilled water for 12 hours, then the mucilage coating was removed by rubbing the seeds between the folds of filter paper. Seeds were first kept in 70 % ethanol for 5 minutes. After that seeds were kept in 25 % commercial bleach for 15

2.4 Explant Selection

The nodes, internodes and leaves were used as explants for callus induction. For direct regeneration nodes, internodes, leaves and petioles were used as explants.

2.5 Media Preparation

2.5.1 Media for callogenesis

For callus culture of *Lallementia royleana* full strength MS medium was used supplemented with different concentrations and combinations of hormones (Table 2.1). The pH of media was adjusted at 5.8 and 2 g/l gelling gum powder was also used to solidify the media.

Table 2.1: Combinations of plant growth regulators for callus induction MS media.

S. No.	Media code	PGR's	Concentration (mg/l)
1	CIM0	-	-
2	CIM1	NAA	0.5
3	CIM2	NAA	1.0
4	CIM3	NAA	2.0
5	CIM4	IAA	0.5
6	CIM5	IAA	1.0
7	CIM6	IAA	2.0
8	CIM7	BAP	0.5
9	CIM8	BAP	1.0
10	CIM9	BAP	2.0
11	CIM10	BAP + IAA	2 + 1
12	CIM11	BAP+IAA	1 + 2
13	CIM12	2,4-D + NAA	0.5 + 1
14	CIM13	2,4-D + NAA	1 + 2

2.5.2 Media for direct regeneration

2.5.2.1 Shooting media:

Explants were shifted to MS media supplemented with different concentrations of BAP and kinetin (Table 2.2). The shooting media was poured in glass bottles inside the laminar flow hood. After inoculation of explants on shooting media the bottles were kept in growth chamber for shoot initiation.

Table 2.2: Shoot induction MS media having different concentrations of plant growth regulators

S. No.	Media Code	PGR's	Concentration (mg/l)
1	S0	-	-
2	S1	BAP	0.5
3	S2	BAP	1.0
4	S3	BAP	2.0
5	S4	BAP	4.0
6	S5	Kinetin	0.5
7	S6	Kinetin	1.0
8	S7	Kinetin	2.0
9	S8	Kinetin	4.0

2.5.2.2 Rooting media:

After shoot formation, these were transferred to rooting medium for root initiation. The rooting media were supplemented with different concentrations of NAA and IAA (Table 2.3).

Table 2.3: Root induction MS media having different concentrations of plant growth regulators.

S. No.	Media code	PGR's	Concentration (mg/l)
1	R0	-	-
2	R1	NAA	0.5
3	R2	NAA	1.0
4	R3	NAA	2.0
5	R4	IAA	0.5
6	R5	IAA	1.0
7	R6	IAA	2.0

2.6 Aseptic Transfer of Explants

Aseptic transfer of explants was accomplished in Laminar flow hood. Before transferring the explants, the laminar flow hood was sprayed with 70 % ethanol. Then explants were transferred on to the medium for callus induction.

2.7 Culture Conditions

The cultures were kept under 16 hrs photoperiod, 55±5 % relative humidity at 25-26 °C and the light intensity was maintained at 1000 lux inside growth chamber for callus induction.

2.8 Maintenance of *In Vitro* Cultures

The explants were sub-cultured on the medium having same concentration of hormones after a regular interval of 25-30 days.

2.9 Somaclonal Variations

Somaclonal variation among the regenerated plants was assessed on the basis of RAPD analysis, for which the following steps were performed:

2.9.1 DNA extraction

Total genomic DNA was extracted from shoots using CTAB (Cetyl Trimethyl Ammonium Bromide) method (Richards, 1997) with few modifications. Approximately 0.3 g of plant material (about four to five leaves) was washed with distilled water. Plant material was then crushed in a mortar by pestle into fine paste using 2 ml of preheated (65 °C) 2 x CTAB (Cetyl Trimethyl Ammonium Bromide) buffer. 2 x CTAB was prepared by adding 100 mM Tris HCL (pH 8.0), 20 mM ethylene diamine tetra acetic acid (EDTA) (pH 8.0), 1.4 M sodium chloride (NaCl) and 2 % w/v CTAB. β-Merceptoethanol (1 %) was also added to warm CTAB before grinding. The homogenized mixture was transferred to autoclaved 1.5 ml eppendorf tubes. The mixture in tubes was incubated at 65 °C for 45 minutes on heating block. The tubes were then centrifuged at 10,000 rpm for 10 minutes after incubation. The supernatant was collected and transferred to new eppendorf tubes. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the supernatant in tubes and inverted gently five to six times for proper mixing. The mixture was centrifuged at 10,000 rpm for 10 minutes to spin down remaining cell debris and supernatant was then collected and shifted to another eppendorf carefully. This step was repeated three to four times, and then an equal volume of chilled isopropanol was added to it to precipitate the DNA. The tubes were slowly inverted for two to three times. These tubes were left overnight at -20 °C. Centrifugation was done the next day at 12,000 rpm for 12 minutes. The DNA in white form precipitated at the bottom of tube, and the aqueous phase was poured out. The pellet was washed with 70 % chilled ethanol. After discarding the 70 % ethanol, the pellet of DNA was air dried at room temperature and then rehydrated in 30-40 µl of 0.1 x TE (Tris EDTA) (Tris ethylene diamine tetra acetic acid) buffer containing 2 µl (0.1 µg/ µl) of RNase. The isolated DNA was preserved at -20 °C.

2.9.2 DNA Quality Test

The quality and integrity of extracted DNA was assessed by agarose gel electrophoresis. For this purpose minigel 25 ml of 1 % agarose solution was prepared in 0.5 x tris acetate ethylene diamine tetra acetic acid (TAE) buffer, these small gels give good photographs and are commonly used. The prepared gel cassette was then placed in gel tank containing 0.5 x TAE as running buffer. The samples were loaded separately in wells along with 6 x bromophenol blue as loading dye. The gel was run for 35 minutes at constant voltage of 85 volts, and then stained in ethidium bromide for 20 minutes. The gel was visualized under ultraviolet (UV) light and photographed using Dolphin Doc ^{plus} gel documentation system (Wealtec).

2.9.3 RAPD analysis

2.9.3.1 Primers:

RAPD amplifications were carried out by using five decamer RAPD primers from OPC series, OPC1-OPC5 (table 2.4), to analyze the somaclonal genetic variability in different samples.

Table 2.4: Sequence of primers used from OPC series.

S. NO.	Name	Sequence
1	OPC1	5'-TTCGAGCCAG -3'
2	OPC2	5'-GTGAGGCGTC -3'
3	OPC3	5'-GGGGGTCTTT -3'
4	OPC4	5'-CCGCATCTAC -3'
5	OPC5	5'-GATGACCGCC -3'

2.9.3.2 Polymerase chain reaction (PCR) amplification:

PCR reaction mixture of 25 µl was prepared by using 25 ng/µl of genomic DNA template, 25 pmol primer, 12.5 µl 2x PCR master mixture and 10.5 µl of PCR water (Fermentas). Different PCR conditions were used for the optimization of amplification, however the best suitable conditions were as follows; initial denaturation at 94°C for 30sec, annealing at 40°C for 1 minute for OPC 1, and 2, 37°C for OPC 4, and 39°C for OPC 5 respectively, and extension at 72°C for 2 minutes. Final cycle was same except extension for 7 minutes at 72°C; whereas no amplification profile was generated for OPC 3. The contents were held at 4°C after the completion of reaction.

2.9.3.3 Electrophoresis of amplimers:

PCR generated amplimers were separated on 1.5 % agarose gel prepared in 0.5x TAE buffer. Gel was stained with ethidium bromide (0.1 mg/10 ml) solution and gel documentation was conducted by using Dolphin Doc Plus Gel Image System (Wealtec).

2.9.3.4 Data scoring and analysis:

Data was scored as presence or absence of bands. The presence of a specific band was marked as 1 and absence as 0, in this way a binary matrix was constructed to compare the patterns. Bands with similar mobility were treated as identical bands. Cluster analysis was then performed based on similarity co-efficient among the samples, based on molecular data by using numerical taxonomy and Multivariate Analysis System (NTSYS) Pc version 2.01 (Rohlf, 2000).

RESULTS AND DISCUSSION

In the present study, tissue culture conditions were optimized for *Lallementia royleana* by using different plant growth regulators. The whole research work was carried out in three steps. In the first step different explants were used for callus induction using a wide range of combinations of plant growth regulators. The second step comprised of direct regeneration by using various explants on different concentrations of plant growth regulators. In the third step plants of *Lallementia royleana* grown in soil and in MS media along with regenerated plants were assessed for somaclonal variation, produced as a result of artificial propagation, using RAPD markers.

3.1 Seed Germination

Sterilized seeds were shifted to full strength MS medium for germination. Seed germinated within two weeks of inoculation. Five weeks old plants were used for explant source.

3.2 Indirect Regeneration

3.2.1 Callogenesis

For callus induction thirteen different types of media were used with different combinations of plant growth regulators. IAA, NAA and BAP were analyzed for their callogenic ability. It was seen that MS medium without any growth regulator did not promote callus induction. Callus was induced in nine out of these thirteen callus induction media and survived for about three weeks. The callus produced was of very small size and degenerated after some time due to which it could not be further used for organogenesis.

Maximum callus induction percentage i.e. 100 % was obtained on nodes and internodes on CIM11 which contained 1 mg/l BAP in combination with 2 mg/l IAA (Table 3.1). The callus was of larger size as compared to the others, green in colour and of fragile texture which survived for three weeks (Fig. 3.1). The combination of BAP and NAA has been reported earlier to induce maximum callus in *Lycopersicon esculentum* (Jatoi *et al.*, 1999).

Table 3.1 Callus induction response of explants on MS media with different plant growth regulators.

Media Type	PGRs	Conc. (mg/l)	Explant	Weekly Response					Texture	Colour	Callus Induction %
				1	2	3	4	5			
CIM0	-	-	Nodes	-	-	-	-	-	-	-	-
			Internodes	-	-	-	-	-	-	-	-
			Leaves	-	-	-	-	-	-	-	-
CIM1	**NAA**	0.5	Nodes	-	-	+	-	-	Fragile	Brown	70
			Internodes	-	-	-	-	-	-	-	-
			Leaves	-	-	-	-	-	-	-	-
CIM2	**NAA**	1	Nodes	-	+	+	-	-	Fragile	Green	50
			Internodes	-	+	+	-	-	Fragile	White	90
			Leaves	-	-	-	-	-	-	-	-
CIM3	**NAA**	2	Nodes	-	+	-	-	-	Fragile	Green	90
			Internodes	-	+	++	++	-	Fragile	Green	90
			Leaves	-	-	-	-	-	-	-	-
CIM4	**IAA**	0.5	Nodes	-	+	+	-	-	Fragile	Brown	50
			Internodes	+	-	-	-	-	Fragile	Brown	40
			Leaves	-	-	-	-	-	-	-	-
CIM5	**IAA**	1.0	Nodes	-	-	-	-	-	-	-	-
			Internodes	-	-	-	-	-	-	-	-
			Leaves	-	-	-	-	-	-	-	-
CIM6	**IAA**	2	Nodes	-	+	-	-	-	Fragile	Brown	40
			Internodes	-	-	-	-	-	-	-	-
			Leaves	-	-	-	-	-	-	-	-

CIM7	**BAP**	0.5	Nodes	-	-	-	-	-	-	-	-
			Internodes	-	-	-	-	-	-	-	-
			Leaves	-	-	-	-	-	-	-	-
CIM8	**BAP**	1.0	Nodes	-	-	-	-	-	-	-	-
			Internodes	-	-	-	-	-	-	-	-
			Leaves	-	-	-	-	-	-	-	-
CIM9	**BAP**	2.0	Nodes	-	-	-	-	-	-	-	-
			Internodes	-	-	-	-	-	-	-	-
			Leaves	-	-	-	-	-	-	-	-
CIM 10	**BAP + IAA**	0.5 + 1	Nodes	+	+	-	-	-	Fragile	Green	90
			Internodes	-	+	+	-	-	Fragile	Green	60
			Leaves	-	-	-	-	-	-	-	-
CIM 11	**BAP + IAA**	1 + 2	Nodes	++	++	++	-	-	Fragile	Green	100
			Internodes	++	++	++	-	-	Fragile	Green	100
			Leaves	-	-	-	-	-	-	-	-
CIM 12	**2,4-D + NAA**	2 + 1	Nodes	+	+	+		-	Fragile	Green	90
			Internodes	+	+	++	++	-	Fragile	Green	90
			Leaves	-	-	+	++	-	Fragile	Pale Green	20
CIM 13	**2,4-D + NAA**	1 + 2	Nodes	+	+	+	++	-	Fragile	Green	90
			Internodes	-	-	+	+	-	Fragile	Green	80
			Leaves	-	-	-	-	-	Fragile		0

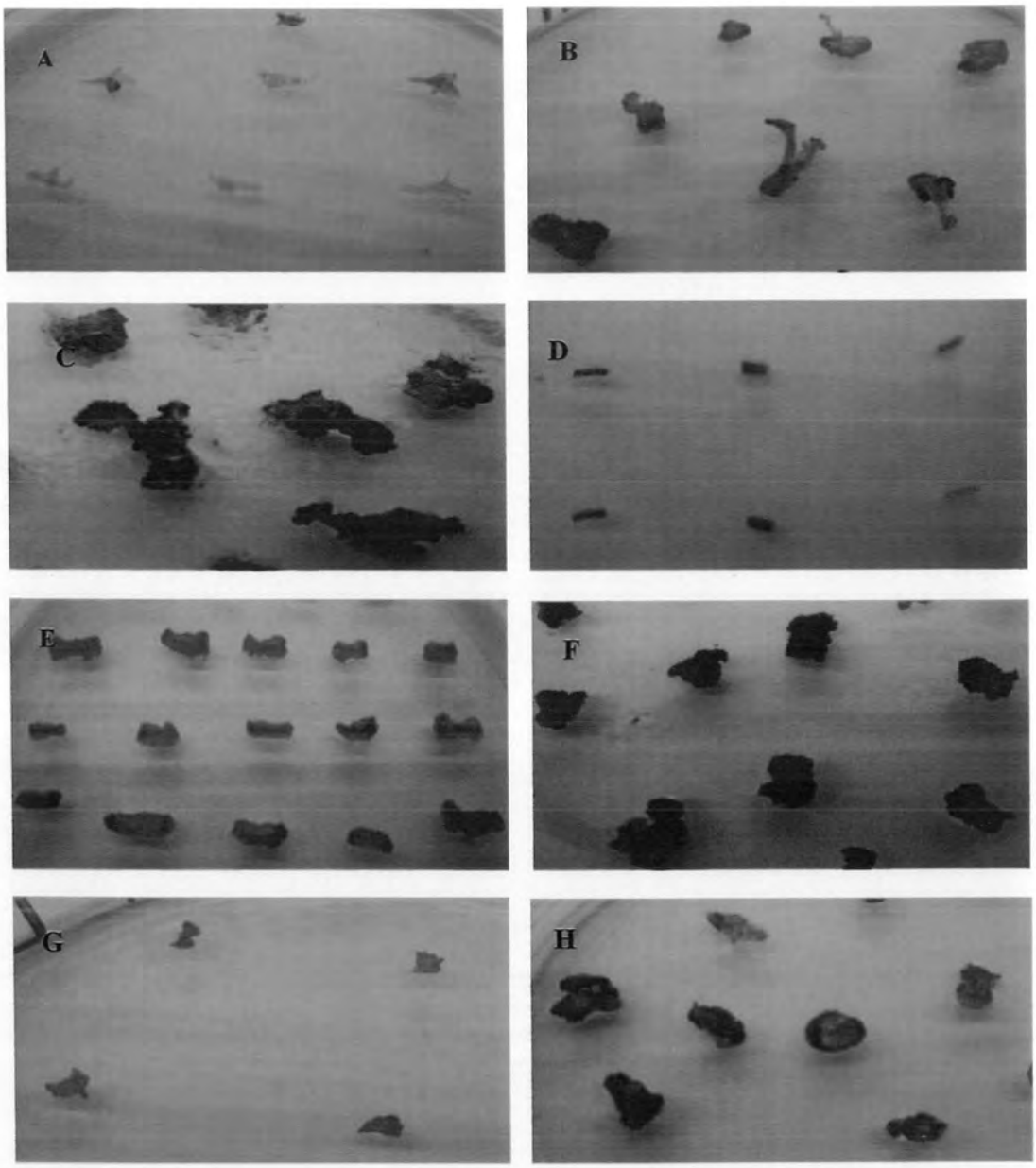

Figure 3.1 Callus induction response of different explants on CIM11.

A: nodal explants inoculated on media. **B:** 2 week old node derived callus. **C:** 4 week old node derived callus on. **D:** internodes inoculated on media. **E:** 2 week old internode derived callus. **F:** 4 week old internode derived callus. **G:** leaf explants inoculated on media. **H:** 4 week old leaf explants.

In the present study auxin, cytokinins and combinations of these were used for callus induction. When used alone, the auxins were found to be more effective in callus induction as compared to cytokinins. Some callogenic activity was observed on nodal explants cultured on media containing auxins. These included CIM1 (NAA 0.5 mg/l), CIM2 (NAA 1.0 mg/l), CIM3 (NAA 2.0 mg/l), CIM4 (IAA 0.5 mg/l) AND CIM6 (IAA 2.0 mg/l) (Fig. 3.2 B, 3.3 B, 3.4 B, 3.5 B and 3.6 B). CIM2 (NAA 1.0 mg/l), CIM3 (NAA 2.0 mg/l) and CIM4 (0.5 mg/l IAA) also promoted some callus induction on internodal explants as well (Fig. 3.3 D, 3.4 D and 3.5 D). Among the concentrations of auxins used CIM3 i.e. 2.0 mg/l NAA was more effective in callus induction as callus induction percentage was observed to be 90 %. Callus obtained on internodal explants on CIM3 was fragile and green in colour. However it differed from all of the other calli obtained as it was hairy in texture (Fig. 3.4 D). Whereas, some auxin containing media also promoted the development of adventitious roots on nodal and intermodal explants. These included nodes on CIM1, CIM4 and CIM5, and internodes on CIM4 (Fig. 3.2 B, 3.5 B, 3.5 D and 3.7 B). Among the plant growth regulators used NAA was found to be more effective in callus induction as compared to IAA. This observation was in accordance with Arya *et al.* (2008) as NAA was found to be best suited for producing callus in *Pluchea lanceolata*. Likewise, in another report NAA was found to be most effective for callus induction in *Salvia canariensis* (Molina, 2004). Cytokinins did not promote callogenic activity in *Lallementia royleana* as CIM7 (BAP 0.5 mg/l), CIM8 (BAP 1.0 mg/l) and CIM9 (BAP 2.0 mg/l) did not induce callus on any type of explant (Fig. 3.8, 3.9 and 3.10). Conversely, direct regeneration was observed on these concentrations of BAP.

Callus induction media with combinations of auxins, i.e. CIM10 (BAP 0.5 mg/l + IAA 1.0 mg/l), CIM11 (BAP 1.0 mg/l + IAA 2.0 mg/l), CIM12 (2,4-D 2.0 mg/l + NAA 1.0 mg/l) and CIM13 (2,4-D 1 mg/l + NAA 2 mg/l), were found to be more efficient in inducing callus as compared to media containing single plant growth regulator (Fig. 3.11, 3.1, 3.12, 3.13). Graphical representation of their callus induction percentage is given in Fig. 3.14.

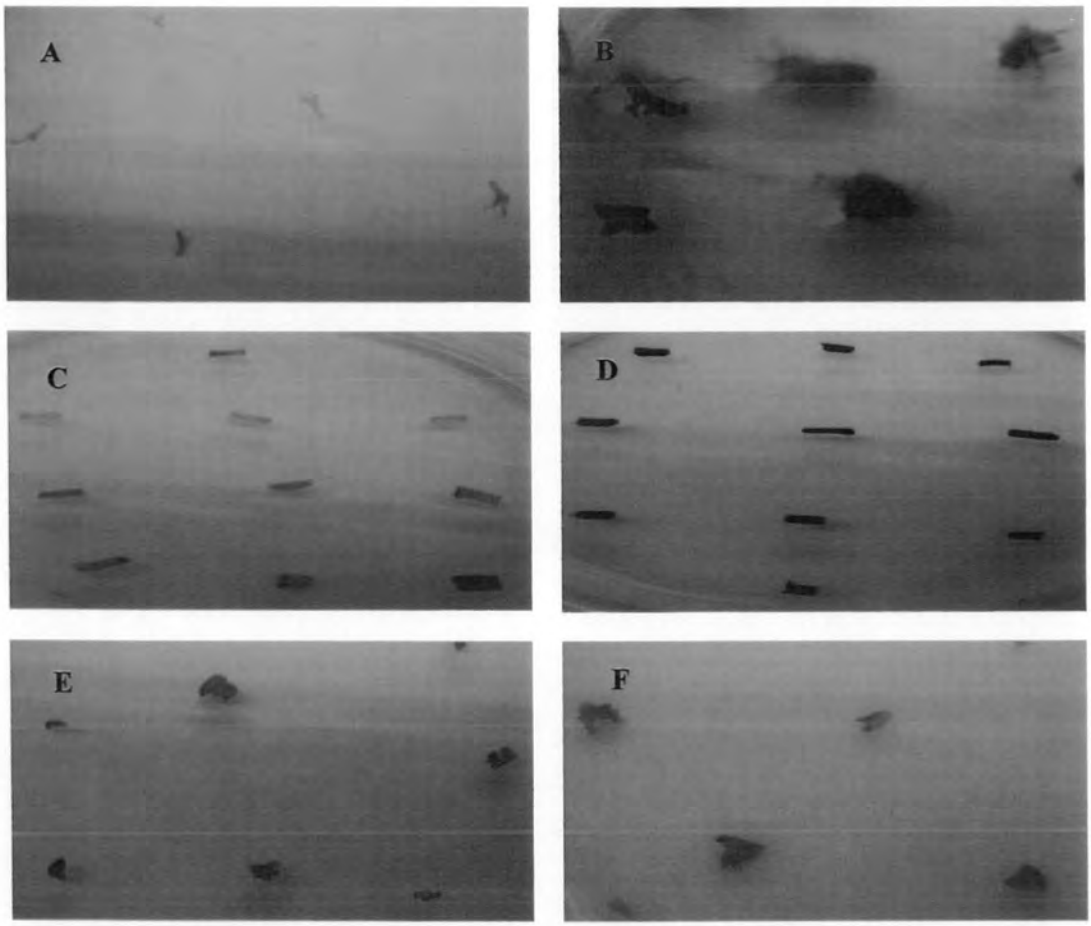

Figure 3.2 Callus induction response of different explants on CIM1.

A: nodal explants inoculated on media. **B:** 4 week old node derived callus. **C:** internodes inoculated on media. **D:** 4 week old internodal explants. **E:** leaf explants inoculated on media. **F:** 4 week old leaf explants.

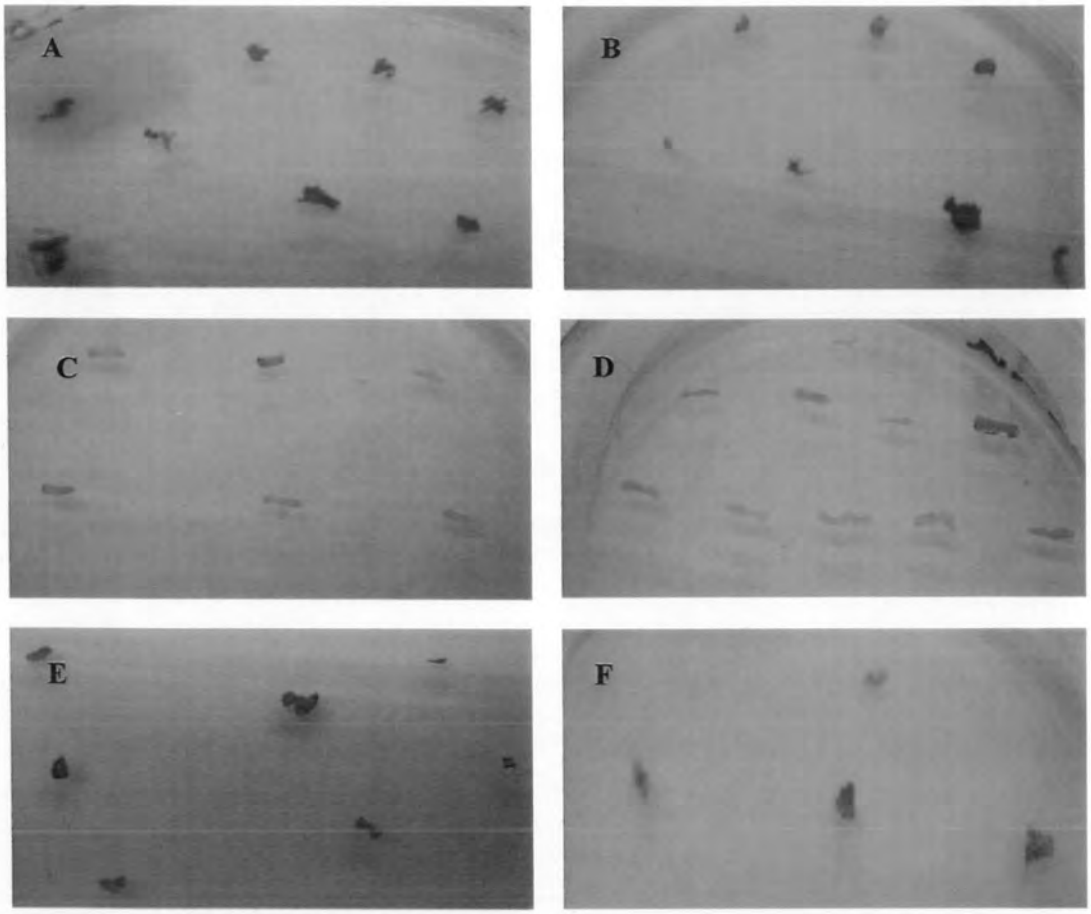

Figure 3.3 Callus induction response of different explants on CIM2.

A: nodal explants inoculated on media. **B:** 4 week old node derived callus. **C:** internodes inoculated on media. **D:** 4 week old internode derived callus. **E:** leaf explants inoculated on media. **F:** 4 week old leaf explants.

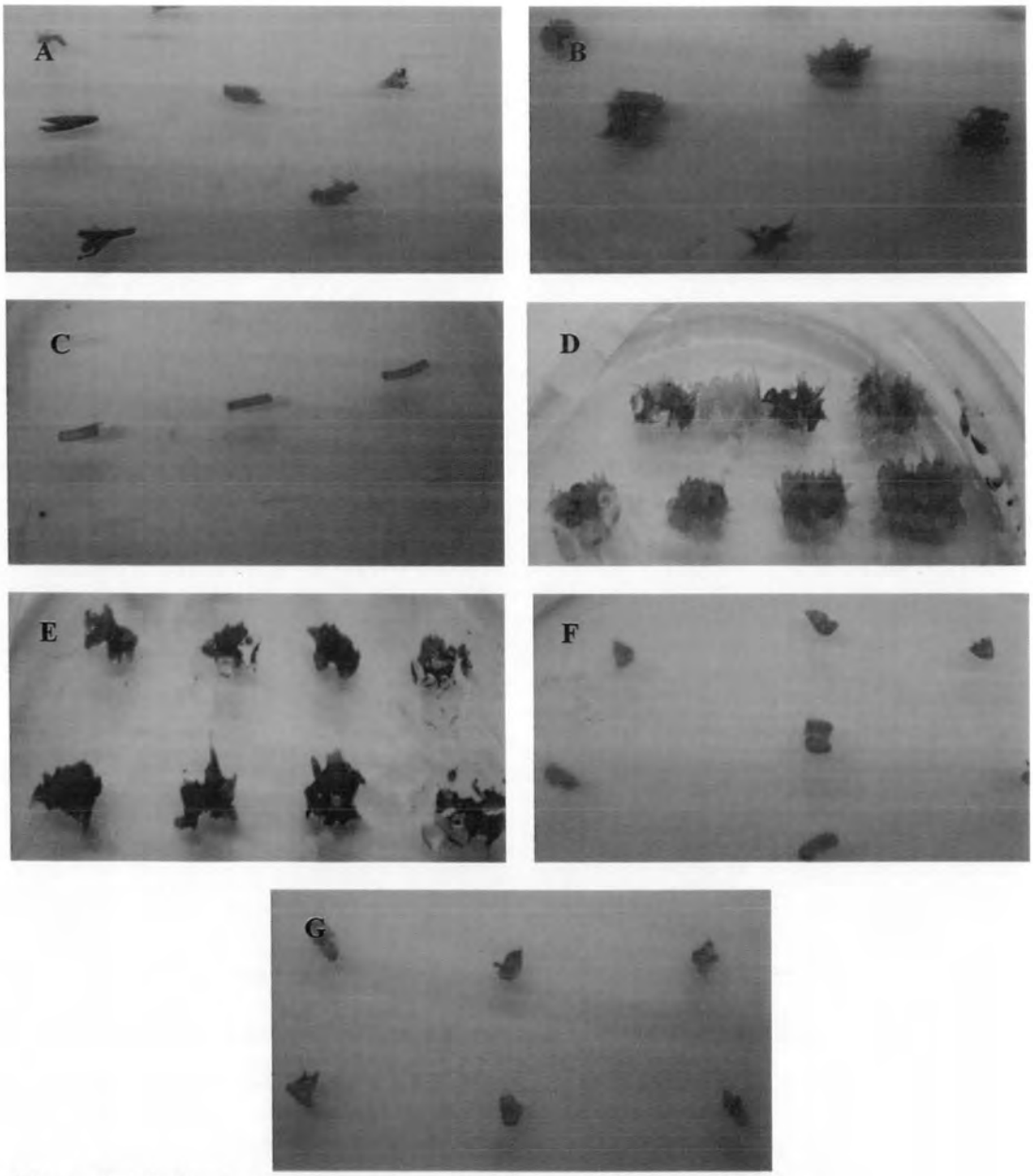

Figure 3.4 Callus induction response of different explants on CIM3.
A: nodal explants inoculated on media. **B:** 4 week old node derived callus. **C:** internodes inoculated on media. **D:** 2 week old internode derived callus. **E:** 4 week old internode derived callus. **F:** leaf explants inoculated on media. **G:** 4 week old leaf explants.

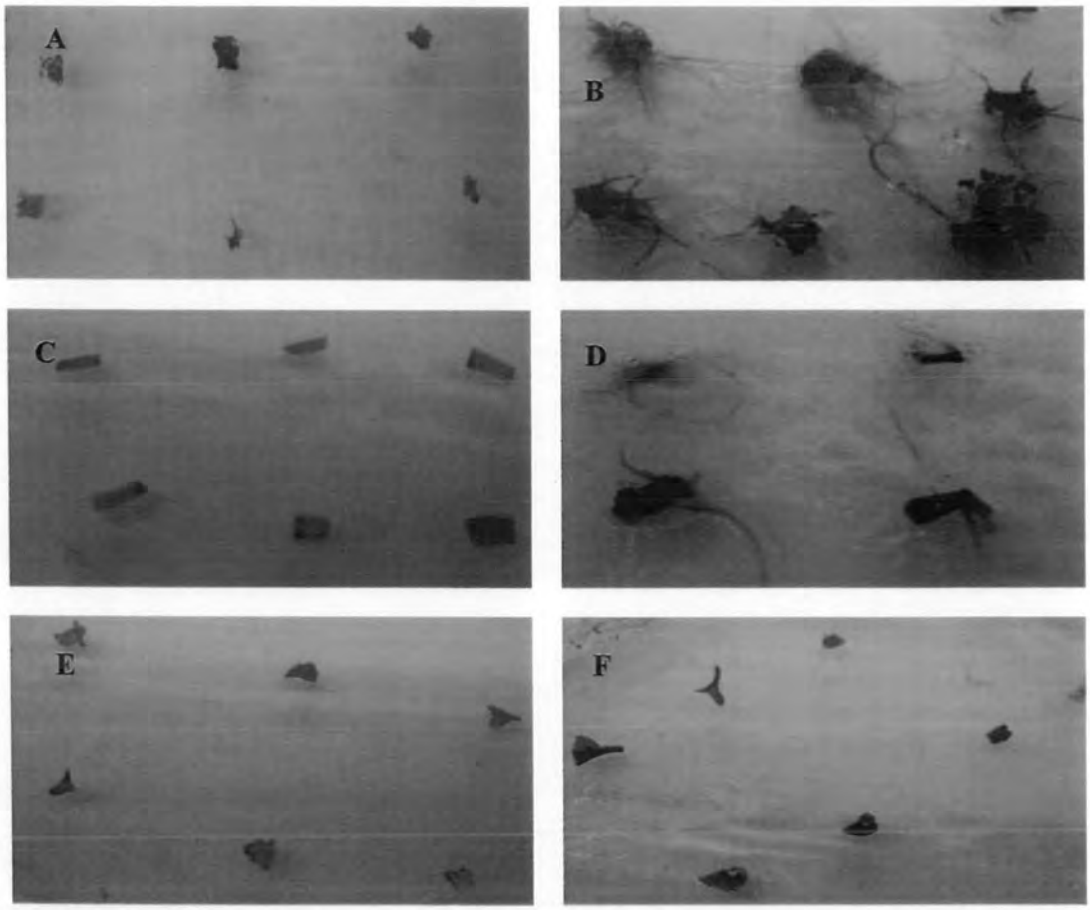

Figure 3.5 Callus induction response of different explants on CIM4.

A: nodal explants inoculated on media. **B:** 4 week old node derived callus. **C:** internodes inoculated on media. **D:** 4 week old internode derived callus. **E:** leaf explants inoculated on media. **F:** 4 week old leaf explants.

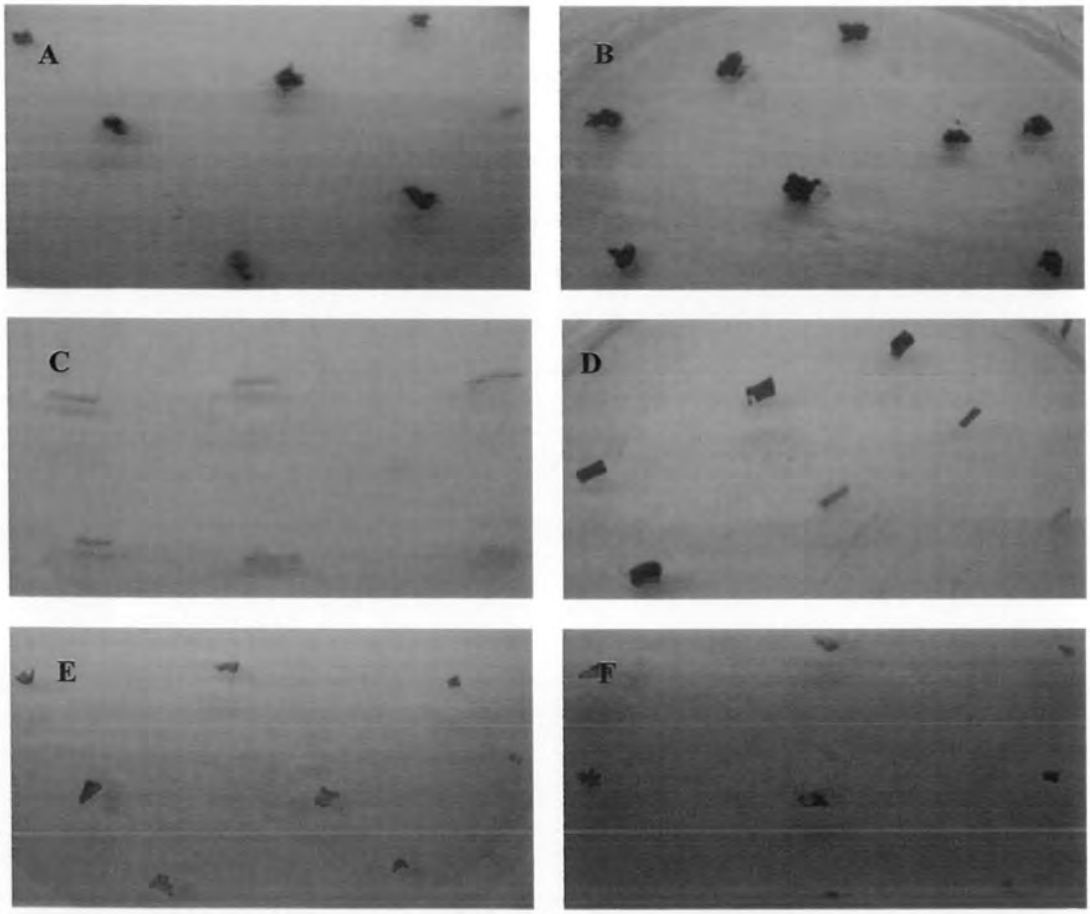

Figure 3.6 Callus induction response of different explants on CIM6.

A: nodal explants inoculated on media. **B:** 4 week old node derived callus. **C:** internodes inoculated on media. **D:** 4 week old internode derived callus. **E:** leaf explants inoculated on media. **F:** 4 week old leaf explants.

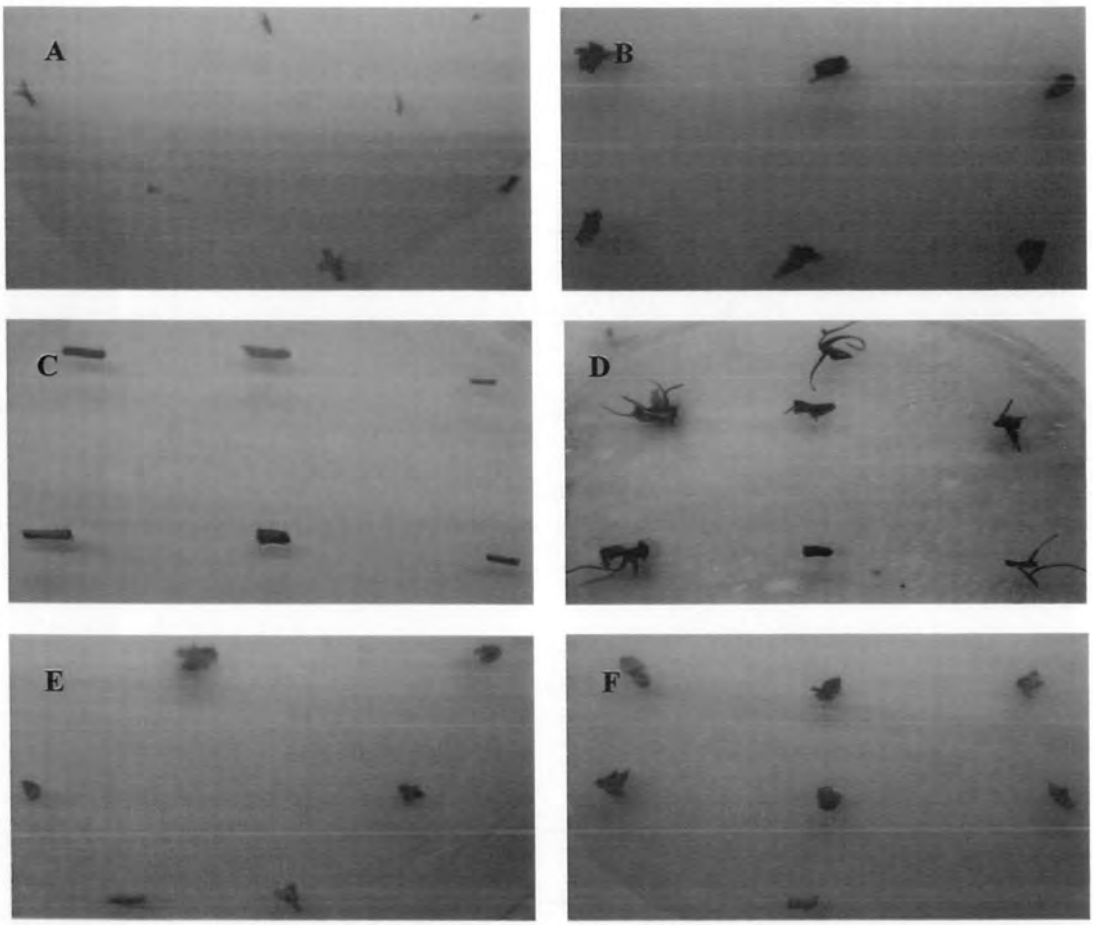

Figure 3.7 Callus induction response of different explants on CIM5.

A: nodal explants inoculated on media. **B:** 4 week old nodal explants. **C:** internodes inoculated on media. **D:** 4 week old intermodal explants. **E:** leaf explants inoculated on media. **F:** 4 week old leaf explants.

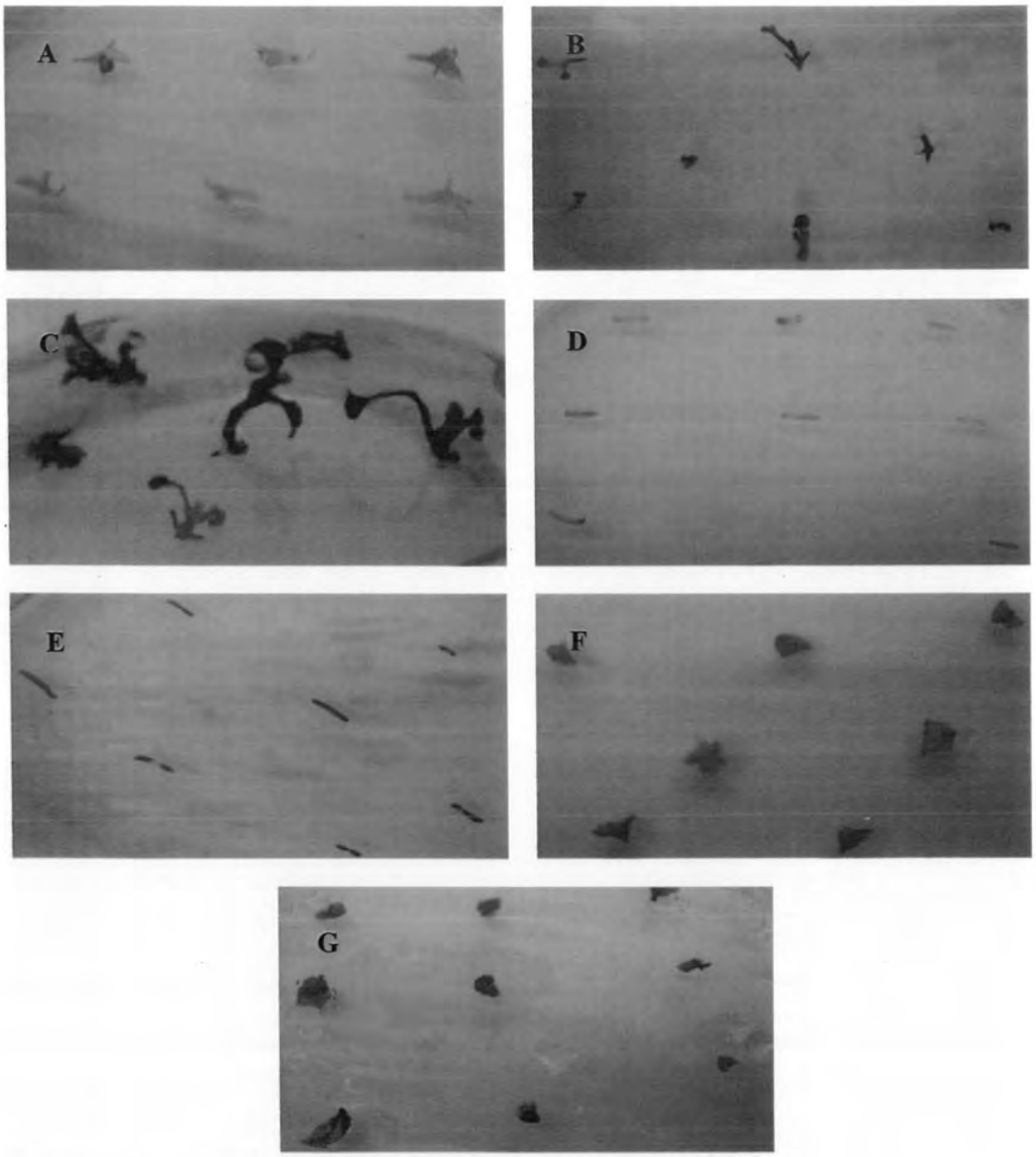

Figure 3.8 Callus induction response of different explants on CIM7.

A: nodal explants inoculated on media. **B:** 2 week old node derived callus. **C:** 4 week old nodal explants. **D:** internodes inoculated on media. **E:** 4 week old intermodal explants. **F:** leaf explants inoculated on media. **G:** 4 week old leaf explants.

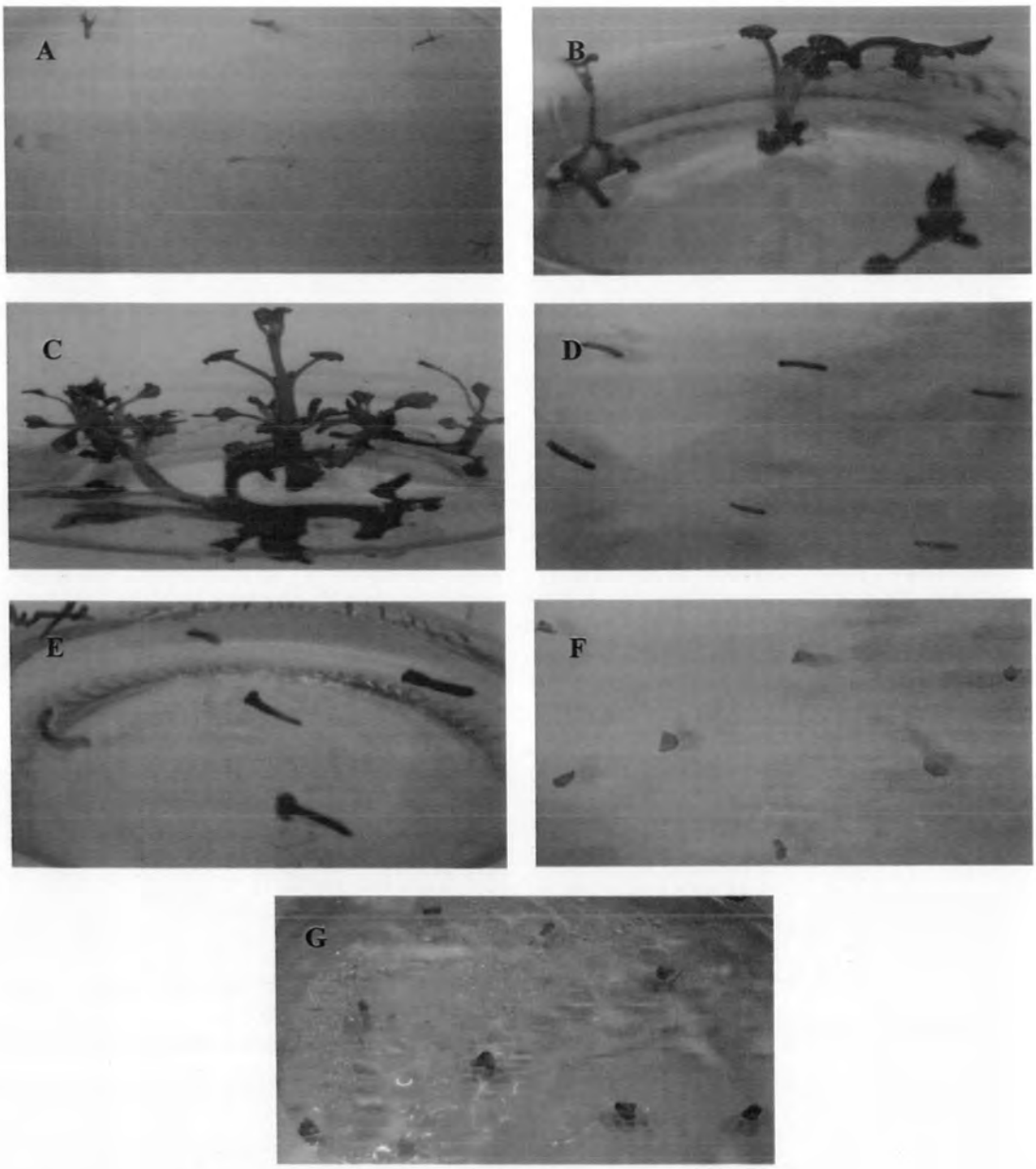

Figure 3.9 Callus induction response of different explants on CIM8.

A: nodal explants inoculated on media. **B:** 2 week old nodal explants. **C:** 4 week old nodal explants. **D:** internodes inoculated on media. **E:** 4 week old internode derived callus. **F:** leaf explants inoculated on media. **G:** 4 week old leaf explants.

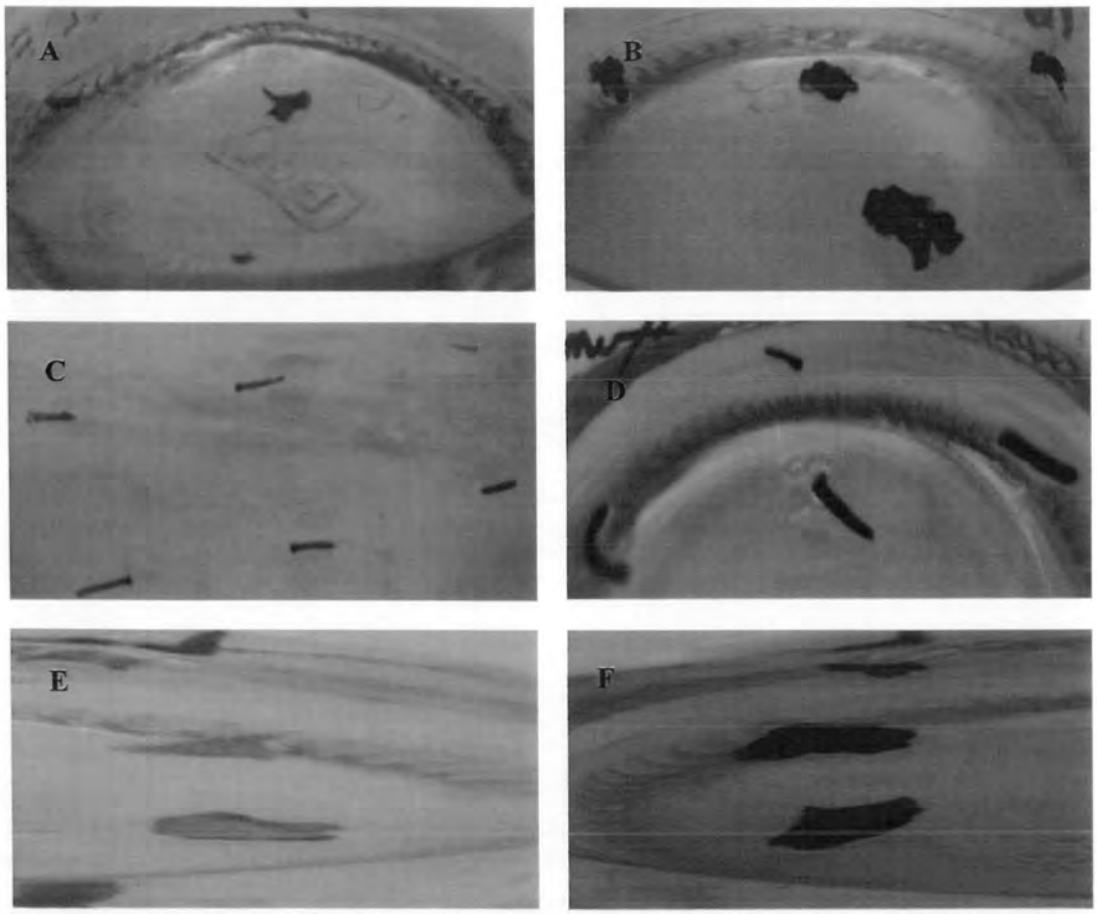

Figure 3.10 Callus induction response of different explants on CIM9.

A: nodal explants inoculated on media. **B:** 4 week old node derived callus. **C:** internodes inoculated on media. **D:** 4 week old internode derived callus. **E:** leaf explants inoculated on media. **F:** 4 week old leaf explants.

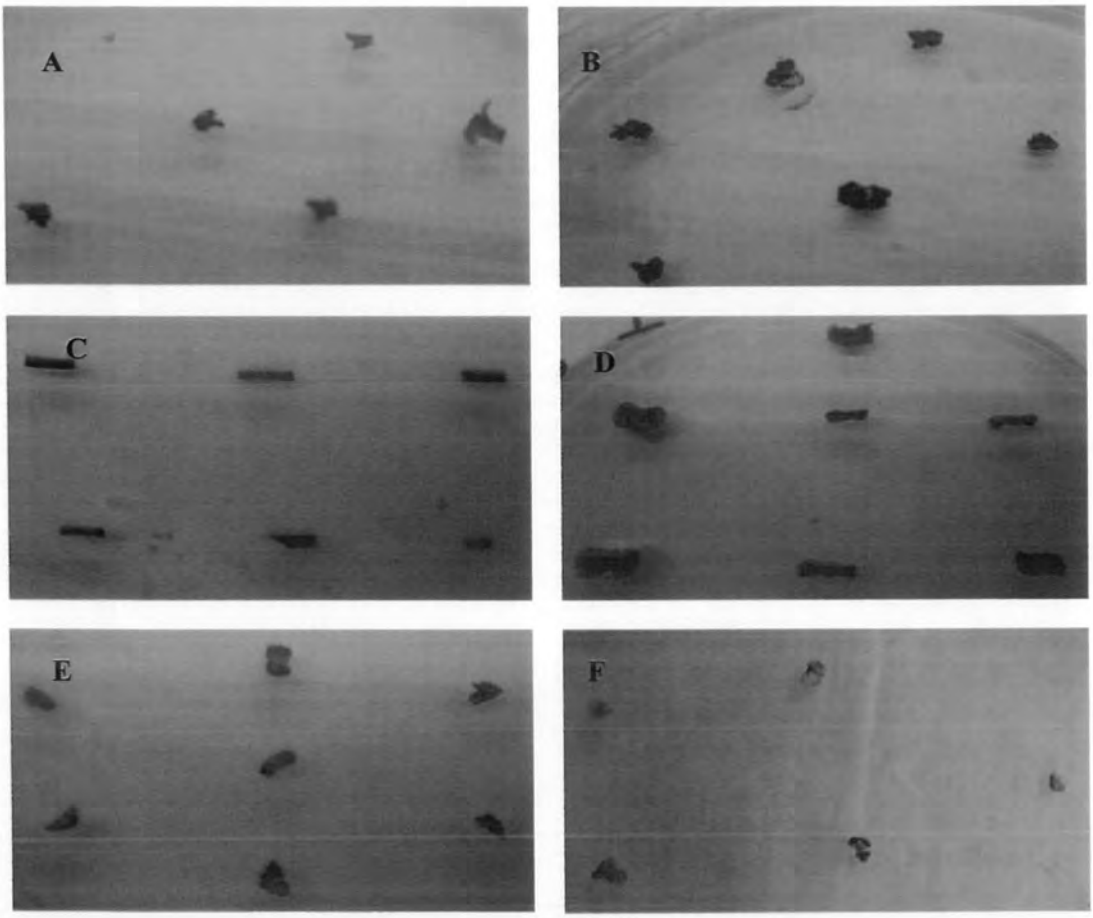

Figure 3.11 Callus induction response of different explants on CIM10.

A: nodal explants inoculated on media. **B:** 4 week old node derived callus. **C:** internodes inoculated on media. **D:** 4 week old internode derived callus. **E:** leaf explants inoculated on media. **F:** 4 week old leaf explants.

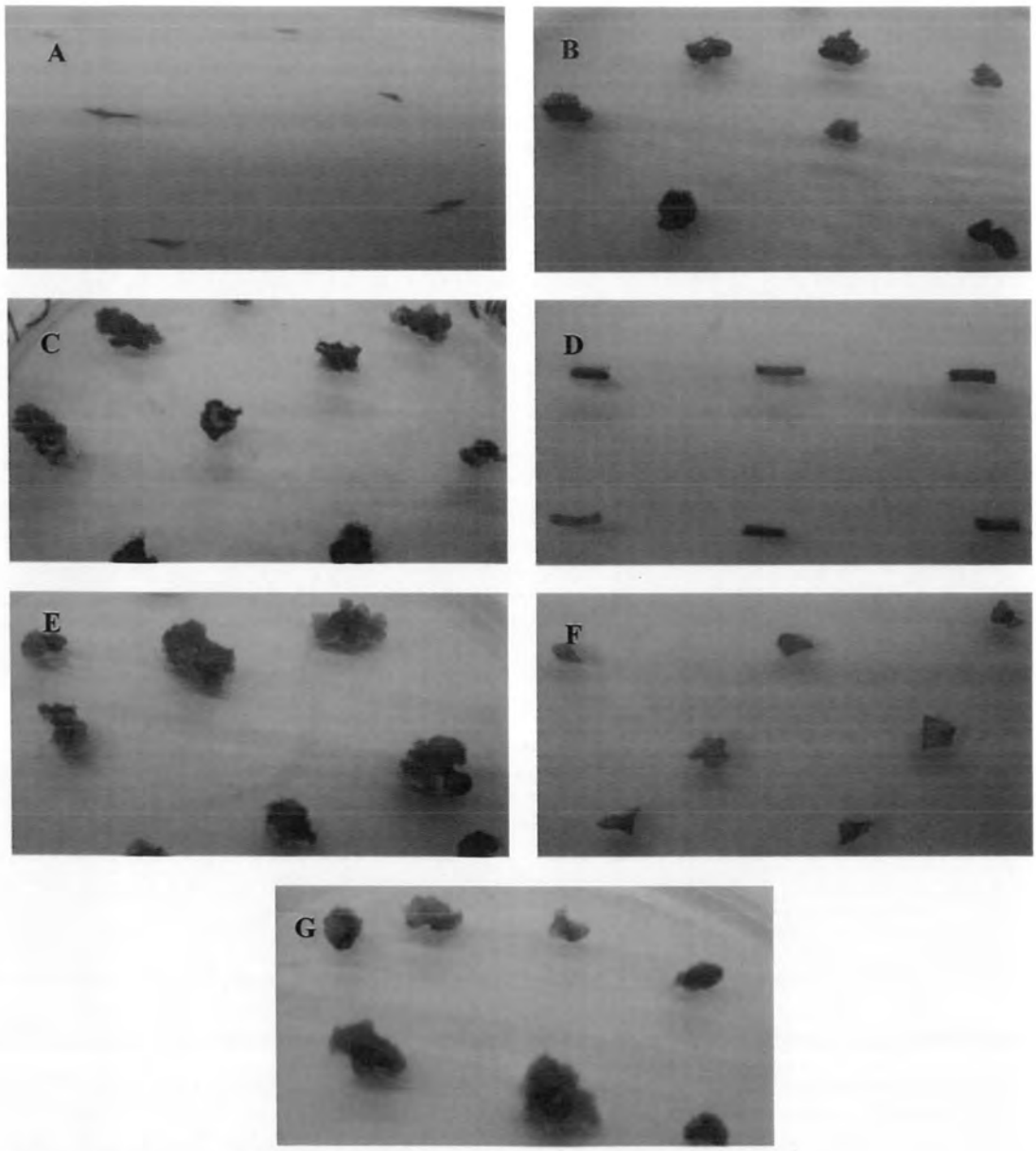

Figure 3.12 Callus induction response of different explants on CIM12.

A: nodal explants inoculated on media. **B:** 2 week old node derived callus. **C:** 4 week old node derived callus. **D:** internodes inoculated on media. **E:** 4 week old internode derived callus. **F:** leaf explants inoculated on media. **G:** 4 week old leaf derived callus.

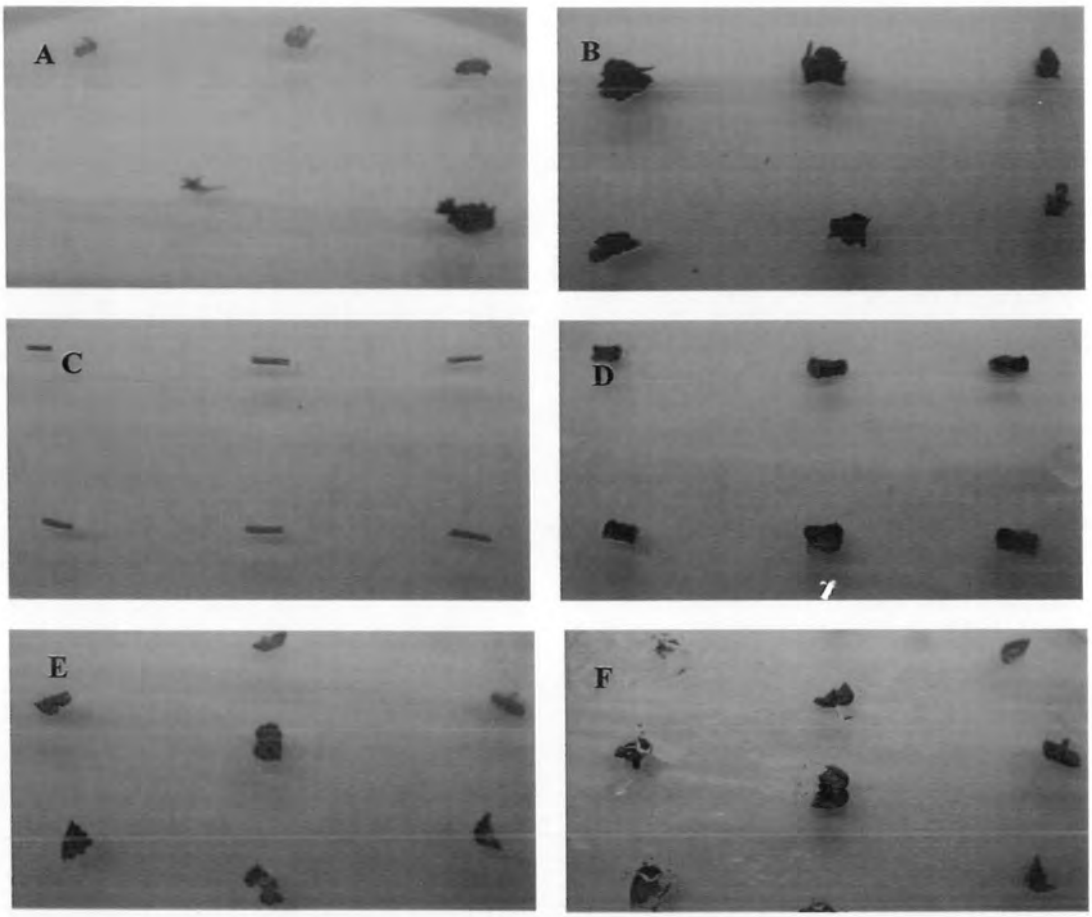

Figure 3.13 Callus induction response of different explants on CIM13.

A: nodal explants inoculated on media. **B:** 4 week old node derived callus. **C:** internodes inoculated on media. **E:** 4 week old internode derived callus. **F:** leaf explants inoculated on media. **G:** 4 week old leaf explants.

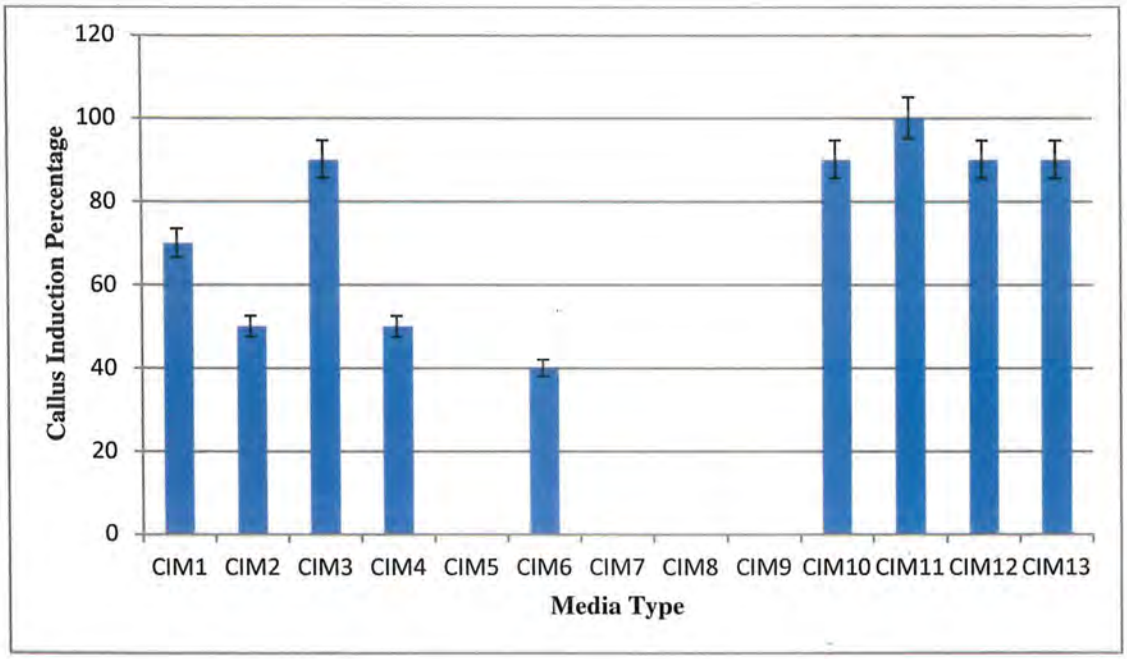

Figure 3.14 Callus induction percentage of *Lallementia royleana* on media having different hormonal combinations. CIM1 70 %, CIM2 50 %, CIM3 90 %, CIM4 50 %, CIM5 0 %, CIM6 40 %, CIM7 0 %, CIM8 0 %, CIM9 0 %, CIM10 90 %, CIM11 100 %, CIM12 90 % and CIM13 90 %.

Similarly, the combinations of auxins gave good callus in case of *Ocimum tenuiflorum* (Gogoi and Kumaria, 2011), *Ocimum americanum* and *Ocimum sanctum* (Pattnaik and Chand, 1996). Callus induction percentage was observed to be 90 % in case of nodal explants cultured on CIM12 (2,4-D 2.0 mg/l along with NAA 1 mg/l) and CIM13 (2,4-D 1 mg/l + NAA 2 mg/l). However, the callus produced was of small size (Fig. 3.12 B and 3.13 B) and survived for three weeks. Similarly, green and fragile callus was induced on leaves and internodes as well on CIM12 (Fig. 3.12 E, G) but none of the callus survived for more than 24 days. Degeneration of callus has also been reported for *Quercus rubra* within a period of five months by Gingas (1991).

3.2.1.1 Effect of explant on callogenesis:

Callus induction potential varied with the type of explant used. Nodal segments were found to be more suited for callus induction as compared to the other explants (Fig. 3.15). Similar findings have been reported for *Ocimum tenuiflorum* (Gogoi and Kumaria, 2011) and *Salvia officinalis* (Tawfik and Mohamed, 2007) which have been regenerated from nodal explants. Frequent callus induction occurred at the cut end of nodal and internodal segments of stem as compared to leaves which showed poor callus induction potential. Callus on leaves was induced on CIM12 only. On the contrary, in some members of Lamiaceae leaves have been found to be of considerable regenerative potential. Recently, Sujana and Naidu (2011) have reported regeneration of *Mentha piperita* from leaf derived callus. In some other reports, *Ocimum basilicum* (Gopi and Ponmurugan, 2006) and *Ocimum sanctum* (Lim *et al.*, 2009) were also regenerated from leaf derived callus.

3.3 Direct Regeneration

3.3.1 Shoot induction

MS media with different concentrations of BAP and Kinetin were used for shoot induction. Shoots were induced initially on 0.5 mg/l BAP (S1), 1.0 mg/l BAP (S2), 2.0 mg/l BAP (S3), 4.0 mg/l BAP (S4), 0.5 mg/l Kinetin (S5), 1.0 mg/l Kinetin (S6) and 4.0 mg/l Kientin (S8) within 2 weeks of incubation (Fig. 3.16 A-D, 3.17 A, B, D).

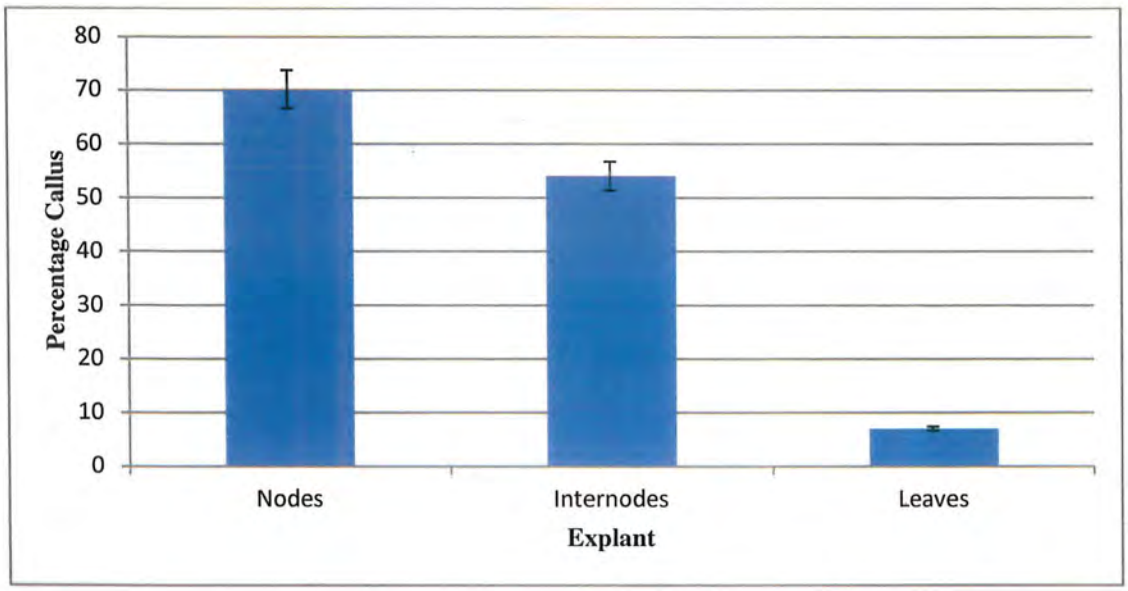

Figure 3.15 Callus induction response of different explants. Nodes 70 %, internodes 54 % and leaves 7 %.

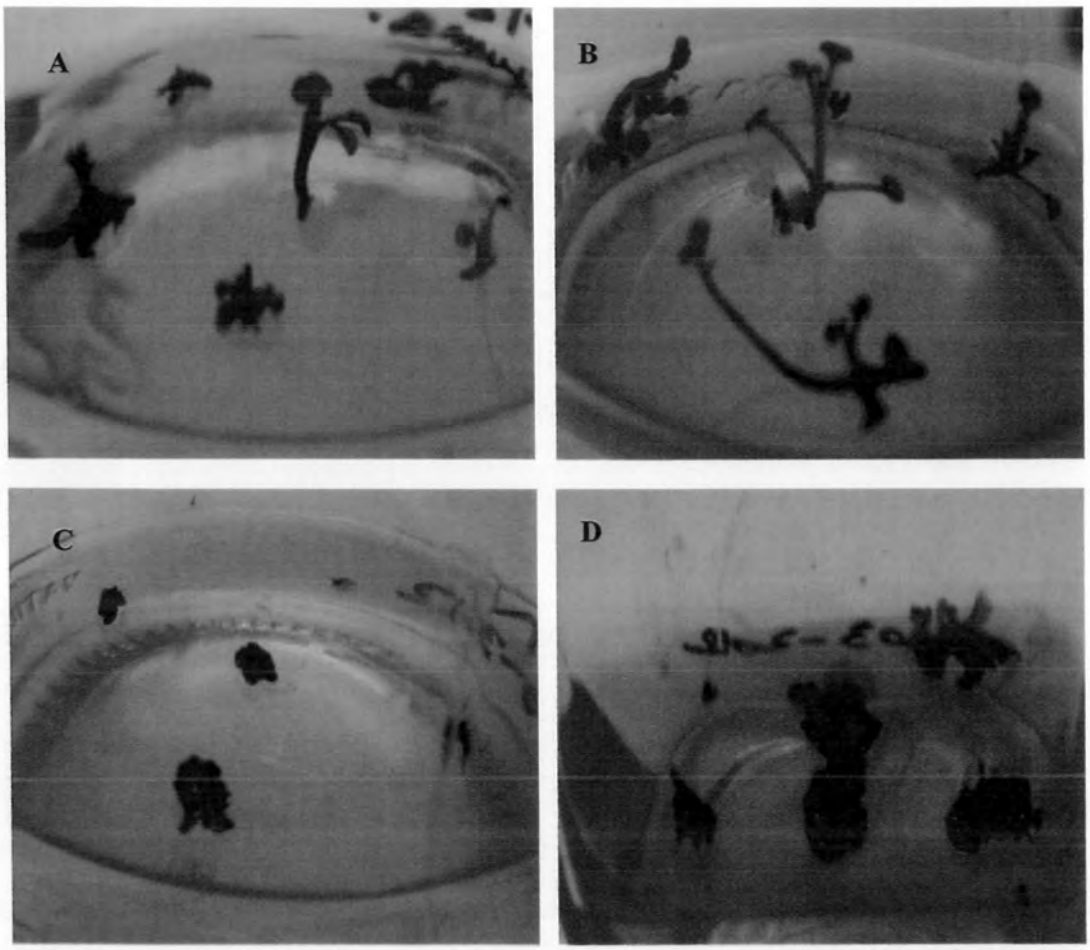

Figure 3.16 Shooting response of nodal explants on different media after two weeks. **A:** S1 (0.5 mg/l BAP) **B:** S2 (1.0 mg/l BAP) **C:** S3 (2.0 mg/l BAP) **D:** S4 (4.0 mg/l)

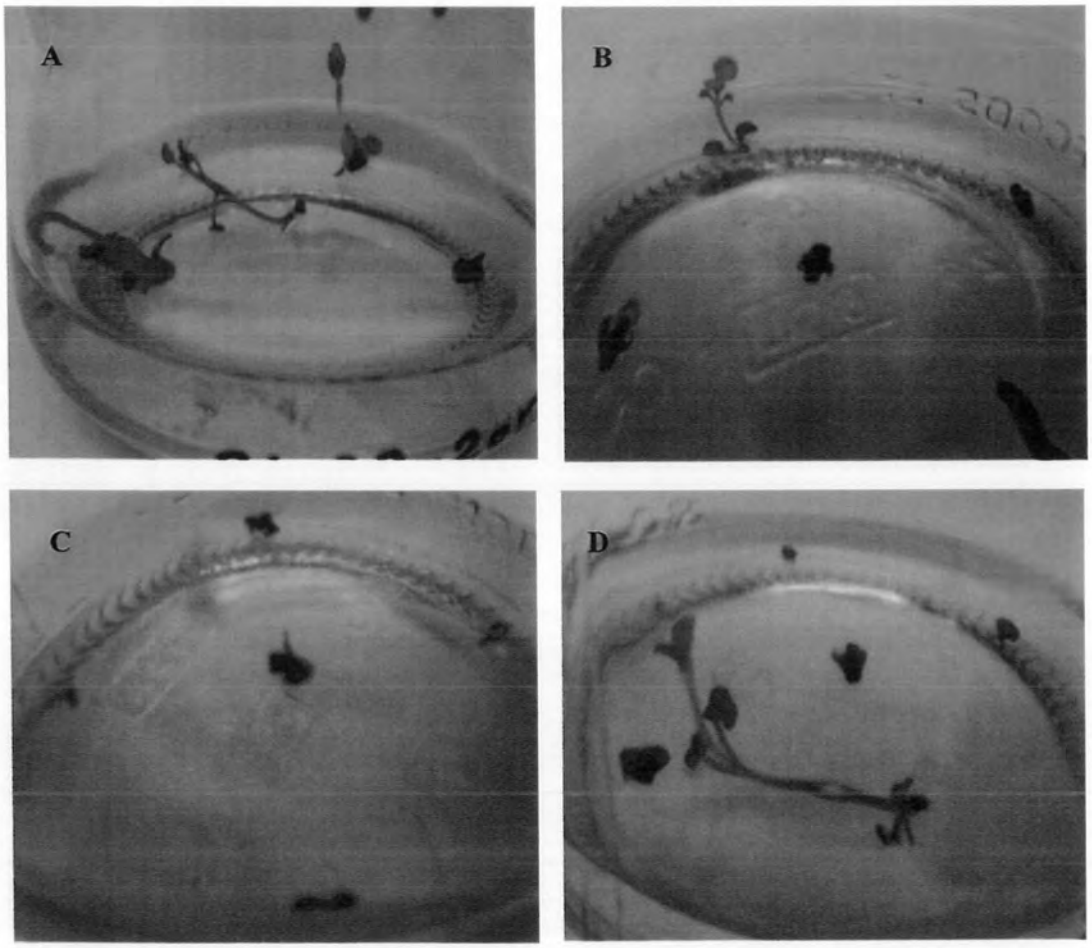

Figure 3.17 Shooting response of nodal explants on different media after two weeks.
A: S5 (0.5 mg/l Kinetin) **B:** S6 (1.0 mg/l) **C:** S7 (2.0 mg/l) **D:** S8 (4.0 mg/l)

After 5 weeks shoots formed on S2, S5, S6, S8 media (Fig. 3.18 A-D) survived whereas, shoots on S1, S3 and S4 media (Fig. 3.19 A-C) were lost to degeneration. Whereas, no shooting was induced in 2.0 mg/l Kinetin i.e. S7 media (Fig. 3.19 D). In the present study BAP was observed to be best suited for shoot induction as maximum shooting percentage (80 %) was observed on S2 media supplemented with 1.0 mg/l BAP (Fig. 3.20) producing 17.75 shoots per explant with average length of 3.8 cm (Table 3.2). These results were in accordance with the findings of Raja and Arockiasamy (2008); they also found BAP producing maximum number of shoots in *Mentha viridis*. It has been reported that in addition to BAP, Kinetin also promotes shoot proliferation (Gopi and Ponmurugan, 2006). In our study Kinetin also promoted shoot proliferation in addition to BAP, as 60 % shoot induction was observed on S5 supplemented with 0.5 mg/l kinetin (Fig. 3.18 B); but, with further increase in kinetin concentration the shooting percentage was reduced up to 20 % as in media S6 and S8 supplemented with 1.0 mg/l kinetin and 4.0 mg/l kinetin respectively (Fig. 3.21). However shoot length was increased at higher concentration of Kinetin (4.0 mg/l) producing average shoot length of 8.5 cm.

3.3.1.1 Effect of explant on shoot induction:

Among the explants used (leaves, petioles, nodes and internodes) shooting was induced only on nodal explants whereas internodes, leaves and petioles did not show any regeneration potential. High regenerative nature of nodal segments is an established fact in Lamiaceae, as the phenomena has been observed in many members, such as *Ocimum gratissimum, Ocimum basilicum, Ocimum grtissimum, Ocimun viride* and *Mentha viridis* have been regenerated by nodal explants (Ahuja *et al.*, 1982; Gopi and Ponmurugan, 2006; Raja and Arockiasamy, 2008 and Siddique and Anis, 2008). In the present study leaves were found to be of minimum regeneration potential but in some members of Lamiaceae for example *Salvia nemorosa* and *Ocimum basilicum*, leaves were found to be of adequate regeneration potential (Skala and Wysokinsa, 2004 and Phippen and Simon, 2000). Similarly, in another study *Salvia canariensis* was regenerated from petiolar explants (Molina, 2004) but in the present study petioles failed to produce any shoot. Earlier, Shasany *et al.* (1998) have reported regenerative nature of internodal segments of *Mentha arvensis* but no shooting was observed on the internodal explants in case of *Lallementia royleana*.

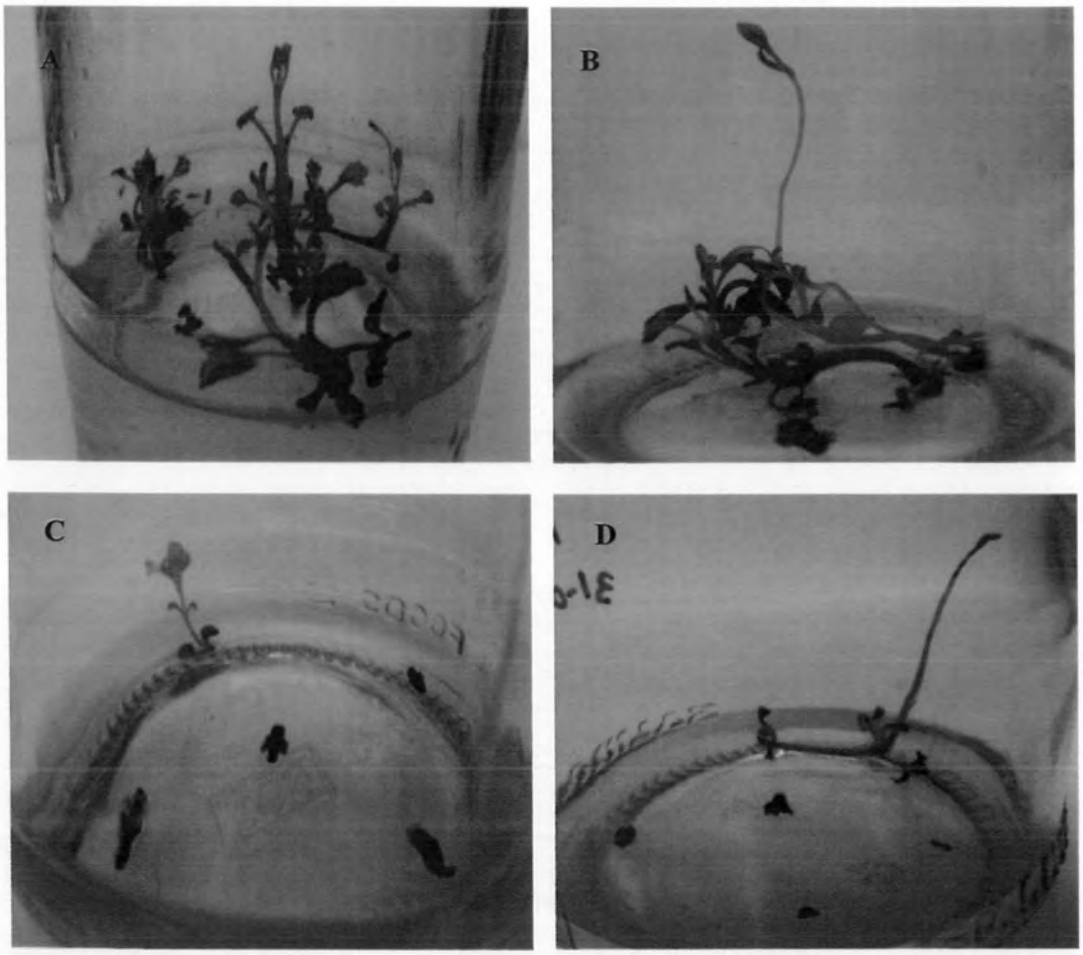

Figure 3.18 Shooting response of nodal explants on different media after five weeks.

A: S2 (1.0 mg/l BAP) **B:** S5 (0.5 mg/l BAP) **C:** S6 (1.0 mg/l Kinetin) **D:** S8 (4.0 mg/l Kinetin)

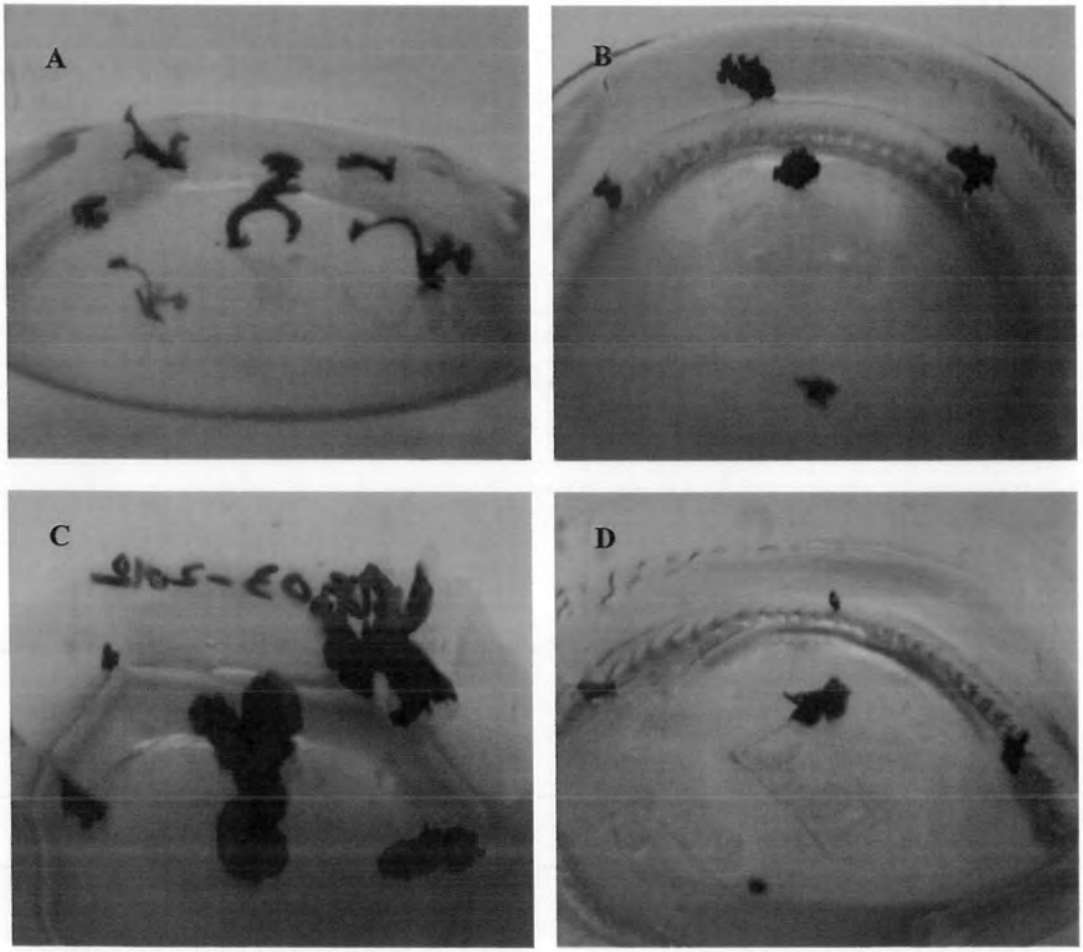

Figure 3.19 Shooting response of nodal explants on different media after five weeks.

A: S1 (0.5 mg/l BAP) **B:** S3 (1.0 mg/l 2.0 mg/l BAP) **C:** S4 (4.0 mg/l Kinetin) **D:** S7 (2.0 mg/l Kinetin)

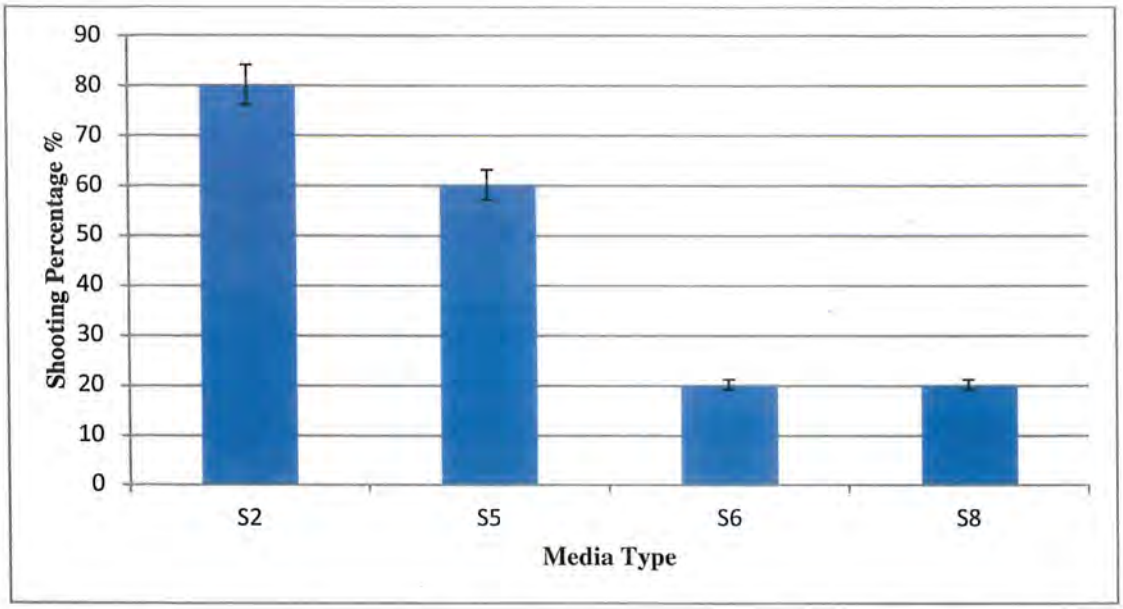

Figure 3.20 Percentage shooting response of explants on MS media with different plant growth regulators. S2 80 %, S5 60 %, S6 20 % and S8 20 %.

Table 3.2 Shoot induction on MS media with different plant growth regulators. Any two means sharing a letter in common are not significantly different according to LSD test at the level of P>0.05.

PGR Concentration (mg/L)	Shooting %age		Average Number of Shoots		Average Shoot Length (cm)	
	BAP	Kinetin	BAP	Kinetin	BAP	Kinetin
Control	-	-	-	-	-	-
0.5	-	60a	-	7.3a	-	4.5b
1	80	20b	17.75	4b	3.8	1.1b
2	-	-	-	-	-	-
4	-	20b	-	4b	-	8.5a
LSD	-	4.21	-	2.7	-	7.55

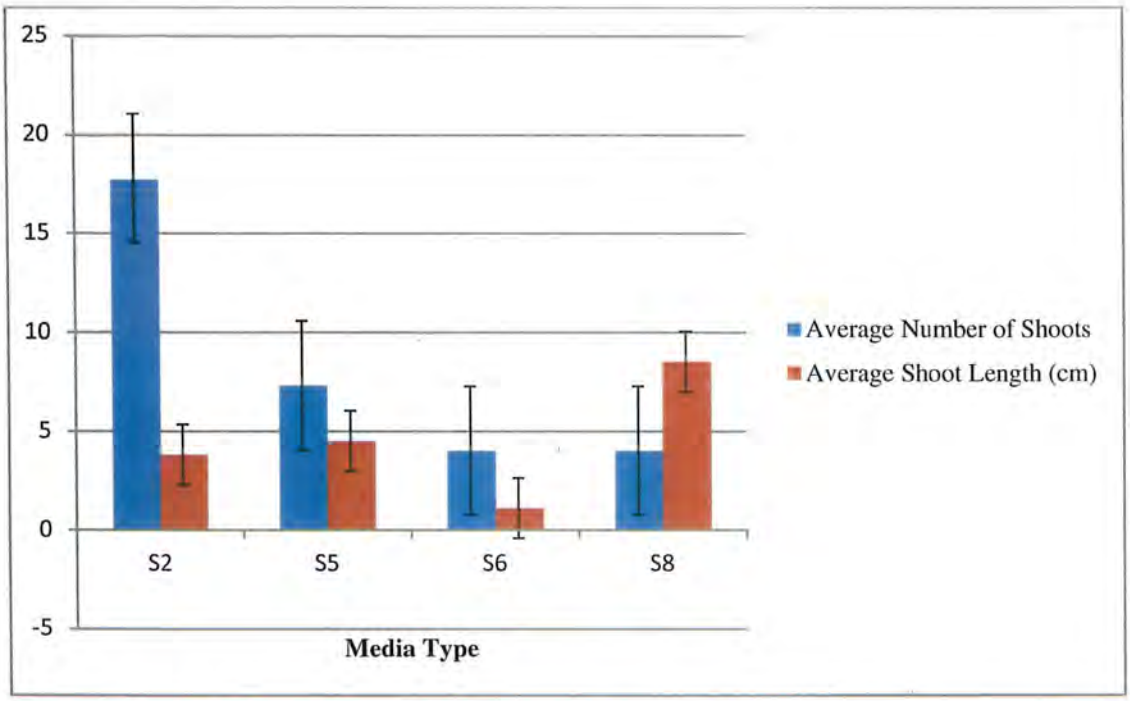

Figure 3.21 Average number of shoots and average shoot length on MS media with different plant growth regulators. S2 average shoots 17.75 and 3.8 cm average shoot length, S5 average shoots 7.3 and 4.5 cm average shoot length, S6 average shoots 4 and 1.1 cm average shoot length, S8 average shoots 4 and 8.5 cm average shoot length.

3.3.2 Root induction

The fully established shoots from S2 medium were separated and shifted to rooting media for root induction. It was observed that MS media lacking plant growth regulators failed to produce any roots. Whereas, roots were induced after 2 weeks of incubation in the presence of NAA and IAA. Maximum number of roots were induced on R2 medium having 1.0 mg/l NAA (Fig. 3.22 B) producing 24 roots on average (Table 3.3), and maximum root length (3.8 cm) was observed on R4 medium having 0.5 mg/l IAA (Fig. 3.22 D). It was also observed that NAA was more effective in root induction as compared to IAA (Fig. 3.23). Similar findings were reported by Lal *et al.* (1988) as NAA was found most effective for root induction in *Picrorhiza*. In another report, NAA was observed to be effective for root induction in *Ocimum sanctum* as well (Shilpa *et al.*, 2010). On the other hand these were contrary to the findings of Saha *et al.* (2010) where NAA was unable to induce rooting in *Ocimum kilimandscharicum.*

3.3.2.1 Effect of rooting media on shoot elongation:

All rooting media also caused increase in shoot number and length along with root induction. Among all rooting media, R2, R3 and R4 media also caused increase in number of shoots (up to 35 shoots) in addition to root induction (Table 3.4). Furthermore, increase in shoot length was observed with increase in concentration of NAA as maximum increase in shoot length (5.8 cm) was caused by R3 media supplemented with 2.0 mg/l NAA (Fig. 3.22 C). Whereas, R1 media having 0.5 mg/l NAA had minimum effect on both shoot length as well as on shoot number (Fig 3.22 A). Earlier, Little and McDonald (2003) have reported that exogenous IAA and NAA did not promote growth of stem units in scots pine and white spruce of Pinaceae. The current observations were found contrary to these findings as exogenous auxins caused considerable increase in growth of stem units.

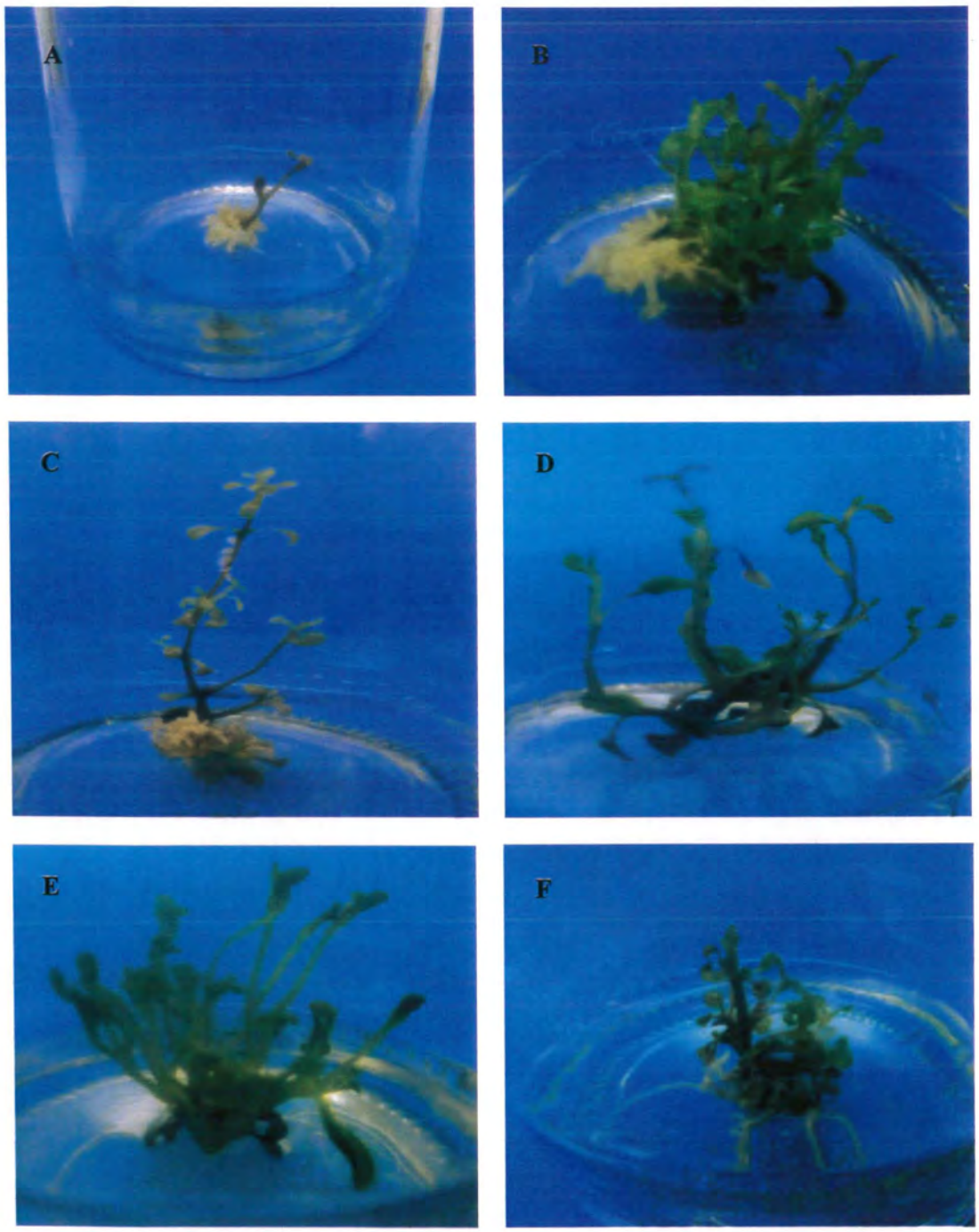

Figure 3.22 Rooting of differentiated shoots on MS media with different plant growth regulators after 4 weeks.

A: R1 (0.5 mg/l NAA) **B:** R2 (1.0 mg/l NAA) **C:** R3 2.0 mg/l NAA) **D:** R4 (0.5 mg/l IAA) **E:** R5 (1.0 mg/l IAA) **F:** R6 (2.0 mg/l IAA)

Table 3.3 Root induction in differentiated shoots using MS media with different plant growth regulators. Any two means sharing a letter in common are not significantly different according to LSD test at the level of P>0.05.

PGR Concentration (mg/L)	Number of roots		Root length (cm)	
	NAA	IAA	NAA	IAA
Control	-	-	-	-
0.5	9b	4b	0.9a	3.8a
1	24a	-	2.7a	-
2	9b	11a	0.7a	3.4a
LSD (0.05)	4.41	5.3	4.74	3.74

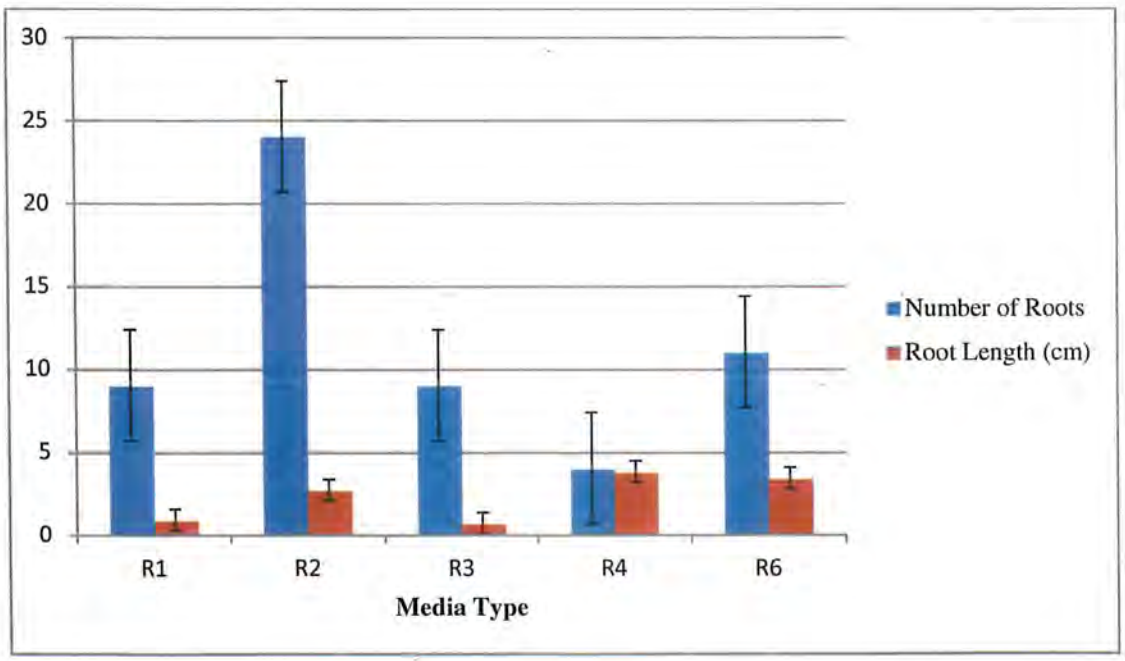

Figure 3.23 Average number of roots and average root length on MS media supplemented with different plant growth regulators. R1 average number of roots 9 and 0.9 cm average root length, R2 average number of roots 24 and 2.7 cm average root length, R3 average number of roots 9 and 0.7 cm average root length, R4 average number of roots 4 and 3.8 cm average root length, R6 average number of roots 11 and 3.4 cm average root length .

Table 3.4 Effect of rooting media on shoot elongation. Any two means sharing a letter in common are not significantly different according to LSD test at the level of P>0.05.

PGR Concentration (mg/L)	**Number of shoots**		**shoot length**	
	NAA	**IAA**	**NAA**	**IAA**
0.5	4b	35a	2.3a	5.5a
1	35a	29b	3.2a	4.6a
2	35a	22c	5.8a	4.1a
LSD (0.05)	12.86	3.3	5.78	2.54

3.4 Molecular Analysis of Somaclonal Variants of *Lallementia royleana* using OPC Primers

PCR based Random Amplification of Polymorphic DNA (RAPD) decamer primers from OPC series i.e. OPC 1-OPC 5 were used in this study to detect somaclonal variation produced at DNA level in 11 samples of *Lallementia royleana*.

3.4.1 Isolation of genomic DNA and polymerase chain reaction

DNA isolation was done following the protocol designed by Richards *et al.* (1997) with some modifications. The quality of genomic DNA was checked by running it on 1% agarose gel (Fig. 3.24). RAPD amplifications were carried out by using five decamer RAPD primers from OPC series. Amplified products were separated on 1.5 % agarose gel prepared in 0.5 X TAE buffer.

3.4.2 RAPD analysis using OPC 1

The amplification profile of OPC1 for eleven samples of *Lallementia royleana* taken at different stage of regeneration and of seed derived control plants revealed 4.35 % variation among the samples. It generated 23 bands in total (Fig. 3.25). Out of these only one band was found to be polymorphic whereas 22 bands were monomorphic. The smallest band obtained was of ~300 bp whereas the largest band was of ~590 bp size. Monomorphic bands of ~350 bp and ~590 bp were found in all of the samples. Seed derived plant of *Lallementia royleana* grown on MS medium (sample 2) showed a unique band of ~300 bp size which was absent in all of the other samples.

3.4.3 Cluster analysis of OPC 1

NTSYS version 2.0 (Exeter Software, Setanket, NY) statistical package was used for cluster analysis of eleven samples of *Lallementia royleana*, which revealed 33 % variation among these samples and formed one group in the cladogram (Fig. 3.26).

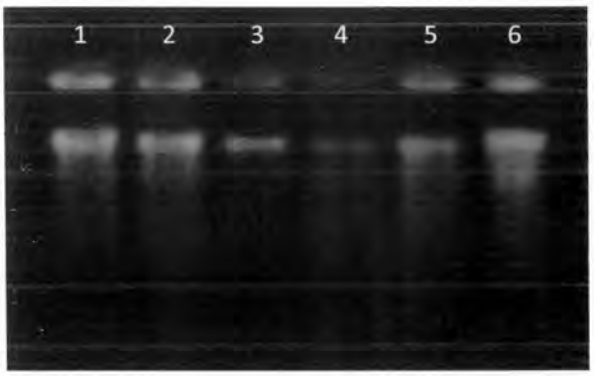

Figure 3.24 Representative picture of isolated genomic DNA from different samples of *Lallementia royleana.*

1: Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA.

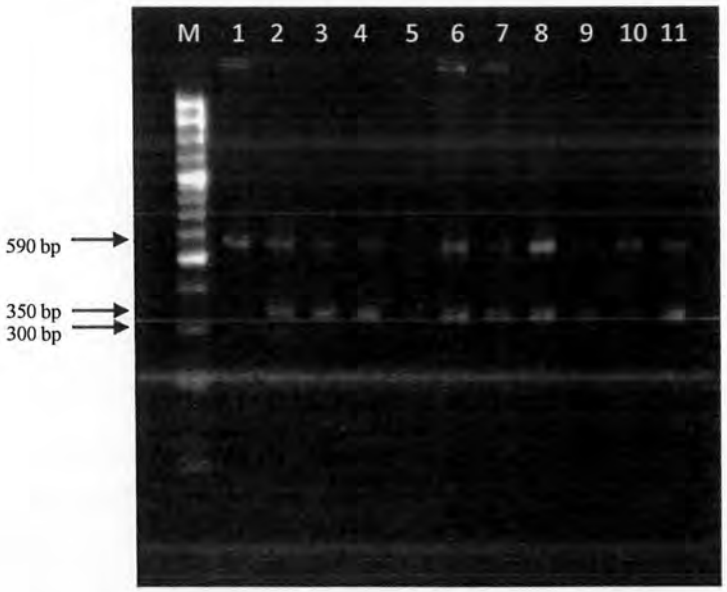

Figure 3.25 Amplification profile of OPC 1 for *Lallementia royleana.*

M: DNA ladder marker (100 bp plus Fermentas), **1:** Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** rooted plant at 1.0 mg/l NAA, **8:** rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

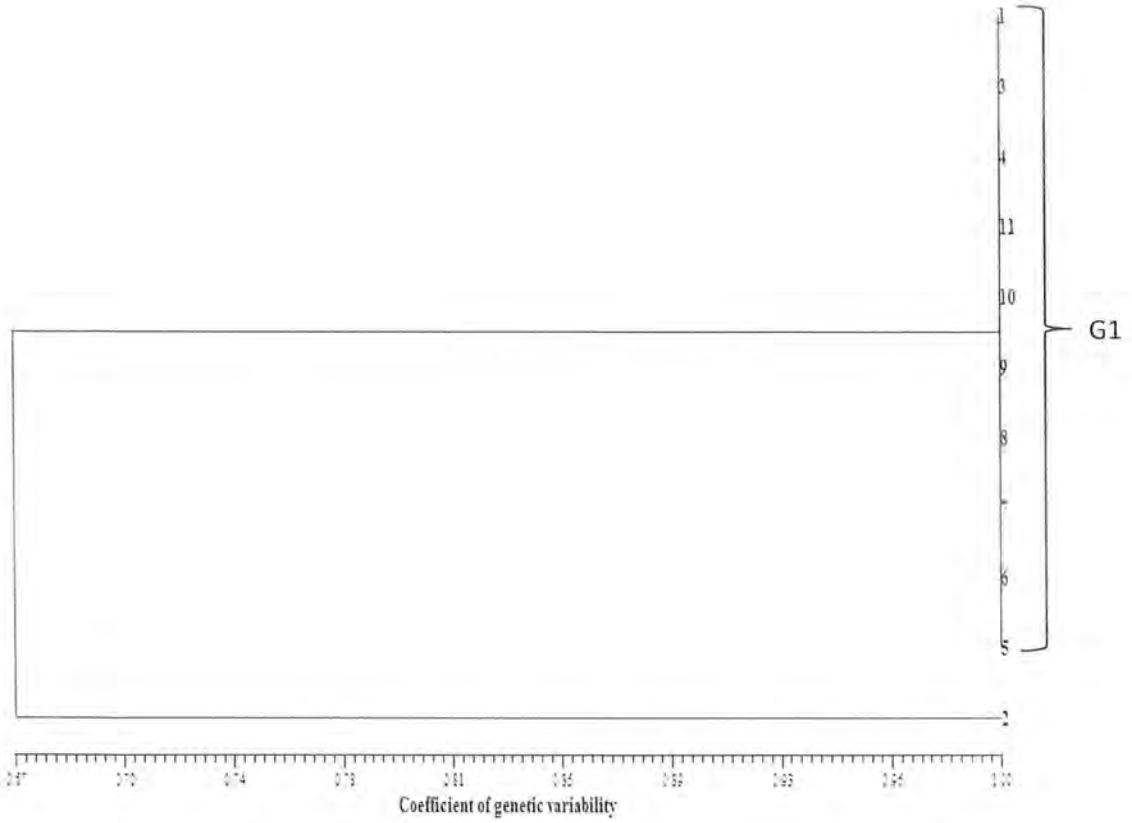

Figure 3.26 Dendrogram representing the genetic relationship among 11 samples of *Lallementia royleana* using NTYSYS clustral analysis generated from RAPD primer OPC 1.

1: Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** Rooted plant at 1.0 mg/l NAA, **8:** Rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

Group 1 was characterized by seed derived plant of *Lallementia royleana* grown in soil (sample 1), and directly regenerated plants cultured on different media which included shooted plants at 0.5 mg/l BAP (sample 3), 4.0 mg/l BAP (sample 4), 4.0 mg/l Kinetin (sample 5), and rooted plants at 0.5 mg/l NAA (sample 6), 1.0 mg/l NAA (sample 7), 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9), 1.0 mg/l IAA (sample 10) and 2.0 mg/l IAA (sample 11). Sample 6 and 7 showed 100 % genetic similarity with respect to OPC 1. Seed derived plant of *Lallementia royleana* grown in MS media (sample 2) was found to be 33 % different from the other samples due to which it remained unresolved.

3.4.4 RAPD analysis using OPC 2

Samples of *Lallementia royleana* at different stages of regeneration and of seed derived plants were analyzed using OPC 2 primer and 46.7 % polymorphism was found among these samples. Amplification profile for OPC 2 consisted of 62 DNA bands out of which, 29 bands were found to be polymorphic whereas 33 bands were monomorphic (Fig. 3.27). The smallest band was of ~100 bp and the largest band was of ~600 bp size. DNA bands of ~200 bp, ~325 bp and ~600 bp were monomorphic. The band of ~100 bp size was present in all of the samples except for shooted plant at 1.0 mg/l BAP (sample 3). DNA band of ~240 bp size was absent in seed derived plant of *Lallementia royleana* grown in soil (sample 1) and was present in all of the plants grown *in vitro*. Shooted plant at 4.0 mg/l Kinetin (sample 5) and rooted plant at 0.5 mg/l NAA (sample 6) lacked band of ~400 bp size which was otherwise present in rest of the samples.

3.4.5 Cluster analysis of OPC 2

The tree constructed for OPC 2 was characterized by one main cluster (Fig. 3.28). The shooted plant at 4.0 mg/l Kinetin (samples 5) and rooted plant at 0.5 mg/l NAA (sample 6) having 100 % similarity with each other and 20 % variation from the rest of the samples were grouped as G1.

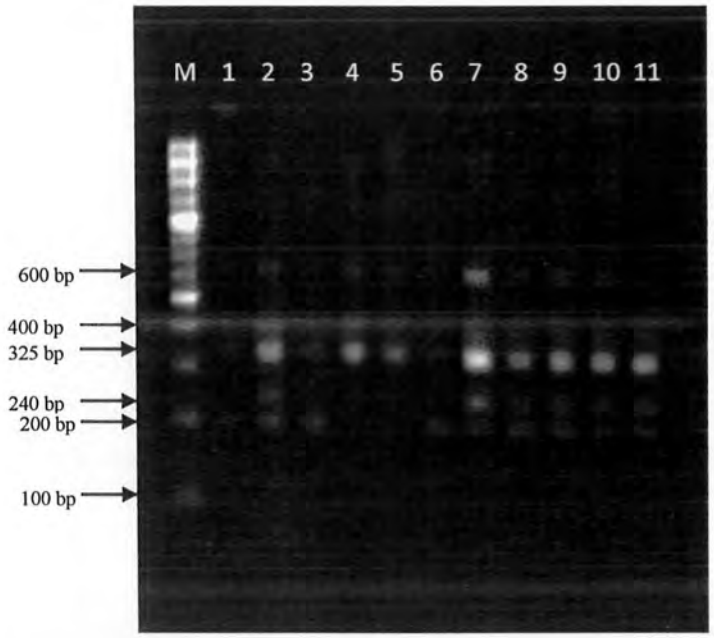

Figure 3.27 Amplification profile of OPC 2 for *Lallementia royleana.*

M: DNA ladder marker (100 bp plus Fermentas), **1:** Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** Rooted plant at 1.0 mg/l NAA, **8:** Rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

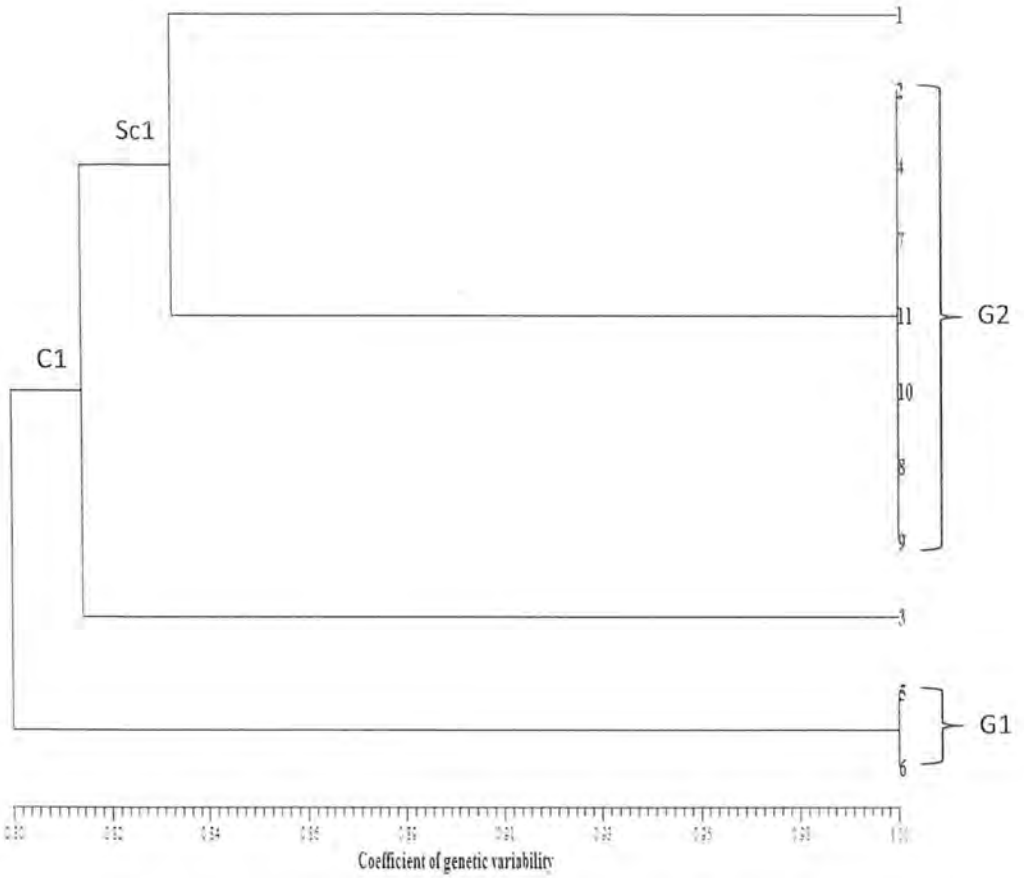

Figure 3.28 Dendrogram representing the genetic relationship among 11 samples of *Lallementia royleana* using NTYSYS clustral analysis generated from RAPD primer OPC 2.

1: Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** Rooted plant at 1.0 mg/l NAA, **8:** Rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

Cluster 1:

The seed derived plants of *Lallementia royleana* grown in soil (samples 1), in MS medium (sample 2), shooted plants at 1.0 mg/l BAP (sample 3), 4.0 mg/l BAP (sample 4), rooted plants at 1.0 mg/l NAA (sample 7), 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9), 1.0 mg/l IAA (sample 10) and 2.0 mg/l IAA (sample 11) were found to be 83 % similar to each other forming a cluster. Shooted plant at 1.0 mg/l BAP (sample 3) showed 19 % variation from the other samples thus forming an independent lineage.

Sub cluster 1

Sub cluster 1 was characterized by seed derived plants of *Lallementia royleana* grown in soil (sample 1) and MS medium (Samples 2), shooted plant at 4.0 mg/l BAP (sample 4), rooted plants at 1.0 mg/l NAA (sample 7), 2.0 mg/l NAA (sample 8), 0.5 mg/l NAA (sample 9), 1.0 mg/l NAA (sample 10) and 2.0 mg/l NAA (sample 11) having 83 % genetic similarity. *Lallementia royleana* grown in MS medium (Samples 2), shooted plant at 4.0 mg/l BAP (sample 4), rooted plants at 1.0 mg/l NAA (sample 7), 2.0 mg/l NAA (sample 8), 0.5 mg/l NAA (sample 9), 1.0 mg/l NAA (sample 10) and 2.0 mg/l NAA (sample 11) having 100% similarity were grouped together as G2. Soil grown plant of *Lallementia royleana* (sample 1) was found to be 17 % different from these samples hence it remained unresolved.

3.4.6 RAPD analysis of OPC 4

Amplification profile of OPC 4 generated a total of 42 bands of which 20 bands were polymorphic and 22 bands were monomorphic (Fig. 3.29). Polymorphism was found to be 47.6 % among the eleven samples of *Lallementia royleana*. The smallest band was of ~290 bp whereas largest band was of ~1500 bp size. It was observed that the bands of ~490 bp and ~550 bp size are monomorphic. However, bands with size of ~600 bp, ~1000 bp and ~1500 bp were unique as they were found only in seed derived plant of *Lallementia royleana* grown in soil (sample 1) and were absent in all of the plants regenerated *in vitro*. DNA band of ~390 bp size was present only in rooting sample at 0.5 mg/l NAA (sample 6). A band of ~450 bp band was absent in shooting sample at 0.5 mg/l BAP (sample 3) and shooting sample of 4.0 mg/l Kinetin (sample 5). Moreover, samples

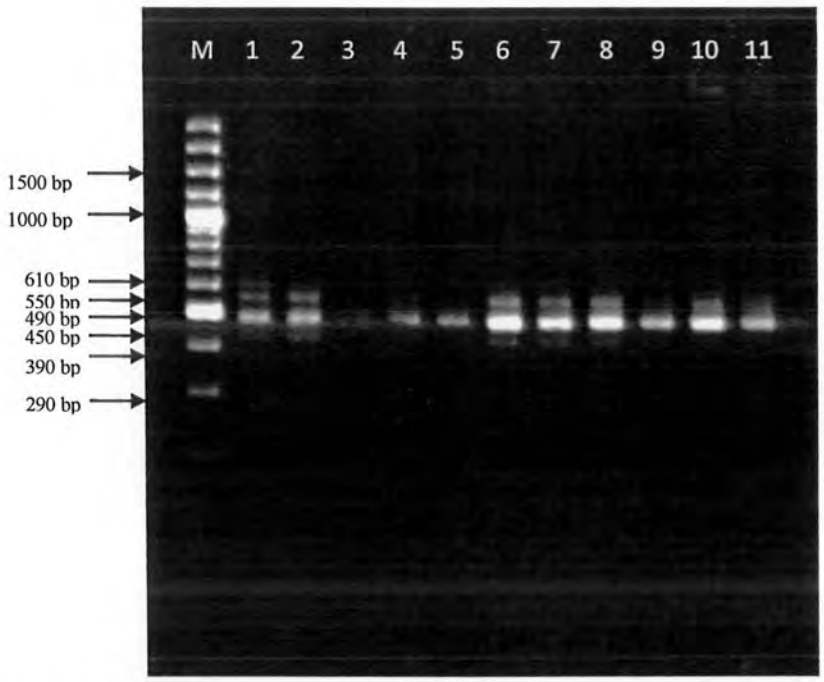

Figure 3.29 Amplification profile of OPC 4 for *Lallementia royleana.*

M: DNA ladder marker (100 bp plus Fermentas), **1:** Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** Rooted plant at 1.0 mg/l NAA, **8:** Rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

from 0.5 mg/l BAP (sample 3), 2.0 mg/l BAP (sample 4), 4.0 mg/l Kinetin (sample 5) and 2.0 mg/l IAA (sample 11) lacked the band of ~290 bp in size.

3.4.7 Cluster analysis of OPC 4

Based on data generated by OPC 4 a dendrogram was generated which revealed one cluster with two sub clusters (Fig. 3.30). Seed derived plant of *Lallementia royleana* grown in soil (sample 1) showed 46 % genetic variation from the *in vitro* samples therefore it was placed separately while all of the other samples were grouped in a single cluster.

Cluster 1:

Cluster 1 comprised of seed derived plant of *Lallementia royleana* grown in MS medium (sample 2), shooted plants at 1.0 mg/l BAP (sample 3), 4.0 mg/l BAP (sample 4), at 4.0 mg/l BAP (sample 5), rooted plants at 0.5 mg/l NAA (sample 6), 1.0 mg/l NAA (sample 7), 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9), 1.0 mg/l IAA (sample 10) and 2.0 mg/l IAA (sample 11) sharing up to 79 % genetic similarity. The cluster was further divided into two sub clusters.

Sub cluster 1

Sub cluster 1 comprised of *Lallementia royleana* grown in MS medium (sample 2), rooted plants at 0.5 mg/l NAA (sample 6), 1.0 mg/l NAA (sample 7), 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9) and 1.0 mg/l IAA (sample 10) as these showed 87 % genetic similarity. *Lallementia royleana* grown in MS medium (sample 2), rooted plants at 1.0 mg/l NAA (sample 7), 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9) and 1.0 mg/l IAA (sample 10) were 100 % similar to each other due to which these were grouped together as G1. Rooted plant at 0.5 mg/l IAA (sample 6) was found to be 12 % different from these samples and was placed as a parallel lineage.

Sub cluster 2

The shooted plants at 1.0 mg/l BAP (sample 3), 4.0 mg/l BAP (sample 4), 4.0 mg/l Kinetin (sample 5) and rooted plant at 2.0 mg/l IAA (sample 11) shared 88 % similarity and were grouped together in sub cluster 2. Shooted plants at 0.5 mg/l BAP (sample 3) and 4.0 mg/l Kinetin were 100 % similar and were grouped together (G2).

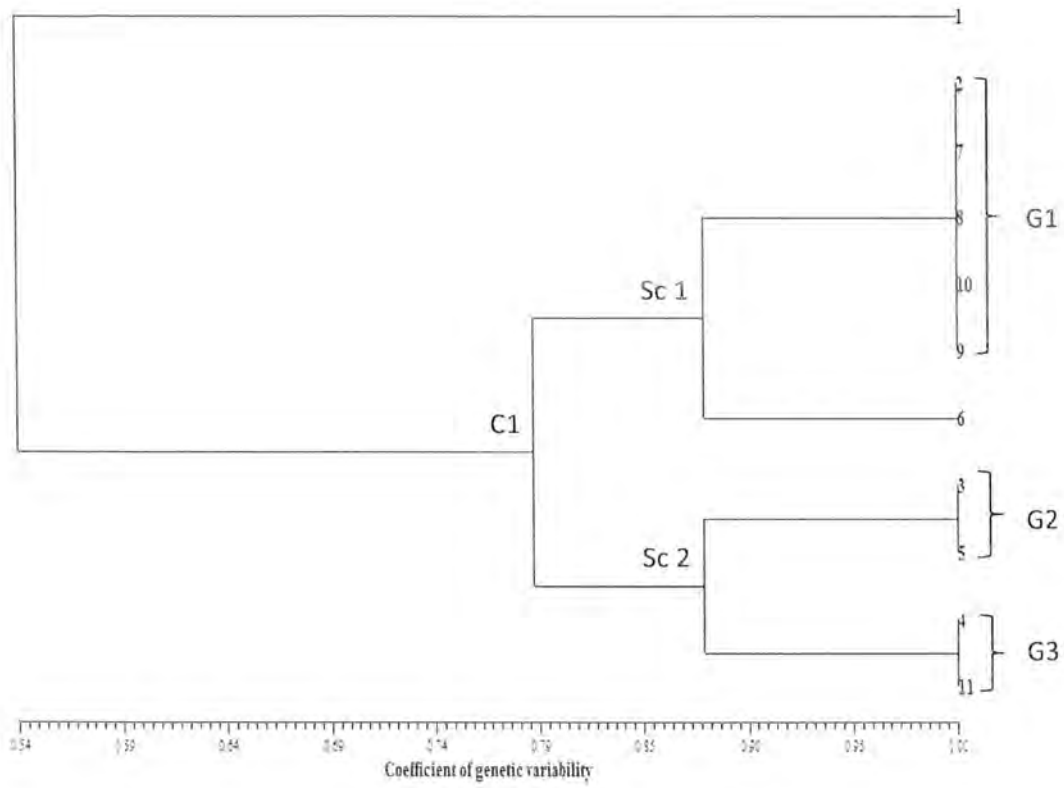

Figure 3.30 Dendrogram representing the genetic relationship among 11 samples of *Lallementia royleana* using NTYSYS clustral analysis generated from RAPD primer OPC 4.

1: Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** Rooted plant at 1.0 mg/l NAA, **8:** Rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

Shooted plant at 4.0 mg/l BAP (sample 4) and rooted plant at 2.0 mg/l IAA (sample 11) were also similar to each other consequently forming a group (G3).

3.4.8 RAPD analysis of OPC 5

OPC 5 generated 47 bands in total out of which 14 bands were polymorphic and 33 bands were monomorphic (Fig. 3.31). Polymorphism was calculated to be 29.7 % among the samples. The smallest band was of ~350 bp whereas largest band was of ~2500 bp. The bands of ~510 bp, ~600 bp and ~700 bp size were observed to be monomorphic. The ~350 bp band was absent only in rooted plants at 2.0 mg/l IAA (sample 11). The bands of ~1100 and ~2500 bp were found only in rooted plants at 0.5 mg/l NAA (sample 6) and 1.0 mg/l NAA (sample 7) due to which these were categorized as polymorphic.

3.4.9 Cluster analysis of OPC 5

Cluster analysis of OPC 5 showed one major cluster (Fig. 3.32). The samples shares up to 65 % genetic similarity. Rooted plants at 0.5 mg/l NAA (sample 6) and 1.0 mg/l NAA (sample 7) were found to be 35 % variant from the other samples. Samples 6 and 7 sharing 100 % similarity with each other were grouped together (G1).

Cluster 1:

Cluster 1 consisted of seed derived plant of *Lallementia royleana* grown in soil (sample 1), *Lallementia royleana* grown in MS medium (sample 2), shooted plants at 1.0 mg/l BAP (sample 3), 4.0 mg/l BAP (sample 4), 4.0 mg/l Kinetin (sample 5), rooted plants at 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9), 1.0 mg/l IAA (sample 10) and 2.0 mg/l IAA (sample 11) which were 65 % similar to each other. Among these sample seed derived plants of *Lallementia royleana* grown in soil (sample 1), in MS medium (sample 2), shooted plants at 1.0 mg/l BAP (sample 3), 4.0 mg/l BAP (sample 4), 4.0 mg/l Kinetin (sample 5), rooted plants at 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9) and 1.0 mg/l IAA (sample 10) were 100 % similar to each other hence grouped together as group 1. The rooted plant at 2.0 mg/l IAA (sample 11) showed 83 % similarity to these samples thus forming a parallel lineage within cluster 1.

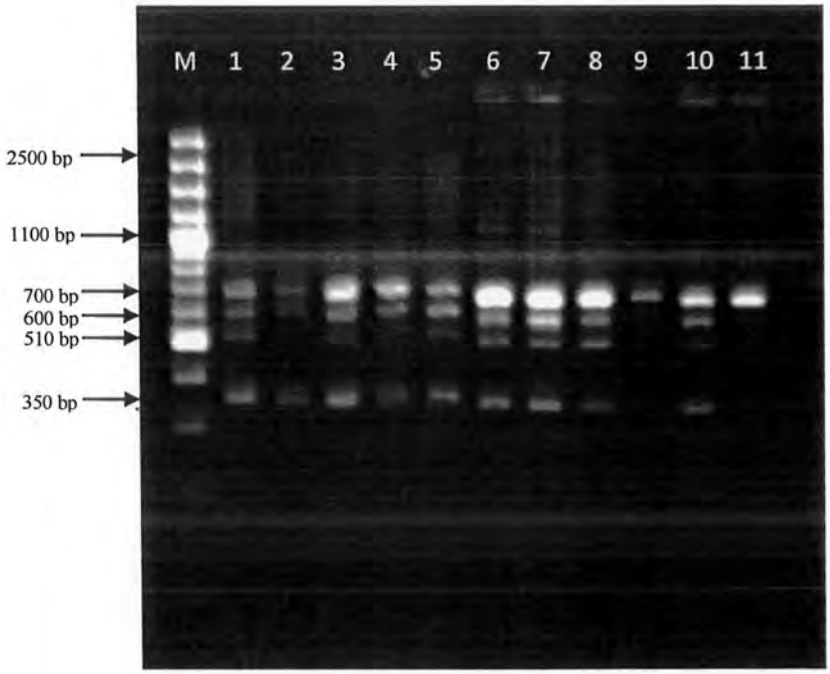

Figure 3.31 Amplification profile of OPC 5 for *Lallementia royleana.*

M: DNA ladder marker (100 bp plus Fermentas), **1:** Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** Rooted plant at 1.0 mg/l NAA, **8:** Rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

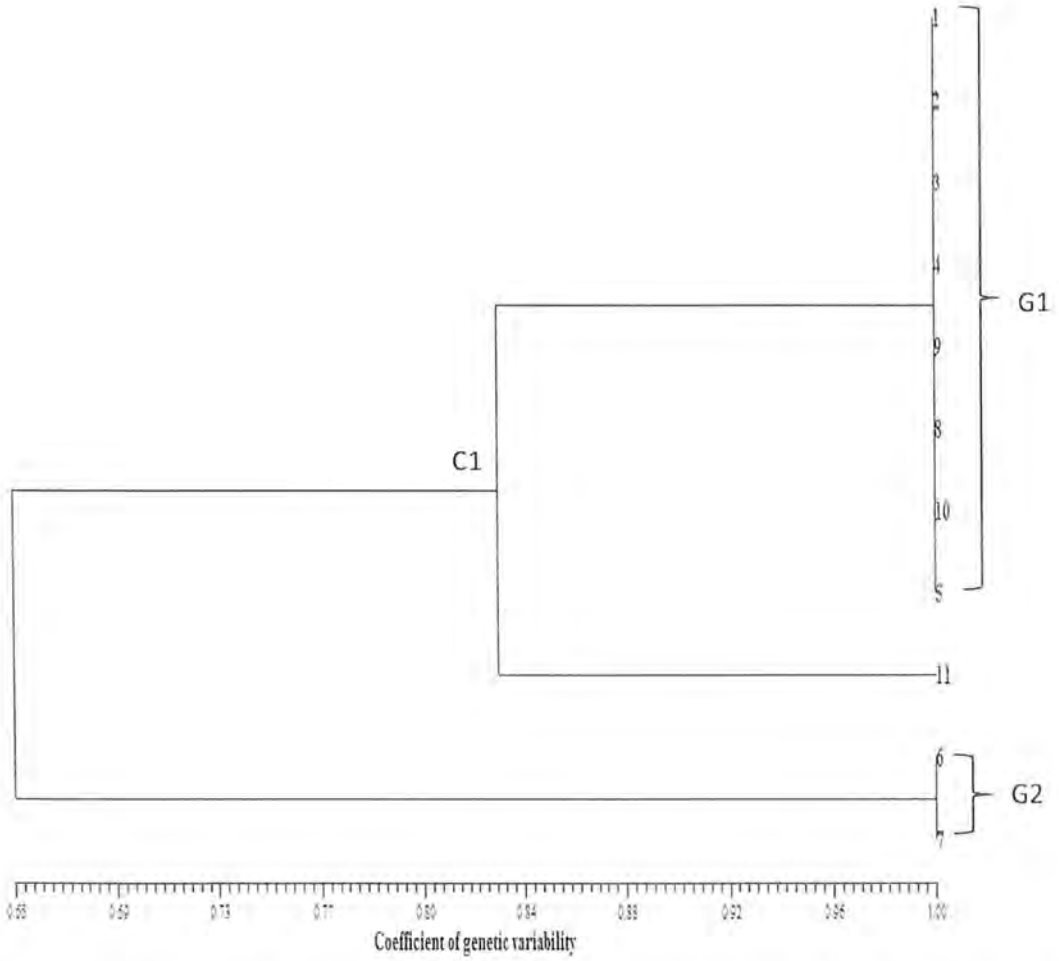

Figure 3.32 Dendrogram representing the genetic relationship among 11 samples of *Lallementia royleana* using NTYSYS clustral analysis generated from RAPD primer OPC 5.

1: Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** Rooted plant at 1.0 mg/l NAA, **8:** Rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

3.4.10 RAPD analysis of all primers

Out of the five primers used OPC1, 2, 4 and 5 gave polymorphic profiles whereas OPC 3 was unable to produce amplification even after extensive optimization. The highest number of scorable bands was obtained with OPC 2 while the lowest number was obtained with OPC 1 primer. Different primers showed variation in their ability to detect polymorphism. Primer OPC 4 showed maximum polymorphism whereas the lowest was observed on OPC 1 (Table 3.5). The amplification products ranged ~100-2500 bp in size. A total of 174 DNA bands were generated out of which 110 bands were monomorphic and 64 bands were polymorphic. Seed derived plant of *Lallementia royleana* grown in soil (Sample 1), rooted plant at 0.5 mg/l NAA (sample 6) and 1.0 mg/l NAA (sample 7) generated maximum number of bands i.e. 18, whereas minimum number of bands was 13 which were observed for shooted plant at 1.0 mg/l BAP (sample 3) and 4.0 mg/l Kinetin (sample 5).

3.4.11 Cluster analysis of all OPC primers

Cluster analysis of eleven samples of *Lallementia royleana* revealed two clusters in the cladogram (Fig. 3.33). It was observed that all of the samples shared up to 76 % similarity. The soil grown plant of *Lallementia royleana* showed 24 % variation from the rest of the samples due to which it remained ungrouped.

Cluster 1:

Seed derived plant of *Lallementia royleana* grown in MS medium (sample 2), shooted plants at 1.0 mg/l BAP (sample 3), 4.0 mg/l BAP (sample 4), 4.0 mg/l Kinetin (sample 5) and rooted plants at 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9), 1.0 mg/l IAA (sample 10) and 2.0 mg/l IAA (sample 11), having 18 % variation from others therefore forming cluster 1. Cluster 1 was further subdivided into two sub clusters as 14.5 % variation was found among these samples.

Table 3.5 Number of bands generated and polymorphism percentage as revealed by RAPD primers among 11 samples of *Lallementia royleana.*

Sr. No.	Primers	Total Bands	Monomorphic Bands	Polymorphic Bands	Unique Bands	Percentage Polymorphism
1	OPC1	23	22	1	1	4.35 %
2	OPC2	62	33	29	0	46.7 %
4	OPC4	42	22	20	4	47.6 %
5	OPC5	47	33	14	2	26.86 %
Total		174	110	64	7	36.8 %

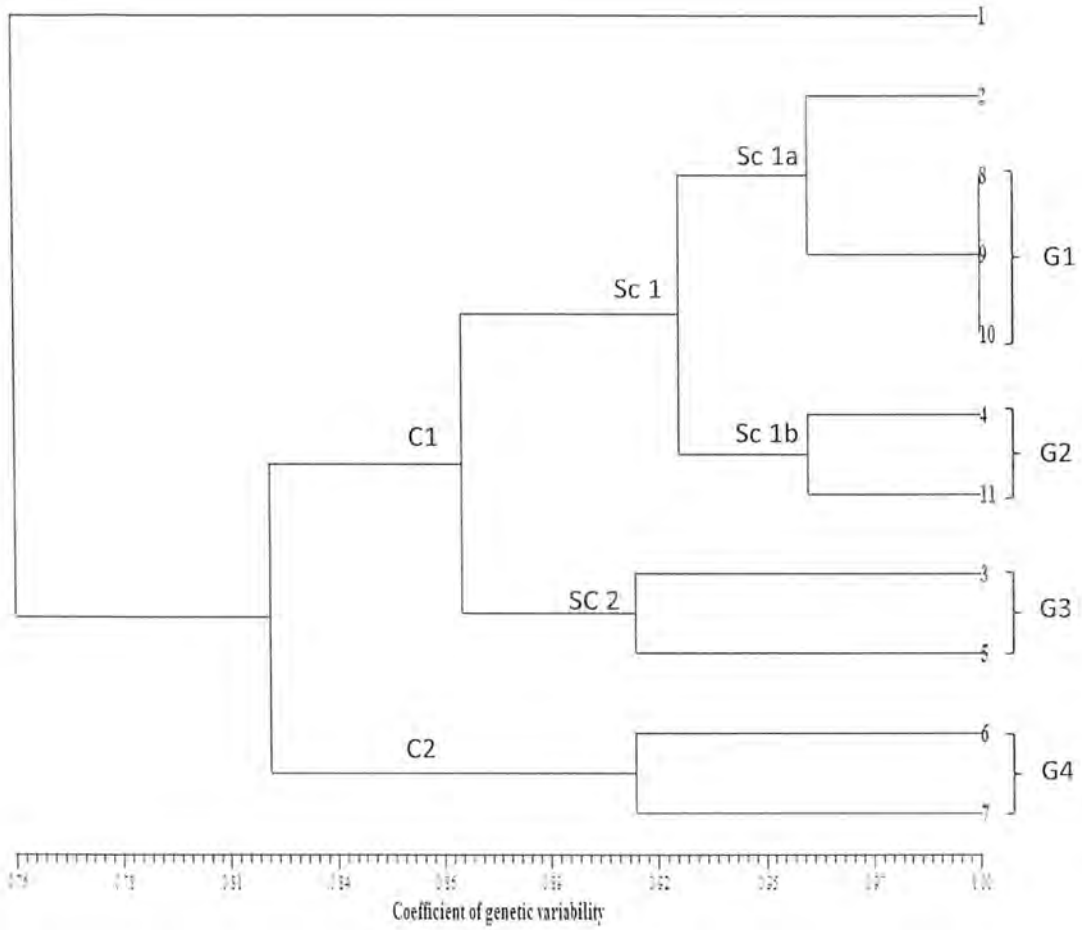

Figure 3.33 Dendrogram representing the genetic relationship among 11 samples of *Lallementia royleana* using NTYSYS clustral analysis generated from RAPD primers OPC1, OPC 2, OPC4 and OPC5.

M: DNA ladder marker (100 bp plus Fermentas), **1:** Seed derived plant of *Lallementia royleana* grown in soil, **2:** Seed derived plant of *Lallementia royleana* grown in MS medium, **3:** Shooted plant at 1.0 mg/l BAP, **4:** Shooted plant at 4.0 mg/l BAP, **5:** Shooted plant at 4.0 mg/l Kinetin, **6:** Rooted plant at 0.5 mg/l NAA, **7:** Rooted plant at 1.0 mg/l NAA, **8:** Rooted plant at 2.0 mg/l NAA, **9:** Rooted plant at 0.5 mg/l IAA, **10**: Rooted plant at 1.0 mg/l IAA, **11:** Rooted plant at 2.0 mg/l IAA.

Sub cluster 1

Sub cluster 1 consisted of shooted plants at 1.0 mg/l BAP (sample 2) and 4.0 mg/l Kinetin (sample 4), rooted plants at 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9), 1.0 mg/l IAA (sample 10) and 2.0 mg/l IAA (sample 11) sharing up to 93 % genetic similarity. Sub cluster 1 was further divided into sub cluster 1a and sub cluster 1b due to 7 % variation among the samples.

Sub cluster 1a comprised of the shooted plant at 1.0 mg/l BAP (sample 2), rooted plants at 2.0 mg/l NAA (sample 8), 0.5 mg/l IAA (sample 9) and 1.0 mg/l IAA (sample 10) sharing 96 % similarity. Rooted plants at 2.0 mg/l NAA (sample 8), 0.5 mg/l NAA (sample 9) and 1.0 mg/l IAA (sample 10) were 100 % similar to each other therefore were placed together in G1. Sample 2 differed from sample 8, 9 and 10 by 4 % due to which it formed an independent lineage from G1.

Sub cluster 1b consisted of shooted plant at 4.0 mg/l Kinetin (sample 4) and rooted plant at 2.0 mg/l IAA (sample 11) were found to be 96 % similar to each other therefore these were placed together in the same sub cluster 1b. These were 7 % different from the other samples of sub cluster 1.

Sub cluster 2

Shooted plant at 1.0 mg/l BAP (sample 3) and 4.0 mg/l Kinetin (sample 5) shared up to 91.5 % genetic similarity and were grouped together as sub cluster 2.

Cluster 2:

Rooted plants at 0.5 mg/l NAA (sample 6) and 1.0 mg/l NAA (sample 7) sharing 91.5 % genetic similarity were grouped together forming cluster 2. These samples were observed to be varying up to 18 % from the samples of cluster 1.

The similarity matrix was also generated from RAPD amplification data using Simqual subprogram of NTSYS-pc software, to estimate genetic difference and relatedness among 11 samples of *Lallementia royleana*. The similarity coefficients ranged from 0.652 (65.2 %) to 1.00 (100 %) (Table 3.6).

Table 3.6 Similarity index showing co-efficient of similarity among 11 samples of *Lallementia royleana* using RAPD markers.

	1	2	3	4	5	6	7	8	9	10	11
1	1										
2	0.783	1									
3	0.696	0.826	1								
4	0.783	0.913	0.913	1							
5	0.696	0.826	0.913	0.913	1						
6	0.652	0.783	0.696	0.783	0.783	1					
7	0.739	0.87	0.783	0.87	0.783	0.913	1				
8	0.826	0.957	0.87	0.957	0.87	0.826	0.913	1			
9	0.826	0.957	0.87	0.957	0.87	0.826	0.913	1	1		
10	0.826	0.957	0.87	0.957	0.87	0.826	0.913	1	1	1	
11	0.739	0.87	0.87	0.957	0.87	0.739	0.826	0.913	0.913	0.913	1

Minimum genetic similarity of 65.2 % was shown by sample 6 (rooted plant at 0.5 mg/l NAA) with soil grown control plant, whereas maximum genetic similarity (100 %) was found between the rooted plants at 2.0 mg/l NAA, 0.5 mg/l IAA and 1.0 mg/l IAA. The similarity coefficients of rest of the samples were found to lie between 0.696 (69.6 %) to 0.957 (95.7 %).

Numerous researches have shown that there are many factors that effect *in vitro* propagation of plants thus resulting in somaclonal variation. These factors include explant origin, growth regulators, regeneration pattern and many others. Somaclonal variations basically arise due to epigenetic influence or changes in the genome of the differentiating vegetative cells induced by tissue culture conditions (Muller *et al.* 1990). The causes of somaclonal variations have been reported to be different biochemical and molecular events which include changes in DNA methylation pattern, activation of transposable elements and chromosome remodeling (Price *et al.,* 2002). Therefore, molecular markers have been exploited for the detection of somaclonal variation, including RAPD (Chen *et al.*, 1998) and Rival *et al.*, 1998), methylation sensitive restriction fragment length polymorphism (RFLP) (Jaligot *et al.*, 2000, and Kubis *et al.*, 2003) and microsatellite sequence variation (Alou *et al.,* 2004). The RAPD technique has several advantages such as the ease and rapidity of analysis, availability of a large number of primers and the requirement of a very small amount of DNA for analysis (William *et al.*, 1990). RAPD based detection of somaclonal variation has been found useful in describing somaclonal variability in regenerated plants in several plant species including *Sylibum marianum* (Mahmood *et al.*, 2010), *Phaelenopsis bellina* (Khoddamzadeh *et al.*, 2010), *Musa acuminata* cv. Berangan (Hurirah and Khalid, 2006), date palm (Saker, 2000), *Lilium longiflorum* (Purwantoro *et al.*, 1999) and *Beta vulgaris* (Munthali *et al.*, 1996).

Somaclonal variations have been reported frequently in microproagated plants of *Populus deltoids* (Rani *et al.*, 1995), *Coffea Arabica, Camellia sinensis*, *Camellia assamica* (Devarumath *et al.*, 2002). On the other hand Zhang (2011) has reported no variation among the control and directly regenerated plants of *Populus hopeiensis* whereas plants regenerated through callus induction showed somaclonal variation based upon RAPD analysis. Similarly Harirah and Khalid (2006) have reported regenerated

plants of *Musa acuminate* cv. Berangan to be 100 % similar to each other as monomorphic RAPD profiles were obtained for all of the samples. It has been reported that maximum somaclonal variations are induced in plants regenerated after an intermediate callus phase (Piccioni *et al.*, 1997). Because cells in plant callus are in a changeable state and can differentiate into different organ primordia. The differentiating cells are exposed to unstable aberration rate therefore somaclonal variation could appear easily (Zhang, 2011). Earlier, Jain *et al.* (1998) have stated that hardly any variation occurs in plants regenerated directly. However, the current study has revealed 36.8 % variation among the directly regenerated and control plants of *Lallementia royleana*. Therefore, it can be inferred that plant growth regulators and tissue culture conditions are capable of causing genetic variation even if the callus stage has been avoided. It was observed that among the plant growth regulators used, NAA was responsible for inducing maximum polymorphism. Similar findings have also been observed in case of eggplant by Zayova *et al.* (2010).

Conclusion

Present research work shows that different explants of *Lallementia royleana* have poor callogenic ability; however, they have the ability to regenerate through direct regeneration without going through the callus stage. Therefore, an *in vitro* protocol for direct regeneration of *Lallementia royleana* from nodal explants was developed. Furthermore, RAPD markers were used to study the somaclonal variation in regenerated plants in comparison with soil grown control plant. In spite of direct regeneration, 36.8 % genetic variation was found among the samples, thus contradicting the earlier concepts that somaclonal variation does not occur in directly regenerated plants.

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