Somatic embryogenesis in black pepper (*Piper nigrum* L.)

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Somatic embryogenesis in black pepper (*Piper nigrum* L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Afna Mol O.P

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LIST OF ABBREVIATIONS AND SYMBOLS USED

2, 4-D	2, 4-Dichlorophenoxyacetic acid
BA	N^6- benzyl adenine
$CaCl_2$	Calcium chloride
Cm	Centimeter
et al.	and others
Fig.	Figure
G	Gram
GA	Gibberellic acid
H	Hour
HCL	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
M	Molar
Mg	Milligram
Ml	Milliliter
Min	Minute
Mm	Millimolar
MS	Murashige and Skoog, 1962
NaCl	Sodium chloride
NaOH	Sodium hydroxide
µM	Micromolar
pH	Potential of hydrogen
PGR	Plant growth regulator
PVP	Polyvinylpyrrollidone
Rpm	Revolutions per minute
S	Second
SA	Sodium alginate
sp.	Species
TDZ	Thidiazuron
V	Volt
viz.	Namely
Picloram	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
NAA	1-Naphthaleneacetic acid
SH	Schenk & Hildebrandt Medium
°C	Degree Celsius
%	per cent
±	plus or minus
i.e.	That is
Sl. No	Serial number
Sp. or spp.	Species (Singular and plural)
Ppm	Parts per million
Ha	Hector
T	Tonnes

Wt.	Weight
RH	Relative humidity

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Introduction

INTRODUCTION

Black pepper is the most important and widely used among the spices in the world. The black pepper of commerce comprises the dried fruits of the tropical, perennial climbing plant *Piper nigrum* L., which comes under the family Piperaceae. It is having several medicinal properties such as defensive role against infection by insects, animals and microbes. (Scott *et al.*, 2008; Ahmad *et al.*, 2011),treating digestive disorders, gastric problems, diarrhea, ingestion and respiratory problems (Parganiha *et al.*, 2011).

India is a major producer and exporter of black pepper, the annual export being over Rs. 4000 million. The global production is estimated as 409,000 tons, in which India ranks third after Vietnam and Indonesia. Indian black pepper production had dropped to 45,000 ton in 2014 (International Pepper Community, 2015). The area under cultivation in Kerala is reduced from 2.02 lakh ha in 2000-01 to 1.22 lakh ha in 2013-14.The production in Southern and Northern part of Kerala showed a decline from 11.2 thousand ton (1982) to 5.4 tonnes (2009) as reported by Sajitha (2014). In spite of its great economic importance, the production and productivity of black pepper in India is declining day by day, mainly because of lack of adequate planting material of high yielding varieties and also crop losses due to diseases, nematodes and insect pests.

Vegetative propagation through conventional techniques is inadequate to meet the increase in demand for planting material. Propagation through seed is not advisable as it yields only a few heterogeneous progenies and also due to their poor viability and high sterility in post-fertilization stages (Ravindran *et al.* 2000).

In vitro propagation methods provide a strapping tool for the mass multiplication and germplasm conservation of this economically important species. Plantlets regenerated from seedling derived callus and shoot apices had

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been reported, but most attempts to regenerate plants from mature vine were unsuccessful (Mathews and Rao, 1984).

Protocols for regeneration through shoot tip (Philip *et al.*, 1992; Bhat *et al.*, 1995; Ahmad *et al.*, 2011) as well as through somatic embryogenesis (Joseph *et al.*, 1996; Nair and Gupta, 2006) have been reported. Somatic embryogenesis is the most appealing method for mass cloning of plants since a very large number of somatic embryos can be produced using a limited volume of medium in a short period (Choi *et al.*, 2002; Nair and Gupta, 2006) .

Micropropagation through somatic embryogenesis is mostly preferred over organogenesis, as more regenerated plants will be obtaining from few or single cells. Direct somatic embryogenesis through vegetative and seed tissues other than zygotic embryos and endosperm can be utilized as a method for large-scale clonal propagation system for multiplying elite genotypes and high yielding varieties. There are very few reports on the induction of somatic embryogenesis in black pepper. (Nair and Gupta, 2005; Joseph *et al.*, 1996). All these reports comment on the low per cent of regeneration of the embryos. No studies are reported on somatic embryogenesis from mature explants.

In the present study an attempt was made on standardization of reliable protocol for somatic embryogenesis in black pepper (*Piper nigrum* L.)

Review of Literature

REVIEW OF LITERATURE

Piper is the most popular and diverse genera among the angiosperm (Gentry *et al.*, 1990). It comes under the family Piperaceae and consists of more than one thousand species, throughout the tropical and subtropical regions (Parthasarathy *et al.*, 2006).

2.1 BLACK PEPPER

The genus Piper includes *Piper nigrum* (black pepper), *Piper longum*, *Piper colubrinum*, *Piper chaba, Piper brachystachyum* etc., which are generally used in our endogenous system of medicine (Nazeem *et al.*, 2003).Black pepper (Piper nigrum L.) described as the king of spices is the oldest and most widely used spice in the world (Pradeepkumar *et al.*, 2001). It is believed to have originated from the sub-mountanious tracts of Western Ghats (Rahiman *et al.*, 1987; Joseph and Skaria, 2001).

India is a major producer, consumer and exporter of black pepper in the world(George *et a*l.,2005).Besides India, black pepper is widely cultivated throughout Vietnam, Indonesia , Malaysia, Thailand, Tropical Africa, Brazil, Sri Lanka, and China (Joseph and Skaria, 2001). The area under cultivation in Kerala is reduced from 2.02 lakh ha in 2000-01 to 1.54 lakh ha in 2009-10. The productivity has also come down from 376 kg per ha during 1998-99 to 221 kg per ha during 2009-10. The production in Southern and Northern part of Kerala showed a decline from 11.2 thousand tones (1982) to 5.4 tones (2009) as reported by Sajitha (2014).

2.1.1 Morphology of plant

Pepper plant is a perennial woody climber with small cordate leaves when young. The plant exhibits dimorphic branching; the straight monopodial climbing vegetative orthotropic branches and laterally growing, sympodial non climbing

reproductive plagiotropic branches. Leaves are simple, alternate, cordate, varying in breadth, broadly ovate, 5-9 nerved. It is a dioecious plant with minute flowers in spike which vary in length. Fruit is glibose one seeded drupe, bright red when ripe and seed is globose (Joseph and Skaria, 2001).

2.1.2 Uses of black pepper

Black pepper is used in human dietaries, medicine, and preservative, and in perfumery (Srinivasan, 2007). Black pepper is used in all stages of the cooking process and as a table condiment. It is used commonly in sauces, poultry, gravies, processed meats, snack foods etc. (Vasanthakumar, 2006). Pepper forms an important ingredient of several indigenous medicines of India (Vasasnthakumar, 2006), as well as Chinese and African systems. In Ayurvedic system of medicine black pepper is known by different names such as Maricam (killer of poison), Krishna (corrosive), Ooshana (giving burning sensation and vellayam (antihelminthic). The whole plant as such can be used as medicine for various ayurvedic preparations (Nybe and Sujatha, 2001). Black pepper can be used as a stimulant, digestive stomachic, carminative, nervine to calm nerves, deobstruent (to open the natural ducts in the body) resolvent, cholagogue, diuretic,and emmenagogue and antiperiodic. Black pepper is used as an aromatic stimulant, as an antiperiodic for malarial fever and alternative to arthritis disease (Joseph and Skaria, 2001).

2.1.3 Value added products

Pepper is a spice from which a number of value-added products have been developed. Black pepper contains chiefly (chavicin), volatile oil and alkaloid piperine. The presence of resin gives the stimulant nature, while volatile oil gives the odour and aromatic taste and the alkaloid piperine imparts the febrifuge property.

2.1.3.1 Pepper oil

Is a volatile oil commercially extracted from berries by steam distillation. It is used in flavoring and perfumery (Vasanthakumar,2006).The analysis of volatile components and the odour characteristics of Japanese pepper using gas chromatography reveals that citronellal, linalool, geraniol and methyl cinnamate were perceived to be important to the basic flavor (Jiang and Kubota,2004).

2.1.3.2 Black pepper oleoresin

Oleoresin from black pepper is a mirror image of the flavor, pungency, and aroma components and is obtained by extraction of pepper powder using organic solvents viz., ethyl acetate, ethylene chloride, hexane, ethanol, acetone etc. Freshly prepared oleoresin will be dark green, viscous, heavy liquid with strong aroma. One kilogram of oleoresin dispersed on an inert base can replace fifteen to twenty kilogram of spice used for flavoring purposes (Vasanthakumar, 2006).

2.1.3.3 Piperine

Piperine is a product obtained from oleoresin through centrifugation and its contents varies from four to six percent in dry pepper and thirty five to fifty per cent in oleoresin (Vsasanthakumar, 2006). According to Srinivasan (2007) piperin in diet enhances the digestive capacity and significantly reduces the gastrointestinal food transit time. It is also having inhibitory influence on enzymatic drug bio transforming reactions in the liver. It intensifies the bioavailability of a number of therapeutic drugs. It is non genotoxic and having anti mutagenic and anti tumor influences.

2.1.4 Popular varieties of black pepper

Twelve improved varieties have been released and a few more are in the work at AICRPS centre at Panniyur as well as at Indian Institute of Spice Research (IISR), Kozhikode. Panniyur 1,2,3,4,5,6 and 7 from Pepper Research

Station (PRS), Panniyur yielding between 1.27-2.57 tonnes/ha and Pancahami, Pournami, Sreekara, Shubhakara with 2.3-2.8 tonnes/ha, IISR Malabar Excel,IISR Girimunda and IISR Sakthi with 1.05-2.1 tonnes/ha from IISR and PLD-2 with 2.4 tonnes /ha fom Central Plantation Crop Research institute (CPCRI), Palode are promising varities both in research and in farmers field with respect to yield and other spike quality parameters.Of this Panniyur 1,3 and IISR Malabar Excel and Girimunda are hybrids and others are clonal selection from landraces (Ravindran *et al.*,2006).

Almost sixteen improved varieties are developed or recommended for release so far (Saji and Sasikumar, 2006).

2.1.5 Panniyur 5

Panniyur 5 is an open pollinated progeny of 'perumkodi'. It is a high yielding and regular bearing variety and suited to all pepper growing tracts. It is tolerant to nursery diseases and gives approximately 1098 kg/ha berries per year. There will be 51 fruits per spike and 104 cc volumes for 1000 fruit. Individual vine yields approximately 2 kg with 5.3per cent piperine content and 12.33 per cent oleoresin. The most important characteristic is its adaptability to shady areas, droughty areas and high altitudes. Leaf is elliptical in shape with a spike length of 13.1 cm. Fruits are round in shape with vigorous growth habit (Edison *et al.*, 1998).

2.2 METHOD OF PROPAGATION IN BLACK PEPPER

Majority of flowering plants combine sexual reproduction with some mode of asexual reproduction through various vegetative propagation methods like rhizome, bulbils, cutting, layering, tillering or rooting of surface runners (Cook, 1983; Eckert *et al.*, 1999). In angiosperms, vegetative propagation is common and widely used. (Albert *et al.*, 2003).Vegetative propagation can ensure true to type off spring so can ensure the genetic fidelity.

2.2.1 Vegetative propagation in black pepper

Usually, Black pepper is propagated through pre-rooted cuttings. It develops various types of aerial shoots.

1. Primary stem or Climbing stem
2. Runner shoots which originate from the base of the vines
3. Fruit bearing lateral branches
4. Hanging shoots

Difference in planting material produce differences in plant produced. Generally, cuttings from the runner shoots are used for rising rooted planting materials. Runner shoots with high yield and healthy vine in the garden are selected and cuttings with 2-3 nodes are planted either in nursery beds or in polythene bag filled with fertile potting mixture. (Package of practices, KAU, 2015).

2.3 INVITROPROPAGATION

In vitro cultures are used as tools for the study of various basic problems in plant sciences. It is able to propagate all plants of economic importance in large numbers by tissue cultures (Altman, 2000).

Black pepper is propagated by seeds, layering, cutting, and grafting mainly. Seed propagation will leads to genetic variations due to the formation of recombinants. Other methods of propagations are slow and time consuming (Atal and Banga, 1962). So there is need to introduce efficient and reliable methods for rapid propagation of black pepper. In this aspect, plant tissue culture is the most efficient and reliable method for faster and large scale production of disease free, genetically stable and identical progeny throughout the year (Hu and Wang, 1983).

Tissue culture techniques have played important roles in clonal propagation, conservation of germplasm and plant improvement in black pepper

(Bat *et al.*, 1995: Sajc *et al.*, 2000). *In vitro* propagation is a superior and alternate method in black pepper than traditional methods (Abbasi *et al.*, 2007). Different types of *in vitro* culture techniques are there including organs, embryos, tissues, single cells, and protoplasts cultures.

Technologies for micropropagation of black pepper using various explants were reported (Babu *et al.*, 2012).A commercial viable protocol for large scale invitro multiplication of black pepper was reported be Nazeem *et al.*, 2004.

Babu *et al.*, 2012; joseph *et al.*, 1996; Yamuna (2007) reported the somatic embryogenesis in black pepper from zygotic embryos while Nair and Gupta (2006) reported the cyclic somatic embryogenesis from the maternal tissues, which have tremendous potential for automated micro propagation.

Nair and Gupta (2007) reported a successful encapsulation technique for somatic embryos of black pepper to produce synthetic seed using 4per cent sodium alginate (w/v) and (100 mM) calcium chloride

2.4 SOMATIC EMBRYOGENESIS

According to Williams and Maheswaran (1986) somatic embryogenesis is a process in which bipolar structure, resembling a zygotic embryo, develops from a non zygotic cell without vascular connection with the original tissue.

Somatic embryos closely resembles their zygotic counterparts with appropriate root, shoot and cotyledons and do not have vascular connection with mother tissues (Ammirato, 1983). However, they are found to mature incompletely without entering the rest phase unlike zygotic embryos (Gray, 1987). Somatic embryoids are clonal materials compared to zygotic embryos which bring about new recombination (Kester, 1983).

According to Sharp *et al.* (1980) somatic embryogenesis can be direct or indirect as initiated from pre-embryogenic determined cells (PEDCs) or induced embryogenic determined cells (IEDCs). In PEDCs the embryogenic pathway is predetermined and the cells need only the synthesis of an inducer or removal of an inhibitor to express their potential. The IEDCs on the other hand require an induction treatment to the embryogenic state by exposure to specific auxins. Once the embryogenic state has been reached, both the cell types proliferate in a similar manner.

Isolated cells have shown capacity to produce embryos (Backs-Husemann and Reinert, 1970) even though determined cells may operate singly or in groups to form somatic embryos (Williams and Maheswaran, 1986). Multicellular aggregates are produced before embryo formation (Reinert, 1973). But the pattern of early segmentation differs from the typical embryogenesis of an egg cell (Mc Williamn *et al.*, 1974). Embryogenesis generally proceeds from the globular to the heart, torpedo, cotyledonary and mature somatic embryo stages of development (Tuleke, 1987). During embryogenesis root and shoot develop simultaneously on the same culture medium (Evans *et al.*, 1984).

Potentially embryoidal cells differ from the other cells as revealed by their differential staining properties, conspicuous size and the presence of large number of nucleoli per nucleus (Konar *et al.*, 1972). The embryogenic cells consist of small cells, densely cytoplasmic, rich in ribosomes and containing numerous starch grains (Staritsky, 1970). The developmental activations of somatic embryo involve synthesis of ribosomal RNA and protein (Sussex, 1972). More RNA and proteins are found in embryogenic cells than in non embryogenic cells (Raghavan, 1983)

The initiation of embryos from the somatic cells in culture was first recognized by Steward (1958) and Reinert (1958) in carrot explants derived from storage tap root. Subsequently it has been obtained easily in certain plants but with much more difficulty in others (Ammirato, 1983). Somatic embryogenesis

can be achieved in a wide range of plant species provided that appropriate explants, culture media and environmental conditions are met (Reinert, 1958).

Somatic embryos can be used as a model system in embryological studies. The most importance of somatic embryos is its practical application in large scale vegetative propagation. Somatic embryogenesis is favored over other methods of vegetative propagation because of the possibility to scale up the propagation by using bioreactors. In adition to this, in most cases the somatic embryos or the embryogenic cultures can be cryopreserved, which enables to establish gene banks. Embryogenic cultures are also useful for genetic modification (Arnold *et al.*, 2002). Somatic embryos develop either directly from the explants without an intervening callus phase or indirectly after a callus phase (Williams and Maheswaran, 1986).

The complication of obtaining somatic embryos in certain species, particularly ligneous plants (Bajaj, 1989), or in genotypes having other interesting characteristics (yield, pest and disease resistance), has stimulated studies to enlarge the understanding of mechanisms involved in this process.

The impact of a number of factors on the embryogenic ability of cultured explants has been studied: water parameters (Imamura & Harada, 1980; Etienne et al., 1991a), hormonal balance (Carman, 1989; ElHadrami et al., 1991) mineral balance (Etienne et al., 1991a), addition of polyamines (El Hadrami et al., 1989) or antioxidant agents (Housti et al., 1991) to the culture medium, and carbon sources (Meijer & Brown, 1987; Etienne et al., 1991b). Moreover, the importance of atmospheric gas content (02, CO2, C2H4) in culture vessels (Carman, 1988; Vain et al., 1989; Auboiron et al., 1990), the timing of sub culturing (Michaux-Ferriere & Carron, 1989), the type of medium (solid, liquid or semi-liquid) and the quality of the solid culture medium (gelrite, agar, gelose, cellulose blocks) have also been investigated.

The capacity for somatic embryogenesis is influenced by several factors, of which explants type and genotype are very important. A proper understanding of the influence of these factors in a particular plant species will help to scale up the process of somatic embryogenesis resulting an increased frequency of plant regeneration. (George 1993, Litz and Gray 1995, Arnold *et al.* 2002).

The other key variables influencing somatic embryogenesis include culture medium, exogenous plant growth substances, evolved gases, mode of culture, environmental conditions, density and genetic stability of embryogenic cells and synchrony of development of embryoids (Ammirato, 1983)

There are a few reports on *in vitro* propagation of black pepper through somatic embryogenesis (Joseph *et al.,* 1996 and Nair and Guptha., 2003). Somatic embryogenesis directly from sporophytic tissues of germinating seeds of black pepper and their ontogeny from single cells have been reported by Nair and Guptha (2003). Joseph *et al.,* 1996 have been reported the embryogenic callus derived from the zygotic embryo of black peppe were inuced to form somatic embryos on solid and liquid Schenck and Hildebrandt basal medium.

2.5 CULTURE MEDIA

Basic media that are regularly used in plant tissue culture are Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), Linsmaier and Skoog (LS) medium (Linsmaier and Skoog, 1965), Gamborg (B5) medium (Gamborg *et al.*, 1968) and Nitsch and Nitsch (NN) medium (Nitsch and Nitsch, 1969), SH medium (Schenk and Hildebrant, 1972), Woody Plant Medium (WPM) (Lloyd and Mc Cown, 1980). MS medium characterised by high salt concentration is the most commonly used, often with minor alterations.

The selection of a particular medium is based on the purpose and the plant species or variety to be cultured (Wang and Charles, 1991). The major

components of most plant tissue culture media include mineral salts, sugar as carbon source and water. Other components like organic supplements, growth regulators and a gelling agent also will be there (Gamborg *et al.,* 1968; Gamborg and Phillips, 1995).

Somatic embryo development has been reported on a wide range of media from relatively dilute White's medium (White, 1963) to more concentrated MS medium (Murashige and Skoog, 1962), B-5 medium (Gamborg et al.,1968) and SH medium (Shenk and Hildebrandt,1972).

Callus mediated somatic embryos are induced from the seeds of black pepper using hormone-free solid and liquid SH medium containing 3per cent (w/v) sucrose under darkness. Callus proliferation and germination of the embryos were achieved in 8 months in static culture and within 8 weeks in liquid suspension cultures (Joseph *et al.,* 1996).

Primary embryos were produced from the explants cultured on full-strength SH medium containing 0.8per cent (w/v) agar and sucrose 30 g/l without any plant growth regulators and the initiated somatic embryos were regenerated in to plantlets in liquid SH medium (Nair and Gupta, 2005). In their study, of the various genotypes tested, cultivar 'Karimunda' was found to be highly embryogenic and cultivar 'Kutching' was totally non embryogenic. Other cultivars like Jeerakamundi, Kalluvally, Karimunda, Kutching, Kuthiravally, Narayakodi, Neelamundi, Neyyattinkaramunda, Panniyur-1, Perambramunda, Sreekara, Subhakara, Thevanmundi, Thommenkodi and Vadakkan showed variable degree of embryogenic response (Nair and Dutta Gupta 2005)

Manju and Sonia (2012) reported the induction of somatic embryos in black pepper variety panniyur 1, in both the MS and SH medium without any plant growth regulators.

2.6 NUTRIENT COMPOSITION

The success of plant tissue culture is mainly dependent on the use of suitable nutrient media (Huang and Murashige, 1976). The catalytic functions in enzyme systems are served by vitamins supplied in trace amounts (North *et al.*, 2010). Vitamins may act as limiting factors of growth of cultures in some cases (Torres, 1989). Ramage and Williams (2002) also argue that minerals in the culture medium appear to have an important role in the regulation of plant morphogenesis

Plant tissue culture media generally made up of macro nutrients, micro nutrients, vitamins, amino acids, or other nitrogen supplements, sugar and other undefined organic supplements Plant tissue and cell culture media are generally (Khan et al., 2017).

Halperin and Wetherell, 1965; Reinert et al., 1967 reported that presence of some reduced nitrogen is necessary for somatic embryogenesis in cell and callus cultures also noted enhanced embryogenesis and/or improved embryo growth when media have been supplemented with amino acids in addition to NO_3^- and NH_4^+.

2.6.1 Sucrose concentration

While *in vitro* culturing, plants needs external carbon for energy since the plant cells and tissues in the culture medium lack autotrophic ability. Sucrose is the important carbon energy source in most tissue culture media. The concentration of sucrose varies from 20.0 to 30.0 g L^{-1} in general (Oka and Ohyama, 1982). Sucrose also serves as an osmoticum that can stimulate and regulate morphogenesis (Wethrell, 1984). Many other carbon sources like glucose, maltose, raffinose and fructose were used instead of sucrose, but comparatively less effective. Mannose and lactose were least effective.

Strength of the medium and sucrose concentration are influenced the process of secondary embryogenesis and fresh weight of somatic embryo clumps (Nair and Gupta 2005). Joseph *et al.* (1996) observed that, in callus-mediated somatic embryogenesis of black pepper, the rate of embryo differentiation was enhanced in half-strength basal medium with the sucrose level from 3.0 to 1.5per cent.

Sasi and Bhat (2016) have been reported that tissues showing primary embryo on the micropylar region was inoculated on full strength, hormone-free SH medium containing different sucrose concentration (SH07, SH10, and SH15) and gelled with 0.8per cent agar (Plant tissue culture grade, Hi-media) and incubated in complete darkness for production of secondary somatic embryo (SE).

The influence of sucrose concentration on induction of somatic embryogenesis has also been reported in other species (Chee and Tricoli 1988; Smith and Krikorian 1988; Eapen and George 1993; Weissinger II and Parrott 1993; Choi *et al.* 2002).

2.6.2 Solidifying agents

The most commonly and easily used gelling agent for preparing solid and semi solid plant tissue culture medium is agar. Agar having several advantages when compared to other gelling agents. When mixed with water, it forms a gel that melts at a temperature above 60°C and get solidified at 45°C which makes it stable at feasible incubation temperature. Another benefit of gelling agent is that it doesn't react with media constituents and are not in reaction plant enzymes. Firmness of the culture medium depends on the brand and concentration of agar (0.6 to 0.8 per cent) and the pH (5.7 to 5.8) (Cronauer and Krikorian, 1985; Doreswamy and Sahijram, 1989). Low pH causes acidification of the medium which in turn leads to hydrolysis of sucrose (Marchal, 1990).

Gelrite, is another solidifying agent available under the brand names Applied gel, Gellan and Phytagel. Also common gelling agent used in tissue

culture media. This synthetic agent may be used at concentration range of 1.25 to 2-5 g L^{-1}. Since gelrite provides a clear gel, it is easy to detect contaminations (Krikorian, 1982).

2.6.3 Plant Growth Regulators

The growth, development and morphogenesis of the *in vitro* grown plants are regulated by the interaction and balance between the growth regulators supplied to the medium as well as growth substance produced endogenously by the cultured cell. So, the selection and addition of growth regulators at optimum concentration are essential for successful plant tissue culture.

Root and shoot initiation, and the process of differentiation from unorganized callus tissue, are relatively regulated and controlled by the concentrations of auxins and cytokinin in the medium (Skoog and Miller, 1957; Ammirato *et al.*, 1983; Bajaj *et al.*, 1988; Rout and Das, 1997). Auxin: cytokinin ratio of approximately 10 yields rapid growth of undifferentiated callus, a ratio of approximately 100 favours root development and a ratio of approximately four favours shoot morphogenesis (Murashige, 1979). The gibberellins in medium enhance shoot elongation but not *in vitro* organogenesis (Gaba, 2005).

Ahmed *et al.*,(2011) reported that callogenesis was induced from the petiole explants of the black pepper on medium supplemented with different concentrations of several phytohormones (PGRs) and maximum callus induction was observed on MS-medium supplemented with 0.5 mg l-1 6 – benzyladenine (BA) after 4 weeks of culture. Also studied the effect of IBA on the root organpgenesis of blackpepper and maximum number of root was recorded on 2 mg/l of IBA supplemented medium.

Husain et al., 2011 studied the effect of various PGRs (Auxin and Cytokinin) for callogenesis of black pepper vine from leaf, stem & shoot tip explants and organogenesis from callus to produce disease free plants of *Piper nigrum* L. Babu *et al.*, 2012 reported that multiple shoots can be induced using

BA in the culture medium (MS or SH) either alone or in combination with other auxins. Addition of 1.0 mg/l of BAP in MS medium induced maximum number of shoot regeneration from nodal explants and addition of 1.5 mg/l IBA in to half strength MS medium induced maximum number of healthy root (Khan *et al.*, 2017)

Somatic embryogenesis uses a strong auxin such as 2, 4-D or any other auxin/ cytokinin in a definite concentration in the primary culture medium to support cell proliferation and embryogenesis. The proembryogenic mass within the culture containing auxins, is believed to synthesize all the gene necessary to complete the globular stage of the embryogenesis (Zimmerman, 1993).

2.7 CALLUS INDUCTION

Callus induction from the primary explants is the first step in many tissue culture experiments. The explants may be any plant tissues and structures and the callus is a wound tissue produced in response to injury. The concentration of plant growth regulators (auxin, cytokinins, giberellins, ethylene, etc.) is a major factor that controls callus formation in the culture medium. Concentrations of the plant growth regulators can vary for each plant species and can even depend on the source of the explant or individual plant genotype, age, nutritional status, etc. Culture conditions (temperature, solid media, agar solidified, light, etc.) are also important in callus formation and development. (Husain *et al.*, 2011).

MS medium supplemented with 1.0 mg l^{-1} BA induced 55 per cents of somatic embryos using cotyledon and leaf explants in *Capsicum baccatum* through calli (Venkataiah *et al., 2016*).

Yasudha *et al.*, 1985 reported that *Coffea arabica* leaf explants cultured on medium with 5 micro molar 6-benzyladenine produced white friable callus that lead to the production of somatic embryogenesis.

Embryogenic callus derived from zygotic embryos of black pepper (*Piper nigrum* Linn.) were induced to form somatic embryos on solid and liquid Schenk and Hildebrandt basal medium without growth regulators (Joseph *et al.*, 1996).

Manju and soniya (2012) reported the induction of embryogenic calli from the micropylar region of the black pepper variety panniyur 1 on MS and SH medium without any plant growth regulators under 24 h dark with 90 days of culture.

2.8 REGENERATION OF SOMATIC EMBRYOS

The cyclic somatic embryos of black pepper transferred to SH (liquid) medium with 3.5per cent (SH35) sucrose for further development of embryos into fully developed plantlets. Number of plantlets obtained from 100 mg of cyclic SE varied from variety to variety (Sasi and Bhat , 2016). Secondary somatic embryos showed a high per cent of germination (96.9) in liquid SH30 medium under 24 h darkness and germinated SEs converted in to plants at a rate of 86.3per cent. The highest number of embryos and plantlets was produced from cells grown as suspension cultures raised.

For the conversion of somatic embryos in to plantlets, germinated somatic embryos were grown under 16 h/d diffuse light on filter paper bridges dipped in static liquid SHS30 medium in test tubes (one germinated embryo per tube).The medium was replaced every 5 d by decanting consume medium and adding fresh medium. (Nair and Gupta, 2005).

2.9 HISTOLOGY

Ontogeny of SEs was studied by histological analysis at different stages of secondary embryogenesis. (Nair and Gupta, 2005).

For histological analysis, the PE explants along with developing SEs were fixed in 1:1:18 mixture of formalin–glacial acetic acid–50per cent ethanol (FAA) for 24 h, dehydrated in an ethanol-TBA (tertiary butyl alcohol) series and infiltrated with and embedded in paraffin (56–58◦C, Merck, India) Sections of 8-10 μm were cut using a Leica rotary microtome and stained with Heidenhain's iron–alum–haematoxylin (Johansen, 1940).

Materials and Methods

MATERIALS AND METHODS

The study on "Somatic embryogenesis in black pepper (*Piper nigrum* L.)" was conducted in the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2017. Details of the experimental materials used and the methodology followed in this work are presented in this chapter.

3.1 COLLECTION OF PLANT SAMPLE

Black pepper plants of variety Panniyur-5 were collected from College of Agriculture Padannakkad, Krishi Vikhyan Kendra (KVK), Kannur and Pepper Research Station (PRS), Panniyur. The plants were grown in the Instructional Farm of College of Agriculture, Vellayani. Explants namely leaves, nodes and shoot tips were selected from healthy and disease free plants. Ripened seeds were collected from PRS, Panniyur and KVK, Kannur.

3.2 IN VITRO CULTURE OF BLACK PEPPER

3.2.1 Sterilization of glassware and equipments

All operations for *in vitro* culture were carried out inside a laminar air flow cabinet under aseptic conditions using sterilized plant materials, equipments, glass ware and chemicals. A horizontal laminar flow cabinet with HEPA filter (0.2μ) was used. The hood surface was cleaned by paper towel soaked in 70 per cent ethanol and sterilized by germicidal ultraviolet light for at least 20 min prior to use. Before using the accessories like test tubes, flasks, blade holders, petri plates, bottles etc they were washed and dried in oven. The dried glass wares were wrapped in polypropylene bags and were autoclaved at 6.80 kilogram pressure at 121 ^{0}C temperature for 40 minutes. Surgical instruments like scalpel, forceps, and scissors were sterilized by dipping in 100 per cent ethyl alcohol and flaming prior to use.

3.2.2 Culture Room

The explants were incubated in a culture room where the temperature was maintained at 25-26°C, humidity at 60-70per cent and either under continuous dark or under a photoperiod of 16 h light and 8 h dark

3.3 SURFACE STERILIZATION OF EXPLANTS

3.3.1 Leaf, stem, node

The explants were washed thoroughly with tap water and then with distilled water. They were treated with soap solution (Labolin) for 30 min, followed by washing with distilled water. Explants were treated with 0.3 per cent Bavistin solution for 30 min, followed by washing with distilled water. And then surface sterilization was done with 0.08 per cent mercuric chloride ($HgCl_2$) for 7 minutes followed by rinsing with double distilled water for 3-4 times to remove the traces of mercuric chloride. Then explants were pretreated using PVP, ascorbic acid, $CuSO_4$ and cefotaxime. Then the explants were cut with sterile scalpel and were inoculated on Murashige and Skoog's medium as well as SH medium with different concentrations and combinations of auxins and cytokinins.

3.3.1.1 Pretreatment of explants (leaf, shoot tip, and node)

Table1. Pre treatment procedures followed for *ex vitro* raised explants

Treatment	Protocol
PVP	Explants were pretreated in PVP 50,100,250 and 300 mgl^{-1} for 10,20,50,and1 hour
Ascorbic acid	Explants were soaked in 20,50,100,200,250 mgl^{-1} for 10,30, 50 minutes and 1 hour minutes
Cefotaxime	100,300,600 mgl^{-1} for 30 minutes,1 hour,3 hour and 2 hours

By observing the per cent reduction in contamination and polyphenolics, most suited one is selected and used for the initiation of callusing.

3.3.2 Seeds as explants

Fully ripened seeds were used as explants. Seeds were soaked in tap water for overnight and on the next day itself outer mesocarp of the seed were removed by slight rubbing with hand before surface sterilization. Then seeds were surface sterilized with 0.1per cent mercuric chloride solution for 5 min, followed by repeated washing (3-4 times) with sterile double distilled water. The surface sterilized seeds were allowed to dry on sterile filter paper in laminar air flow for 30 min. Then with a sterile scalpel were inoculated on agar gelled full-strength, hormone-free SH and MS and also on with media contains different concentration of growth regulators.

3.3.2.1 Pre treatment of seeds

Table2. Quantity and time period of GA_3 used

Treatments	**Concentration of GA_3**	**Time**
T1	GA_3 20 ,50,100 and 500 ppm	10 minutes
T2	GA_3 20 ,50,100 and 500 ppm	20 minutes
T3	GA_3 20 ,50,100 and 500 ppm	40 minutes
T4	GA_3 20 ,50,100 and 500 ppm	1 hour
T6	GA_3 20 ,50,100 and 500 ppm	2 hour
T7	GA_3 20 ,50,100 and 500 ppm	5 hour
T8	GA_3 20 ,50,100 and 500 ppm	24 hours

Days taken for the germination of seed in each treatment were recorded. *In vitro* germinated plants were used as explants for the initiation of callusing.

3.4 PREPARATION OF CULTURE MEDIUM

MS (Murashige and Skoog, 1962) and SH (Schenk and Haberland, 1972) media were used as basal media for seed germination, callus induction, callus multiplication, embryo germination and growth. The formulation and composition of MS and SH medium is given below (Apendix I and II)

3.5 PREPARATION OF STOCK SOLUTION

Stock solutions of the major components, such as macronutrients, micronutrients, vitamins, and plant growth regulators of the media were prepared and stored in refrigerator.

Stock solutions of MS and SH media were prepared as per the details given in the Apendix I and II.

3.5.1 Growth regulators

Auxin and cytokinins were the two major phytohormones used in different concentrations and combinations in various medium for induction of callus, somatic embryos and their further development.

2,4 Dichlorphenoxy acetic acid (2,4-D) 200 mg L^{-1} was weighed and dissolved in 1 ml of 90 per cent ethyl alcohol by making up the volume to 100 ml with double distilled water.

Indole-3-Acetic Acid (IAA) 200 mg L^{-1} was weighed and dissolved in 1 ml of 90 per cent alcohol by making up the volume to 100 ml with double distilled water.

Benzyle Amino Purine (BAP) 200 mg L^{-1} was weighed and dissolved in 1 ml of 1 N NaOH by making up the volume to 100 ml with double distilled water.

Picloram 200 mg L^{-1} was weighed and dissolved in 1 ml of 1 N NaOH by making up the volume to 100 ml with double distilled water.

NAA 200 mg L^{-1} was weighed and dissolved in 1 ml of 1 N NaOH by making up the volume to 100 ml with double distilled water.

3.6 PREPARATION OF MEDIA

3.6.1 Preparation of SH medium

For the preparation of SH medium, required volumes of stock solutions were added to the distilled water one by one. Then sucrose 30 gl^{-1} was added and dissolved. Required amount of growth regulators are also added. The pH of the solution was adjusted to 5.9 using 0.1 N NaOH and 0.1 N HCl. Then the volume of the solution was made up to 1 liter. Then 0.43 per cent gelrite or 0.63 per cent agar was added and dissolved by heating. Prepared medium were poured into bottles and autoclaved for 20 min at 15 lb pressure at 121 ^{0}C. Then the media were cooled and kept at room temperature for almost one week for checking the proper setting as well as knowing the contamination. Then the explants were inoculated in to the medium. Observation was made at correct intervals. After 3 weeks sub culturing were done in order to ensure presence of nutrients all the time.

3.62 Preparation of MS medium

For preparing the MS media, working solutions were pipetted out from the stock solutions and added to the distilled water (800 ml) one by one (Table 1). Then sucrose 30 g L^{-1} and inositol 100 mg L^{-1} were added and dissolved. The pH of the solution was adjusted to 5.7 either with 0.1.N HCl or 0.1.N NaOH and the volume was made up to 1 1itre. 0.63 per cent agar was added and dissolved by heating. The prepared medium was dispended into bottles and autoclaved at 15 lb pressure at 121^{0}C for 15 min. Then the medium was cooled to room temperature and kept for a week to check for contamination and then inoculated with explants.

Observations were recorded at different time intervals. After 3 weeks, sub-culturing was done to replenish nutrients to the growing tissues.

3.7 CALLUS INDUCTION

MS (Murashige and Skoog's) and SH (Schenk Hildebrandt) media supplemented with different concentrations of plant growth regulators were used for callus induction experiment. Photoperiod was adjusted to 16 hours light and 8 hours dark or continuous dark as per the requirement. The cultures were kept at 25 ± 2 ^{0}C temperatures and 60 per cent humidity. Time taken for callus induction and callus induction per cent were recorded.

Table3. Growth regulators used for callus induction in MS and SH medium with 3per cent sucrose, explants used were leaf, shoot tip and stem node (*ex vitro*)

Treatment	**PGR conc. (mg L^{-1})**					
	2,4-D	**BA**	**IAA**	**NAA**	**TDZ**	**Picloram**
T1	0.5	-	-	-	-	-
T2	1.0	0.5	-	-	-	-
T3	-	1.0	-	-	-	-
T4	-	1.5	-	-	-	-
T5	-	2.0	-	-	-	-
T6	-	-	1.0	-	-	-
T7	-	-	2.0	-	-	-
T8	-	1.0	1.0	-	-	-
T9	-	1.0	1.5	-	-	-
T10	-	2.0	1.0	-	-	-
T11	-	2.0	1.5	-	-	-

T12	-	-	-	1.0	-	-
T13	-	-	-	1.5	-	-
T14	-	-	-	2.0	-	-
T15	-	-	-	-	0.5	-
T16	-	-	-	-	1.0	-
T17	-	-	-	-	-	1
T18	-	-	-	-	-	1.5
T19	-	-	-	-	-	2
T20	-	1	-	-	-	1
T21	-	1.5	-	-	-	1
Control	-	-	-	-	-	-

Table4. Growth regulators used for callus induction in MS medium with 3per cent sucrose, explants used were leaf, shoot tip and stem node (*in vitro*)

Treatments	**PGR conc. (mg L^{-1})**					
	2, 4-D	**BA**	**IAA**	**Picloram**	**NAA**	**TDZ**
T1	0.5	-	-	-	-	-
T2	1.0	-	-	-	-	-
T3	-	0.5	-	-	-	-
T4	-	1.0	-	-	-	-
T5	-	1.5	-	-	-	-
T6	-	2.0	-	-	-	-
T7	0.5	1.0	-	-	-	-
T8	0.5	1.5	-	-	-	-
T9	0.5	2.0	-	-	-	-
T10	1	0.5	-	-	-	-

T11	1	1	-	-	-	-
T12	1	1.5	-	-	-	-
T13	-	0.5	0.5	-	-	-
T14	-	0.5	1.0	-	-	-
T15	-	0.5	1.5	-	-	-
T16	-	0.5	2.0	-	-	-
T17	-	1	0.5	-	-	-
T18	-	1	1	-	-	-
T19	-	1	1.5	-	-	-
T20	-	1	2.0	-	-	-
T21	-	1.5	0.5	-	-	-
T22	-	1.5	1	-	-	-
T23	-	1.5	1.5	-	-	-
T24	-	1.5	2	-	-	-
T25	-	2	0.5	-	-	-
T26	-	2	1	-	-	-
T27	-	2	1.5	-	-	-
T28	-	2	2	-	-	-
T29	-	-	-	-	0.5	-
T30	-	-	-	-	0.1	-
T31	-	-	-	-	1.5	-
T32	-	-	-	-	2	-
T33	-	0.5	-	-	0.5	-
T34	-	0.5	-	-	1	-
T35	-	0.5	-	-	1.5	-
T36	-	0.5	-	-	2	-
T37	-	1	-	-	0.5	-
T38	-	1	-	-	1	-
T39	-	1	-	-	1.5	-
T40	-	1	-	-	2	-

T41	-	1.5	-	-	0.5	-
T42	-	1.5	-	-	1	-
T43	-	1.5	-	-	1.5	-
T44	-	1.5	-	-	2	-
T45	-	2	-	-	0.5	-
T46	-	-	-	-	-	0.5
T47	-	-	-	-	-	1
T48	-	-	-	0.5	-	0.5
T49	-	-	-	1	-	0.5
T50	-	-	-	0.5	-	-
T51	-	-	-	1	-	-
T52	-	-	-	1.5	-	-
T53	-		-	2	-	-
T54	-		-	4	-	-
T55	-	0.5	-	1	-	-
T56	-	1	-	1	-	-
T57	-	2	-	1	-	-
T58	-	1	-	1.5	-	-
T59	-	2	-	1.5	-	-
T60	-	1	-	2	-	-
T61	-	-	-	-	-	-

Table5. Growth regulators used for callus induction in SH medium with 3per cent sucrose, explants used were leaf, shoot tip and stem node *(In vitro)*

Treatments	PGR conc. (mg L^{-1})					
	2, 4-D	**BA**	**IAA**	**Picloram**	**NAA**	**TDZ**
T1	0.5	-	-	-	-	-
T2			-	-	-	-
T3	-	0.5	-	-	-	-
T4	-	1.0	-	-	-	-
T5	-	1.5	-	-	-	-
T6	-	2.0	-	-	-	-
T7	0.5	1.0	-	-	-	-
T8	0.5	1.5	-	-	-	-
T9	0.5	2.0	-	-	-	-
T10	1	0.5	-	-	-	-
T11	1	1	-	-	-	-
T12	1	1.5	-	-	-	-
T13	-	0.5	0.5	-	-	-
T14	-	0.5	1.0	-	-	-
T15	-	0.5	1.5	-	-	-
T16	-	0.5	2.0	-	-	-
T17	-	1	0.5	-	-	-
T18	-	1	1	-	-	-
T19	-	1	1.5	-	-	-
T20	-	1	2.0	-	-	-
T21	-	1.5	0.5	-	-	-
T22	-	1.5	1	-	-	-
T23	-	1.5	1.5	-	-	-
T24	-	1.5	2	-	-	-
T25	-	2	0.5	-	-	-
T26	-	2	1	-	-	-

T27	-	2	1.5	-	-	-
T28	-	2	2	-	-	-
T29	-	-	-	-	0.5	-
T30	-	-	-	-	0.1	-
T31	-	-	-	-	1.5	-
T32	-	-	-	-	2	-
T33	-	0.5	-	-	0.5	-
T34	-	0.5	-	-	1	-
T35	-	0.5	-	-	1.5	-
T36	-	0.5	-	-	2	-
T37	-	1	-	-	0.5	-
T38	-	1	-	-	1	-
T39	-	1	-	-	1.5	-
T40	-	1	-	-	2	-
T41	-	1.5	-	-	0.5	-
T42	-	1.5	-	-	1	-
T43	-	1.5	-	-	1.5	-
T44	-	1.5	-	-	2	-
T45	-	2	-	-	0.5	-
T46	-	-	-	-	-	0.5
T47	-	-	-	-	-	1
T48	-	-	-	0.5	-	0.5
T49	-	-	-	1	-	0.5
T50	-	-	-	0.5	-	-
T51	-	-	-	1	-	-
T52	-	-	-	1.5	-	-
T53	-	-	-	2	-	-
T54	-	-	-	4	-	-
T55	-	0.5	-	1	-	-
T56	-	1	-	1	-	-

T57	-	2	-	1	-	-
T58	-	1	-	1.5	-	-
T59	-	2	-	1.5	-	-
T60	-	1	-	2	-	-
T61	-	-	-	-	-	-

3.7.1 Callus induction frequency

From the obtained Callus, callus induction frequency was calculated by using the formula,

Callus induction frequency= (No. of explants producing callus x 100/ No. of explanted inoculated)

Calli were transferred to the embryo induction medium for getting somatic embryos

Table6. Combinations of growth regulators and media used for regeneration of embryos from the callus (Basal medium: MS+3per cent sucrose)

Treatments	**PGR conc. (mg L^{-1})**			
	BA	IAA	2-4,D	Picloram
T1	-	-	-	-
T2	1	-	-	-
T3	1.5	-	-	-
T4	2	-	-	-
T5	4	-	-	-
T6	-	1		-
T7	-	1.5	-	-
T8	-	2	-	-

T9	-	4	-	-
T10	1	1	-	-
T11	1	2	-	-
T12	2	1	-	-
T13	2	2	-	-
T14	-	-	1	-
T15	-	-	1.5	-
T16	-	-	2	-
T17	-	-	-	1
T18	-	-	-	1.5
T19	-	-	-	2
T20	-	-	-	4
T21	1	-	-	1
T22	1	-	-	1.5
T23	1	-	-	2
T24	2	-	-	1
T25	2	-	-	1.5
T26	2	-	-	2

Table7: Combinations of growth regulators and media used for regeneration of embryos from the callus (Basal medium: SH+3per centsucrose)

Treatments	**PGR conc. (mg L^{-1})**			
	BA	IAA	2-4,D	Picloram
T1	-	-	-	-
T2	0.5	-	-	-
T3	1	-	-	-
T4	1.5	-	-	-
T5	2	-	-	-
T6	-	-	-	1
T7	-	-	-	1.5
T8	-	-	-	2
T9	-	-	-	4
T10	1	-	-	1
T11	1	-	-	1.5
T12	1	-	-	2
T13	2	-	-	1
T14	2	-	-	1.5
T15	2	-	-	2

Embryo regeneration frequency from callus was calculated by using the formula

$$\text{Embryo induction frequency} = \frac{(\text{No. of explants producing somatic embryo} \times 100)}{\text{No. of explants inoculated}}$$

3.8 SEED INOCULATION

Fully ripened seeds after sterilization were inoculated in both MS and SH medium with or without of growth regulators.

Table8. Combination of growth regulators and medium used for induction of somatic embryos from seeds (Basal medium: SH with different concentration of sucrose)

Treatments	**Media**		**PGR conc. (mg L^{-1})**		
		BA	Picloram	IAA	2,4-D
T1	SH+3% sucrose	-	-	-	-
T2	SH+1.5% Sucrose	-	-	-	-
T3	SH+ 4.5% Sucrose	-	-	-	-
T4	½ SH+3% sucrose	-	-	-	-
T5	SH+3% sucrose	-	1	-	-
T6	SH+3% sucrose	-	1.5	-	-
T7	SH+3% sucrose	-	2	-	-
T8	SH+3% sucrose	1	1	-	-
T9	SH+3% sucrose	1	1.5	-	-
T10	SH+3% sucrose	1	2	-	-
T11	SH+3% sucrose	2	1	-	-
T12	SH+3% sucrose	2	1.5	-	-
T13	SH+3% sucrose	2	2	-	-
T14	SH+3% sucrose	1	-	1	-
T15	SH+3% sucrose	1	-	1.5	-
T16	SH+3% sucrose	1	-	2	-
T17	SH+3% sucrose	2	-	1	-
T18	SH+3% sucrose	2	-	1.5	-
T19	SH+3% sucrose	2	-	2	-
T20	SH semi solid+3%	-	-	-	-

	sucrose				
T21	Liquid SH+3% sucrose	-	-	-	-

Table9. Combination of growth regulators and medium used for induction of somatic embryos from seeds (Basal medium: MS with different concentration of sucrose)

Treatments	**Media**	**PGR conc. (mg L^{-1})**			
		BA	Picloram	IAA	2,4-D
T1	MS+3% sucrose	-	-	-	-
T2	MS+1.5% Sucrose	-	-	-	-
T3	MS+4.5% Sucrose	-	-	-	-
T4	MS+3% sucrose	-	1	-	-
T5	MS+3% sucrose	-	1.5	-	-
T6	MS+3% sucrose	-	2	-	-
T7	MS+3% sucrose	1	1	-	-
T8	MS+3% sucrose	1	1.5	-	-
T9	MS+3% sucrose	1	2	-	-
T10	MS+3% sucrose	2	1	-	-
T11	MS+3% sucrose	2	1.5	-	-
T12	MS+3% sucrose	2	2	-	-
T13	MS+3% sucrose	-	-	-	0.5
T14	MS+3% sucrose	-	-	-	1
T15	MS+3% sucrose	-	-	-	1.5
T16	MS+3% sucrose	-	-	-	2
T17	MS+3% sucrose	-	-	-	4
T18	MS+3% sucrose	1	-	1	-
T19	MS+3% sucrose	1	-	1.5	-

T20	MS+3% sucrose	1	-	2	-
T21	MS+3% sucrose	2	-	1	-
T22	MS+3% sucrose	2	-	1.5	-
T23	MS+3% sucrose	2	-	2	-
T24	MS+3% sucrose	4	-	4	-
T25	MS semi solid+3% sucrose	-	-	-	-
T26	Liquid M+3% sucrose S	-	-	-	-

Embryos germinated from the micropylar region of the seed were transferred in to different medium for further multiplication and growth of the embryos

Table10. Combination of growth regulators and media used for the multiplication and further development of the somatic embryos (Basal medium: SH with different concentration of sucrose)

Treatments	**Media**	**PGR conc. (mg L^{-1})**	
		BA	Picloram
T1	SH+1.5% Sucrose	-	-
T2	SH+4.5% Sucrose	-	-
T3	½ SH+3% sucrose	-	-
T4	SH+3% sucrose	-	-
T5	Liquid SH+3%sucrose	-	-
T6	Liquid SH+3.5%sucrose	-	-
T7	Liquid SH+4%sucrose	-	-
T8	Semi solid SH+3%	-	-

	sucrose		
T9	SH+3% sucrose	1	1
T10	SH+3% sucrose	1	1.5
T11	SH+3% sucrose	1	2
T12	SH+3% sucrose	2	4
T13	SH+3% sucrose	1.5	1
T14	SH+3% sucrose	1.5	1.5
T15	SH+3% sucrose	1.5	2
T16	SH+3% sucrose	3	4
T17	SH+3% sucrose	2	1
T18	SH+3% sucrose	2	1.5
T19	SH+3% sucrose	2	2
T20	SH+3% sucrose		1
T21	SH+3% sucrose	-	1.5
T22	SH+3% sucrose	-	2

Table11. Combination of growth regulators and media used for the multiplication and further development of the somatic embryos (Basal media: MS with different concentration of sucrose)

Treatments	**Media**	**PGR conc. (mg L^{-1})**	
		BA	Picloram
T1	MS+3% sucrose	-	-
T2	MS+1.5% Sucrose	-	-
T3	MS+4.5% Sucrose	-	-
T4	Liquid MS+3% sucrose	-	-
T5	Semi solid MS+3% sucrose	-	-
T6	MS+3% sucrose	1	1
T7	MS+3% sucrose	1	1.5
T8	MS+3% sucrose	1	2
T9	MS+3% sucrose	2	4
T10	MS+3% sucrose	1.5	1
T11	MS+3% sucrose	1.5	1.5
T12	MS+3% sucrose	1.5	2
T13	MS+3% sucrose	3	4
T14	MS+3% sucrose	2	1
T15	MS+3% sucrose	2	1.5
T16	MS+3% sucrose	2	2
T17	MS+3% sucrose	-	1
T18	MS+3% sucrose	-	1.5
T19	MS+3% sucrose	-	2
T20	MS+3% sucrose	-	4

3.9 HISTOLOGY

Histology of the obtained somatic embryo was done at different stages of the growth for confirming the somatic embryo. For this, serial sectioning procedure developed by Sharma and Sharma, 1980 was used.

Steps

1. Fixation in FAA (formaldehyde-acetic acid-ethanol)

   FAA (200 ml)

   - 100 ml 95 per cent ethanol
   - 70 ml dH_2O
   - 20 ml 37per cent formaldehyde solution
   - 10 ml glacial acetic acid

Tissues were cut using a scalpel in to pieces and immerse in cold fixative. Fixation was done in 4^0C for overnight.

2. Dehydration in series of tert-butanol (TBA)

   Dehydrating solution 1 to 8 for 100 ml each was prepared. Tissues to be sectioned were transferred in to each solution for dehydrating for 4 hours

   - 10 ml TBA,40ml 95per cent ethanol,50 ml dH_2O
   - 20 ml TBA,50ml 95per cent ethanol,30 ml dH_2O
   - 35 ml TBA,50ml 95per cent ethanol
   - 55 ml TBA,45 ml 95per cent ethanol
   - 75 ml TBA, 25 ml 95per cent ethanol
   - 100 ml TBA (added one drop of eosin to make the sectioning easier)
   - 100 ml TBA
   - 100 ml TBA

3. Transferred the tissues in to the solution containing liquid paraffin and TBA (1:1) for one hour.
4. Molten wax was prepared and kept in oven at 56-60°C.Transfered the tissues in to the molten wax for 4 hours with 3 changes.
5. Casting wax block

Molten paraffin wax with ceresin was prepared and kept in the oven set at 56-60°C.This molten wax is poured in to casting tray or paper boat and the tissue were transferred in an order in the tray using forceps through flame ensured that bottom layer of wax has solidified and fixed the sample in place. These were done by placing the casting tray on hotplate. After solidifying transferred the mould in to cold water and ice and left it for 10 minutes for complete solidification. Then removed the wax block from the mould and kept in room temperature for further uses.

6. Sectioning

   Trimmed the wax block around the sample with a single edged blade so that the upper and lower edges are parallel and the left and right edges are at an angle, also the lower edges of the block is longer than the upper.

   Trimmed wax was fixed with the sample in a sample holder on microtome. Set the thickness of the section to 10-12 micrometers and cutting angle to 7 degrees. The first few sections were not good. They were removed using a small brush away from the blade. When the surface of the sample evens, started the ribbon formation. Ribbons were fixed on the slide with adhesive.

7. Staining

   Prepared slide passed through a series of xylene and alcohol for the removal of wax and also part of dehydration. After proper drying 1 drop of toludine blue has poured on the slide uniformly covering the entire ribbon on the slide. Then washed off the excess stain using water and pass through the series of xylene and alcohol.

8. Mounting

   Slide containing stained tissues mounted properly using thin cover slip by mounting media.

9. Permanent slides were observed under compound microscope

3.10 HARDENING

In vitro grown plants were thoroughly washed in tap water to remove agar and medium. Old leaves were pinched off. Transplanting was done into small pots with autoclaved sand and coir pith compost. Humidity was maintained around plants. Plants were irrigated 2-3 times during the first 6 days with half strength MS in organic salts. After 10 days plants were acclimatized for natural conditions.

Results

RESULTS

The study entitled "Somatic embryogenesis in black pepper (*Piper nigrum* L.)" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015 to 2017. The results of the study are presented in this chapter.

4.1 ESTABLISHMENT OF *IN VITRO* CULTURE OF BLACK PEPPER

4.1.1 Surface Sterilization

Mercuric chloride and sodium hypochlorite were used for surface sterilization of black pepper explants. The explants from field grown plants were pretreated with ascorbic acid, polyvinylpyrolidone (PVP), cefotaxime and bavistin.

Surface sterilization was found to be most effective when leaves, node and shoot tips were pretreated with Bavistin (0.3 per cent) for 30 min, followed by treatment with Mercuric chloride (0.08 per cent) for 7 minutes.

Surface sterilization of seed was found to be effective when the seeds after removing the outer mesocarp were treated with Bavistin 0.4 per cent for 30 minutes and then by mercuric chloride 0.08 per cent for 10 minutes.

4.1.2 Pretreatment of the *ex vitro* raised explants (Leaf, shoot tip and node)

Different concentration of ascorbic acid, PVP and cefotaxime were tried for reducing the microbial as well as phenolic exudates (4replication each). The pretreatment with 250 mg L^{-1} of ascorbic acid for 50 minutes reduced the browning per cent to 43.75 per cent in leaf tissue and 37.5 per cent in shoot tip and node respectively. Ascorbic acid at 200 mg L^{-1}, reduced the per cent reduction in browning to 37.5per cent in leaf tissues and further decrease of concentration to 100 mg L^{-1}, decreased the per cent reduction in browning to 25per cent in shoot tip and node (Table 12). Pre treatment with PVP (Table 13) 50 mg L^{-1} for 1 hour was found to give the maximum reduction in the phenolic exudates in all the explants. It showed 43.75 per cent reduction in browning from leaf.

Table12. Effect of Ascorbic acid on Phenolic oxidation from explants taken field grown plants

Ascorbic acid mg L^{-1}	Time	Percentage reduction in browning		
		Leaf	Shoot tip	Node
0	10 minutes	0.00	0.00	0.00
20		0.00	0.00	0.00
50		0.00	0.00	0.00
100		0.00	0.00	0.00
200		0.00	0.00	0.00
250		0.00	0.00	0.00
20	30 min	0.00	0.00	0.00
50		0.00	0.00	0.00
100		0.00	0.00	0.00
200		0.00	0.00	0.00
250		0.00	0.00	0.00
20	50 min	0.00	0.00	0.00
50		0.00	0.00	0.00
100		37.50	25.00	25.00
200		37.50	37.50	37.50
250		43.75	37.50	37.50
20	1h	0.00	0.00	0.00
50		0.00	0.00	0.00
100		43.75	18.75	18.75
200		25.00	25.00	18.75
250		18.75	25.00	18.75

tissues and shoot tip and 50 per cent reduction in node. By reducing the time period to 50 min, reduction in browning per cent was noted (25.00per cent, 37.50per cent and 37.50per cent in leaf, shoot tip and node respectively). When the concentration was increased (100,200 and 300 mg L^{-1}) by maintaining the time period as 1 h, also reduced the browning per cent (Table13).

For reducing the microbial load different concentrations of cefotaxime were tried for different periods of time. Maximum per cent reduction in contamination was observed while using cefotaxime at 300 mg L^{-1} for 1 hour. It reduced the per cent of contamination to 56.20 per cent in leaf, 50 per cent in shoot tip and 18.75 per cent in node. When the time of incubation was increased to 2 h, per cent reduction in contamination was decreased to 12.5per cent in leaf, 6.20per cent in shoot tip (Table 14).

4.1.3 Effect of GA_3 on seed germination of black pepper

When different concentration of GA_3 was tried for different time period before inoculation, difference in germination time was observed. While untreated seeds (control) took 73 days for germination, seeds treated with 100 mg L^{-1} of GA_3 for 10 minutes took only 44 days for germination (Table 15). At 50 mg L^{-1}, days taken for germination increased with the increase of time. GA_3 below 50 mgL^{-1} did not have any effect on seed germination. GA_3 at 500 mg L^{-1} prolonged the germination time to105 days. In all the treatments, increase in treatment time above 10min delayed the germination. When the time of GA_3 treatment was increased to 24 hours, there was no germination and the micropylar region of the seed became black.

4.2 CALLUS INITIATION

Twenty one treatments with different combinations of 2, 4-D, BA, IAA, NAA, TDZ and picloram in MS and SH media were tried to study their effect on callus induction from leaves, nodes and shoot tips from *ex vitro* raised plants.

For callus induction from leaves, nodes and shoot tips from *in vitro* raised seedlings, sixty one treatments with different combinations of 2, 4-D, BA, picloram, NAA, TDZ and IAA in MS and SH medium were tried.

Table13. Effect of PVP on polyphenolic oxidation from explants taken field grown explants

PVP mg L^{-1}	Time	Percentage reduction in browning		
		Leaf	Shoot tip	Node
20	10 min	0.00	0.00	0.00
50		0.00	0.00	0.00
100		0.00	0.00	0.00
200		0.00	0.00	0.00
300		0.00	0.00	0.00
20		0.00	0.00	0.00
50		0.00	0.00	0.00
100	20 min	0.00	0.00	0.00
200		0.00	0.00	0.00
300		0.00	0.00	0.00
20	40 min	0.00	0.00	0.00
50		0.00	0.00	0.00
100		0.00	0.00	0.00
200		0.00	0.00	0.00
300		0.00	0.00	0.00
20	50 min	12.50	12.50	18.75
50		12.50	12.50	12.50
100		25.00	37.50	37.50
200		25.00	37.50	43.75
300		25.00	37.50	43.75
20	1h	37.50	37.50	43.75

50		43.75	50.00	50.00
100		25.00	37.50	37.50
200		31.25	25.00	25.00
300		25.00	12.50	18.75

Table14. Effect of Cefotaxime on contamination from explants taken field grown explants

Cefotaxime mg L^{-1}	Time	% reduction in contamination		
		Leaf	Shoot tip	Node
100	30 min	12.50	6.20	0.00
300		43.75	6.20	6.20
600		43.75	12.50	6.20
100	1 h	50.00	43.75	6.20
300		56.20	50.00	18.75
600		50.00	43.75	18.75
100	2 h	12.50	6.20	6.20
300		12.50	6.20	0.00
600		6.20	0.00	0.00

Table15. Effect of different concentration and time period of GA_3 on seed germination of black pepper

Concentration of GA_3 mg L^{-1}	**Days taken for germination in different time period**					
	10 minutes	**20 minutes**	**1 hour**	**2 hour**	**5 hour**	**24 hour**
20	73	73	66	66	90	-
50	63	67	66	69	105	-
100	44	59	71	72	103	-
500	105	107	110	112	-	-
Control	75	-	-	-	-	-

4.2.1 Initiation of callus in SH medium from nursery raised explants

SH medium supplemented with 1 mg $L^{-1}$of picloram showed the highest callus induction (28.50 per cent) in all the three explants (Table 16). Treatment with 1.5 mg $L^{-1}$of picloram also showed similar response. 28.5per cent callus induction from leaf, shoot tip and node, which was on par with the treatment containing 1.5 mg L^{-1} BA, on leaf and shoot tip.

2 mg L^{-1} of picloram did not induce any callusing. Combination of picloram with BA (1 mg L^{-1} each) produced 12.5 per cent callus induction. Similar results were observed BA in combination with 2, 4-D. Treatment contains 0.5 mg L^{-1} 2, 4-D produced 12.5 per cent callus induction from leaf tissue and no callus was formed in shoot tip and node .When the concentration of BA was increased to1 mg L^{-1} along with 0.5 mg L^{-1} of IAA, 12.50 per cent callus was induced in leaf and shoot tip and no callus was induced in node. While trying with BA 1mg L^{-1}, a uniform callus induction frequency of 12.50 per cent was found. When 1 mg L^{-1} IAA was provided, 6.25 per cent of callusing was noticed in leaves. When the concentration was increased to2 mg L^{-1} no callusing was observed.

With a combination of BA and IAA (1 mg L^{-1}each), 6.25 per cent of callusing was observed in leaf and node. With the increase of concentration of IAA to1.5 mg L^{-1} along with 1 mg L^{-1} of BA, 12.5 per cent callusing in leaf and 6.25per cent of callusing in shoot tip were obtained. The remaining combination of BA and IAA did not show any positive response towards callusing in any of the explants tried.

Among the different combinations of NAA tried, the medium supplemented with1.5 mg L^{-1} of NAA showed 6.25per cent callus induction.

When the SH medium was supplemented with 0.5 mg L-1 of TDZ 12.5 per cent callusing was observed in leaf tissues, 6.25 per cent in shoot tip and 6.25 per cent in node. While increasing the concentration to 1 mg L^{-1}, no callusing was observed.

With the combination of BA and picloram, treatment T20 showed maximum callus formation of 12.5 per cent, while this was decreased to 6.25 per cent in treatment T21 (Plate 1) (Table 16).

Table16. Effect of different plant growth regulators on callus induction from field grown explants in SH medium

Treatment	Growth regulators (mg L^{-1})						Percentage of callus induction		
	2,4-D	BA	IAA	NAA	TDZ	Picloram	Leaf	Shoot	Node
T1	0.5	-	-	-	-	-	12.50	6.25	0.00
T2	1.0	0.5	-	-	-	-	12.50	12.50	0.00
T3	-	1.0	-	-	-	-	56.25	43.75	12.50
T4	-	1.5	-	-	-	-	0.00	0.00	0.00
T5	-	2.0	-	-	-	-	0.00	0.00	0.00
T6	-	-	1.0	-	-	-	6.25	0.00	0.00
T7	-	-	2.0	-	-	-	0.00	0.00	0.00
T8	-	1.0	1.0	-	-	-	6.25	0.00	6.25
T9	-	1.0	1.5	-	-	-	12.50	0.00	0.00
T10	-	2.0	1.0	-	-	-	0.00	0.00	0.00
T11	-	2.0	1.5	-	-	-	0.00	0.00	0.00
T12	-	-	-	1.0	-	-	0.00	0.00	0.00
T13	-	-	-	1.5	-	-	6.25	0.00	0.00
T14	-	-	-	2.0	-	-	0.00	0.00	0.00
T15	-	-	-	-	0.5	-	12.50	6.25	6.25
T16	-	-	-	-	1.0	-	0.00	0.00	12.50
T17	-	-	-	-	-	1.0	28.50	28.50	28.50
T18	-	-	-	-	-	1.5	12.5	12.5	12.5
T19	-	-	-	-	-	2.0	0.00	0.00	0.00
T20	-	1.0	-	-	-	1.0	12.5	12.5	12.5
T21	-	1.5	-	-	-	1.0	6.25	6.25	6.25

4.2.2 Initiation of callusing in MS medium from nursery raised explants

MS medium supplemented with 1 mg L^{-1} of picloram showed the highest callus induction per cent (37per cent) in leaf, followed by (28.5per cent) shoot tip and node in (Table 17). Treatment with 1.5 mg L^{-1} of picloram showed 28.5per cent callus induction in leaf, shoot tip and node, which was on par with the treatment containing 1.5 mg L^{-1} BA, on leaf and shoot tip.

With 0.5 mg L^{-1} 2, 4-D, callus formation was observed from leaf and shoot tip as 12.5 per cent and 6.25 per cent respectively. When 1 mg L^{-1} of 2, 4-D was supplemented along with 0.5 mgl^{-1} of BA, 12.5 per cent of callus induction was observed.

With 1 mg L^{-1} of BA, callus induction was 12.5 percent from leaf tissue. Beyond1.5 mg L^{-1} both BA and picloram did not show any response. While trying with1 mg L^{-1} of IAA 6.25per cent of callus induction was recorded and no callusing was induced with 2 mg L^{-1} of IAA.

When BA and IAA were used in combination, 6.25 per cent of callusing was noticed from leaf and node with 1 mg L^{-1}each of BA and IAA. With the increase of concentration of IAA in to1.5 mg L^{-1}, callus induction per cent of 12.5 per cent in leaf and 6.25 per cent in shoot tip were recorded. Callusing was not recorded in any other combination of BA and IAA.

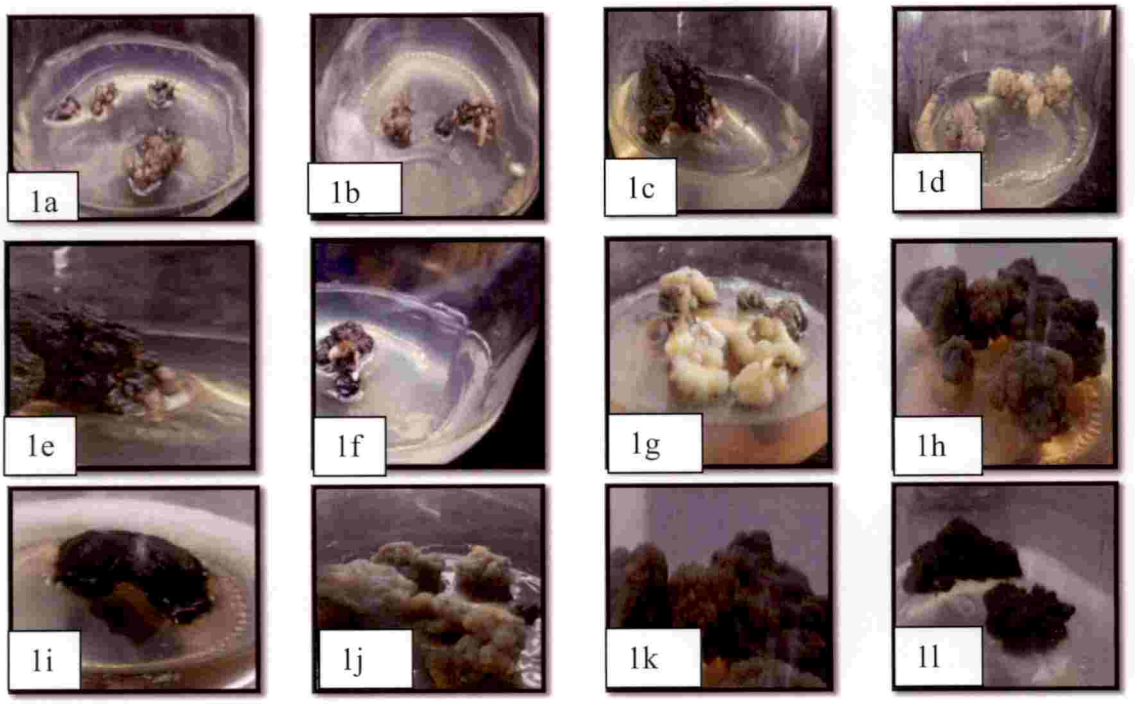
Among the different concentrations of NAA tried, only leaf tissues produced callusing (6.25 per cent) with1.5 mg L^{-1}. While 0.5 mg L^{-1} of TDZ were supplemented in the medium 12.5 per cent of callusing in leaf tissue, and 6.25per cent in shoot tip and node were recorded. When the concentration increased in to 2 mg L^{-1} of TDZ, no callusing was recorded (Plate1).

In this study using *ex vitro* raised explants, treatments given in MS medium produced better response than SH medium.

Table17. Effect of different plant growth regulators on callus induction from field grown explants in MS medium (4 replication)

Treatment	Growth regulators (mg L^{-1})						Percentage of callus induction		
	2,4-D	BA	IAA	NAA	TDZ	Picloram	Leaf	Shoot	Node
T1	0.5	-	-	-	-	-	12.50	6.25	0.00
T2	1.0	0.5	-	-	-	-	12.50	12.50	0.00
T3	-	1.0	-	-	-	-	12.50	6.25	6.25
T4	-	1.5	-	-	-	-	28.50	28.50	12.50
T5	-	2.0	-	-	-	-	0.00	0.00	0.00
T6	-	-	1.0	-	-	-	6.25	0.00	0.00
T7	-	-	2.0	-	-	-	0.00	0.00	0.00
T8	-	1.0	1.0	-	-	-	6.25	0.00	6.25
T9	-	1.0	1.5	-	-	-	12.50	6.25	0.00
T10	-	2.0	1.0	-	-	-	0.00	0.00	0.00
T11	-	2.0	1.5	-	-	-	0.00	0.00	0.00
T12	-	-	-	1.0	-	-	0.00	0.00	0.00
T13	-	-	-	1.5	-	-	6.25	0.00	0.00
T14	-	-	-	2.0	-	-	0.00	0.00	0.00
T15	-	-	-	-	0.5	-	12.50	6.25	6.25
T16	-	-	-	-	1.0	-	0.00	0.00	0.00
T17	-	-	-	-	-	1.0	36.20	28.50	28.50
T18	-	-	-	-	-	1.5	28.50	28.50	28.50
T19	-	-	-	-	-	2.0	0.00	0.00	0.00
T20	-	1.0	-	-	-	1.0	12.50	12.50	12.50
T21	-	1.5	-	-	-	1.0	6.25	6.25	6.25

Plate1. Callus initiated from *ex vitro* raised explants after 40 days of inoculation



1a. MS+2, 4-D 0.5 mg L^{-1}(Node)

1b. MS+BA 0.5 mg L^{-1} (shoot tip)

1c. MS+BA 1.5 mg L^{-1} (leaf)

1d. MS+BA 1 mg L^{-1} (Soot tip)

1e. MS+BA 1 mg L^{-1} (node)

1f. MS+BA 1.5 mg L^{-1} (shoot tip)

1g. MS+Picloram 1 mg L^{-1} (leaf)

1h. MS+Picloram1 mg L^{-1} (Shoot tip)

1i. MS+BA 1.5 mg L^{-1} (node)

1j. MS+TDZ 0.5 mg L^{-1} (shoot tip)

1k. MS+TDZ 0.5 mg L^{-1} (node)

1l. MS+BA1.5 mg L^{-1} (Shoot tip)

4.2.3 Initiation of callusing in MS medium from *in vitro* raised explants

Among the three explants used, leaf showed the best response (Plate 2) in callus induction. MS medium supplemented with 1.5 mg L^{-1} of picloram produced the highest callus induction (68.75per cent) from the leaves, followed by treatments

T49, T50 and T51 which recorded a callus induction per cent of 62.5per cent. While increasing the concentration of picloram to 2 mg L^{-1} and 4 mg L^{-1}, there was a reduction in the callus induction frequency in to 28.5 per cent and 14.28 per cent respectively.

Treatment with BA (1 and 1.5 mg L^{-1}) induced 57.14per cent callus on leaf explants. Similarly BA in combination with IAA (1 mg L^{-1} each) showed good callusing response on leaf (50per cent). Leaf tissues responded to all the treatments with a combination of BA and IAA.

While trying with different combination of picloram and BA, maximum callus induction frequency was found in treatment T56 (42.85 per cent). Other concentrations of BA in this combination reduced the callus induction frequency.

Among the different concentration of NAA tried, no callus induction frequency was observed in 0.5,1 and 1.5 mg L^{-1}. While increasing the concentration to 2 mg L^{-1}, callusing was induced with frequency of 12.5 per cent.

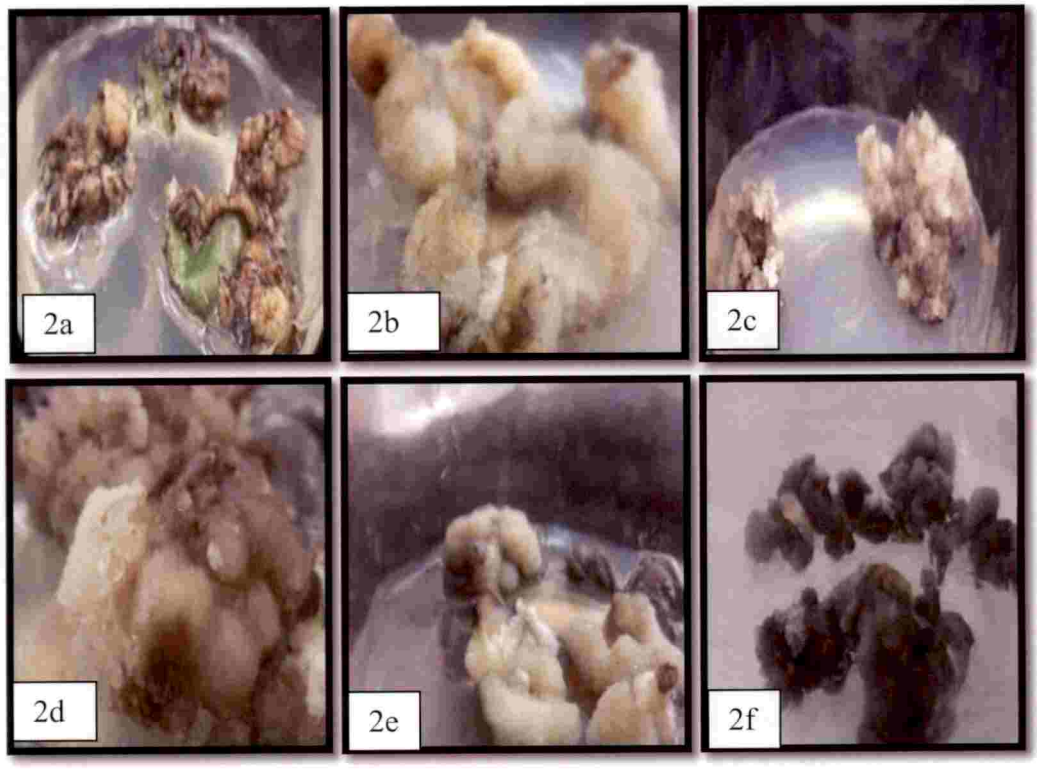
When BA and NAA combinations were tried, treatment T40 produced the maximum callus induction frequency of 28.50 per cent. Other treatments *viz.* T33, T35, T37, T38, T39 and T44 recorded the same (12.50per cent) callus induction frequency.

When the BA was used along with IAA, highest callus induction recorded was 42.84 per cent in T18 and T13.While increasing the concentration of BA to1 mg L^{-1} along with an increase of concentration of IAA to 0.5, 1, 1.5, and 2 mg L^{-1}, there were no response in leaf on callusing. 12.5 per cent of callus induction was found for the treatments T16, T21, T22, T23, T24, and T25 and 28.5 per cent of callusing was recorded in T14, T15, T17, T19 and T20.

When a combination of BA and 2, 4-D was tried, maximum callus induction was found in treatment T12 (12.5per cent). 6.25 per cent of callus

induction was observed in treatment T7, T8, T9, T10, andT11.While BA alone was used, maximum callus induction was observed (57.14per cent) in medium supplemented with 1 mg L^{-1} and 1.5 mg L^{-1} of BA. When the concentration of the BA was increased

Plate 2. Callus initiated on MS medium from *in vitro* raised leaf on 40th day of inoculation and morphological characters



2a. MS+BA1 mg L^{-1} (Dark brown calli)

2b. MS+Picloram 1.5 mg L^{-1}(Creamy white friable calli)

2c. MS+BA0.5 mg L^{-1}(Light brown friable calli)

2d. MS+Picloram 0.5 mg L^{-1}(Light brown friable calli)

2e. MS+BA 1.5 mg L^{-1}(White friable calli)

2f. MS+0.5 mg L^{-1}TDZ+1 mg L^{-1} Picloram (Dark brown calli)

to 2 mg L^{-1}, there was reduction in callus induction frequency to 12.5 per cent. When the concentration of BA was decreased in to 0.5 mg L^{-1}, there was also reduction of callus induction frequency in to 42.84 per cent.

While trying with 2, 4-D alone, highest callus induction was noticed in treatment T1 (28.50per cent) and induction frequency (12.50per cent) was reduced when the concentration was reduced to 0.5 mg L^{-1} (Table 18).

Among the sixty one treatments tried with shoot tip (Plate 3), MS medium supplemented with 1mg L^{-1} picloram gave the highest callus induction (57.14per cent) followed by treatment T52, T48, T49 and T5 (42.85per cent). By increasing the concentration of picloram to 2 and mg L^{-1}, callus induction per cent was reduced to zero. When 1 mg L^{-1} of TDZ was supplemented in MS medium, callus induction frequency of 28.50 per cent was recorded. With a combination of BA and picloram T57 with 1 mg L^{-1} each of both the BA and picloram produced 28.4 per cent of callus induction. And all other combinations contain picloram and BA produced 14.28 per cent of callus induction.

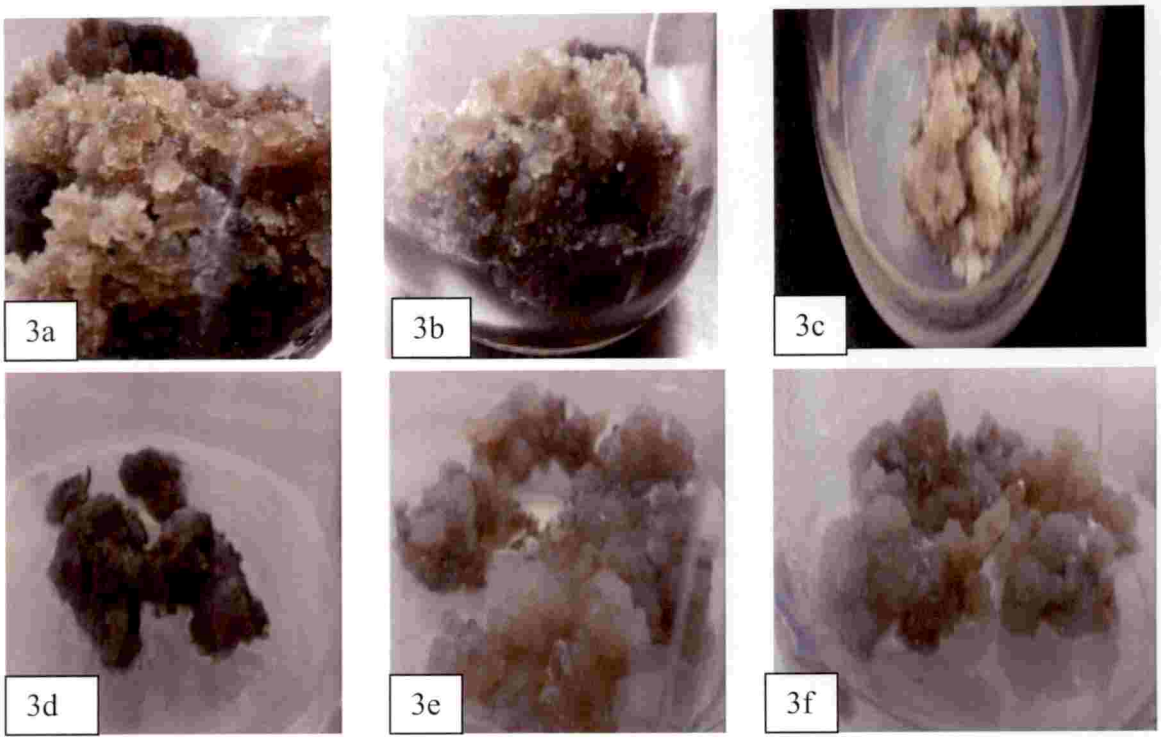
While trying with the different combination of BA and NAA, none of the treatments showed callus induction from shoot tip. Different combinations of BA and IAA, 2, 4-D and BA also did not show any positive response towards callusing from shoot tip.

When the BA alone was provided, 42.84 per cent callus induction was noticed in treatment T5 and 28.50 per cent with 1 mg L^{-1} of BA, 12.5per cent with 0.5 mg L^{-1} and 6.25per cent with 2 mg L^{-1} of BA. In 0.5 and 1 mg L^{-1} of BA, 28.50 per cent and 6.25 per cent of callus induction was recorded (Table 18).

With the 61 treatments tried using node as explants for the induction of callus in MS medium (Plate 4), only the medium supplemented with picloram alone (T50, T51, T52, T53) picloram in combination with TDZ (T48 and T49) and BA (T55, T56 and T57), and BA (T4 and T5) and 2, 4-D (T8) alone were responded to induction. Maximum callus induction frequency of 42.85 per cent was recorded in treatment T51 (MS+1 mg L^{-1}). Lesser (0.5 mgL^{-1}) higher (1.5 mg L^{-1}) concentration of picloram showed reduction in callus induction frequency to 14.28 per cent and 28.50 per cent respectively. With further increase of

concentration to 2, callus induction of 28.50 per cent was obtained and with $4mgL^{-1}$ there was no callus induction. In treatment T49, 7.64 per cent of callus induction frequency was noticed. When BA was added along with picloram, treatment T56 produced callus.

Plate 3. Callus initiated in MS medium from *in vitro* raised shoot tip on 40 th day of inoculation and morphological characters



3a. MS+0.5 mg L^{-1}Picloram(Light to dark brown transparent calli)

3b. MS+1 mg L^{-1} Picloram(Light to dark brown transparent calli)

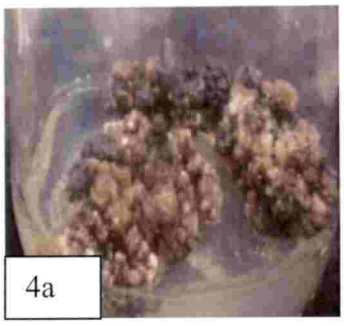
3c. MS+BA mg L^{-1}+ Picloram mg L^{-1}(Light to dark brown transparent calli)

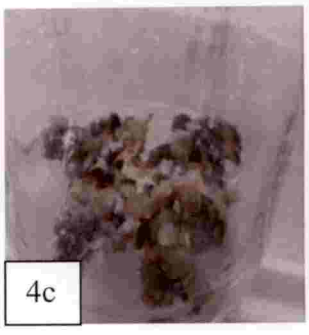
3d. MS+0.5 mg L^{-1} TDZ+1 mg L^{-1} Picloram(Dark brown calli)

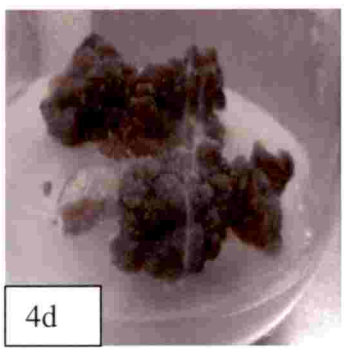
3e. MS+BA 1 mg L^{-1} (Creamy white bubbly calli)

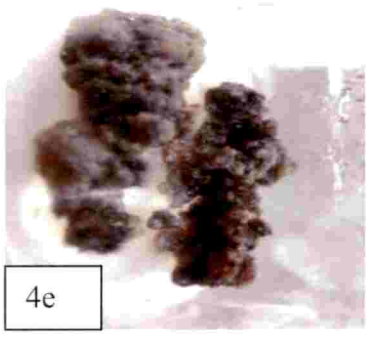
3f. MS+BA 1.5 mg L^{-1}(Creamy white bubbly calli)

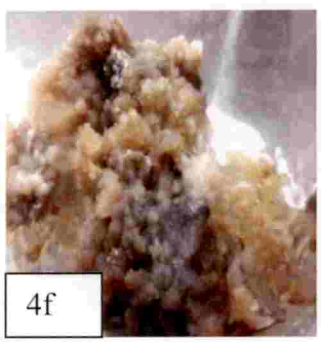
Plate 4. Callus initiated in MS medium from *invitro* raised node on 40th day of inoculation and morphological characters











4a. MS+Picloram 1mg L^{-1} (Brown friable calli)

4b. MS+Picloram 1.5 mg L^{-1}(Brown friable calli)

4c. MS+0.5 mg L^{-1}TDZ+1 mg L^{-1} Picloram(Brown calli)

4d. MS+BA 1 mg L^{-1}(Dark brown bubbly calli)

4e.MS+BA 1.5 mg L^{-1}(Dark brown bubbly calli)

4f. MS+BA 1.5 mg L^{-1}+2, 4-D 0.5 mg L^{-1}(Dark brown bubbly calli)

Table18. Effect of plant growth regulators on callus induction from *in vitro* raised explants of black pepper in MS medium (4 replication)

Treatments	**Growth regulators (mg L^{-1})**						**Percentage of callus induction**		
	2,4-D	BA	IAA	NAA	TDZ	Picloram	**Leaf**	**Shoot tip**	**Node**
T1	0.5	-	-	-	-	-	28.50	28.50	0.00
T2	1.0	-	-	-	-	-	12.50	6.25	0.00
T3	-	0.5	-	-	-	-	42.84	12.50	0.00
T4	-	1.0	-	-	-	-	57.14	28.50	28.50
T5	-	1.5	-	-	-	-	57.14	42.84	28.50
T6	-	2.0	-	-	-	-	12.50	6.25	12.50
T7	0.5	1.0	-	-	-	-	6.25	0.00	0.00
T8	0.5	1.5	-	-	-	-	6.25	6.25	12.50
T9	0.5	2.0	-	-	-	-	6.25	0.00	6.25
T10	1.0	0.5	-	-	-	-	6.25	0.00	0.00
T11	1.0	1.0	-	-	-	-	6.25	0.00	0.00
T12	1.0	1.5		-	-	-	12.50	0.00	6.25
T13	-	0.5	0.5	-	-	-	42.84	0.00	0.00
T14	-	0.5	1.0		-	-	28.50	0.00	0.00
T15	-	0.5	1.5	-	-	-	28.50	0.00	0.00
T16	-	0.5	2.0	-	-	-	12.50	0.00	0.00
T17	-	1.0	0.5	-	-	-	50.00	0.00	0.00
T18	-	1.0	1.0	-	-	-	28.50	0.00	0.00
T19	-	1.0	1.5	-	-	-	28.50	0.00	0.00
T20	-	1.0	2.0	-	-	-	12.50	0.00	0.00
T21	-	1.5	0.5	-	-	-	12.50	0.00	0.00
T22	-	1.5	1.0	-	-	-	12.50	0.00	0.00

T23	-	1.5	1.5	-	-	-	12.50	0.00	0.00
T24	-	1.5	2.0	-	-	-	12.50	0.00	0.00
T25	-	2.0	0.5	-	-	-	0.00	0.00	0.00
T26	-	2.0	1.0	-	-	-	0.00	0.00	0.00
T27	-	2.0	1.5	-	-	-	0.00	0.00	0.00
T28	-	2.0	2.0	-	-	-	0.00	0.00	0.00
T29	-	-	-	0.5	-	-	0.00	0.00	0.00
T30	-	-	-	1.0	-	-	0.00	0.00	0.00
T31	-	-	-	1.5	-	-	0.00	0.00	0.00
T32	-	-	-	2.0	-	-	12.50	0.00	0.00
T33	-	0.5	-	0.5	-	-	12.50	0.00	0.00
T34	-	0.5	-	1.0	-	-	0.00	0.00	0.00
T35	-	0.5	-	1.5	-	-	12.50	0.00	0.00
T36	-	0.5	-	2.0	-	-	0.00	0.00	0.00
T37	-	1.0	-	0.5	-	-	12.50	0.00	0.00
T38	-	1.0	-	1.0	-	-	12.50	0.00	0.00
T39	-	1.0	-	1.5	-	-	12.50	0.00	0.00
T40	-	1.0	-	2.0	-	-	28.50	0.00	0.00
T41	-	1.5	-	0.5	-	-	0.00	0.00	0.00
T42	-	1.5	-	1.0	-	-	0.00	0.00	0.00
T43	-	1.5	-	1.5	-	-	0.00	0.00	0.00
T44	-	1.5	-	2.0	-	-	14.28	0.00	0.00
T45	-	2.0	-	0.5	-	-	0.00	0.00	0.00
T46	-	-	-	-	0-.5	-	28.50	28.50	0.00
T47	-	-	-	-	1.0	-	28.50	28.50	0.00
T48	-	-	-	-	0.5	0.5	42.85	42.85	0.00
T49	-	-	-	-	0.5	1.0	62.50	42.84	7.64
T50	-	-	-	-	-	0.5	62.50	42.84	14.28

T51	-	-	-	-	-	1.0	62.50	57.14	42.85
T52	-	-	-	-	-	1.5	68.75	42.85	28.50
T53	-	-	-	-	-	2.0	28.50	0.00	28.50
T54	-	-	-	-	-	4.0	14.28	0.00	0.00
T55	-	0.5	-	-	-	1.0	28.50	14.28	7.64
T56	-	1.0	-	-	-	1.0	42.85	14.28	14.28
T57	-	2.0	-	-	-	1.0	28.50	28.50	7.64
T58	-	1.0	-	-	-	1.5	28.50	14.28	0.00
T59	-	2.0	-	-	-	1.5	28.50	14.28	0.00
T60	-	1.0	-	-	-	2.0	28.50	14.28	0.00
T61	-	-	-	-	-	-	0.00	0.00	0.00

While trying with different concentration of BA and IAA, maximum callus induction frequency was observed in treatment T13 and T18 (42.85per cent) followed by treatment T14, T15, T17, and T19 (28.50 per cent). 12.5 per cent of callus induction frequency was recorded in treatment T16, T21, T22, T23, T24 and T25 and the remaining treatment containing BA and IAA did not show any positive response towards callusing.

Different concentration of BA and 2, 4-D when tried, 12.5 per cent of callus induction frequency was noticed in treatment T7, T8 and T10. Increasing the concentration of 2, 4-D and BA reduced the callus induction per cent to zero. With the addition of BA 1 mg L^{-1} and 1.5 mgL^{1}, there was 42.80 per cent of callus induction and also reduction in callus induction was noticed when the concentration of BA increases.

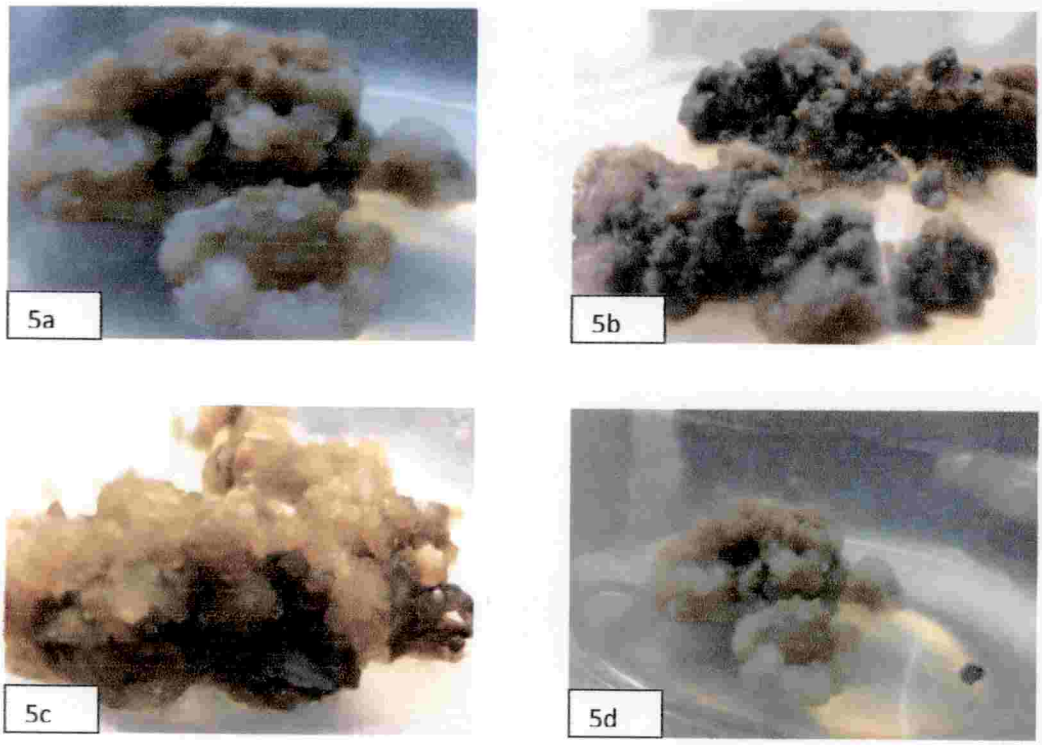
When the medium was supplemented with 0.5 and 1 mg L^{-1} of 2, 4-D, a callus induction frequency of 12.5 per cent was observed in treatment T1 andT2 (Table 19).

Among the sixty one treatments tried in SH medium for obtaining callus from shoot tip, 1 mg L^{-1} picloram (Plate 6) produced the highest callus induction frequency (57.14per cent). When the concentration was increased to 1.5 mg L^{-1} callus induction frequency was decreased into 42.84 per cent and further increase of concentration to, 2 mg L^{-1} also showed 42.84 per cent of callusing. Further increase to 4 mg L^{-1} didn't produced any positive response on callusing. While reducing the concentration to 0.5 mg L^{-1} also reduction in callus induction frequency in to 28.56 per cent was recorded.

While trying picloram and TDZ in different combinations, treatment T48 and T49 produced almost same callus induction frequency of 28.56 per cent. With the addition of 0.5 mg L^{-1} and 1 mgL^{-1} of TDZ alone, a callus induction frequency of 14.28 per cent was noticed.

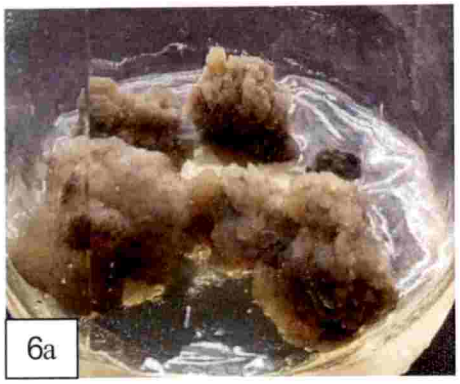
When different concentrations of BA and picloram were tried, treatments T55, T56, T57, T58, T59 and T60 produced same callus induction frequency of 14.28 per cent.

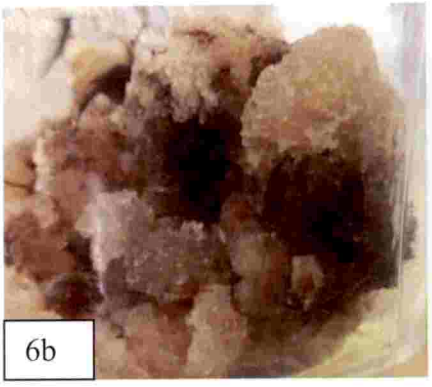
Plate 5. Callus initiated in SH medium from *in vitro* raised leaf

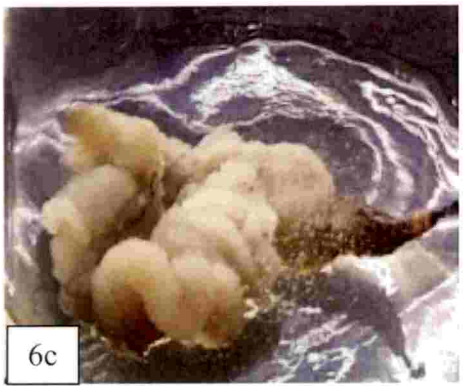


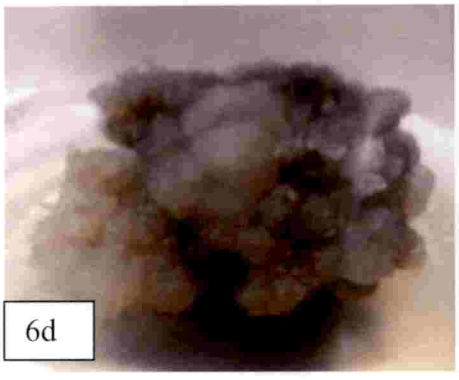
5a. SH+0.5 mg L^{-1} Picloram
5b. SH+1 mg L^{-1} BA
5c. SH+1 mg L^{-1} Picloram
5d. SH+0.5 mg L^{-1} BA+1 mg L^{-1} IAA

Plate 6. Callus initiated in SH medium from *in vitro* raised shoot tip on 40th day of inoculation with morphological characters









6a. SH+ 1mg L^{-1} Picloram (Creamy white friable calli)

6b. SH+1.5 mg L^{-1} Picloram (Brownish white friable calli)

6c. SH+1 mg L^{-1} BA+1 mg L^{-1} IAA (White friable calli)

6d. SH+0.5 mg L^{-1} Picloram+0.5 mg L^{-1} TDZ (Creamy white bubbly calli)

Table19.Effect of plant growth regulators on callus induction from *in vitro* raised explants of black pepper in SH medium

Treatments	**Growth regulators (mg L^{-1})**						**Percentage of callus induction**		
	2,4-D	BA	IAA	NAA	TDZ	Picloram	**leaf**	**shootip**	**node**
T1	0.5	-	-	-	-	-	15.00	0.00	0.00
T2	1.0	-	-	-	-	-	0.00	0.00	0.00
T3	-	0.5	-	-	-	-	30.00	0.00	0.00
T4	-	1.0	-	-	-	-	60.00	0.00	0.00
T5	-	1.5	-	-	-	-	40.00	0.00	0.00
T6	-	2.0	-	-	-	-	30.00	0.00	0.00
T7	0.5	1.0	-	-	-	-	15.00	0.00	0.00
T8	0.5	1.5	-	-	-	-	15.00	0.00	0.00
T9	0.5	2.0	-	-	-	-	0.00	0.00	0.00
T10	1.0	0.5	-	-	-	-	15.00	0.00	0.00
T11	1.0	1.0	-	-	-	-	0.00	0.00	0.00
T12	1.0	1.5	-	-	-	-	0.00	0.00	0.00
T13	-	0.5	0.5	-	-	-	10.00	0.00	0.00
T14	-	0.5	1.0	-	-	-	10.00	0.00	0.00
T15	-	0.5	1.5	-	-	-	10.00	0.00	0.00
T16	-	0.5	2.0	-	-	-	20.00	0.00	0.00
T17	-	1.0	0.5	-	-	-	20.00	0.00	0.00
T18	-	1.0	1.0	-	-	-	20.00	0.00	0.00
T19	-	1.0	1.5	-	-	-	10.00	0.00	0.00
T20	-	1.0	2.0	-	-	-	30.00	0.00	0.00
T21	-	1.5	0.5	-	-	-	0.00	0.00	0.00
T22	-	1.5	1.0	-	-	-	0.00	0.00	0.00
T23	-	1.5	1.5	-	-	-	0.00	0.00	0.00

T24	-	1.5	2.0	-	-	-	20.00	0.00	0.00
T25	-	2.0	0.5	-	-	-	0.00	0.00	0.00
T26	-	2.0	1.0	-	-	-	0.00	0.00	0.00
T27	-	2.0	1.5	-	-	-	0.00	0.00	0.00
T28	-	2.0	2.0		-	-	0.00	0.00	0.00
T29	-	-	-	0.5	-	-	0.00	0.00	0.00
T30	-	-	-	1.0	-	-	0.00	0.00	0.00
T31	-	-	-	1.5	-	-	0.00	0.00	0.00
T32	-	-	-	2.0	-	-	0.00	0.00	0.00
T33	-	0.5	-	0.5	-	-	10.00	0.00	0.00
T34	-	0.5	-	1.0	-	-	10.00	0.00	0.00
T35	-	0.5	-	1.5	-	-	10.00	0.00	0.00
T36	-	0.5	-	2.0	-	-	0.00	0.00	0.00
T37	-	1.0	-	0.5	-	-	0.00	0.00	0.00
T38	-	1.0	-	1.0	-	-	0.00	0.00	0.00
T39	-	1.0	-	1.5	-	-	15.00	0.00	0.00
T40	-	1.0	-	2.0	-	-	0.00	0.00	0.00
T41	-	1.5	-	0.5	-	-	0.00	0.00	0.00
T42	-	1.5	-	1.0	-	-	0.00	0.00	0.00
T43	-	1.5	-	1.5	-	-	0.00	0.00	0.00
T44	-	1.5	-	2.0	-	-	0.00	0.00	0.00
T45	-	2.0	-	0.5	-	-	0.00	0.00	0.00
T46	-	-	-	-	0.5	-	20.00	14.28	0.00
T47	-	-	-	-	1.0	-	20.00	14.28	0.00
T48	-	-	-	-	0.5	0.5	40.00	28.56	0.00
T49	-	-	-	-	0.5	1.0	40.00	28.56	0.00
T50	-	-	-	-	-	0.5	60.00	28.56	14.28
T51	-	-	-	-	-	1.0	70.00	57.14	14.28
T52	-	-	-	-	-	1.5	40.00	42.84	42.85
T53	-	-	-	-	-	2.0	20.00	42.84	28.56

T54	-	-	-	-	-	4.0	10.00	0.00	0.00
T55	-	0.5	-	-	-	1.0	30.00	14.28	0.00
T56	-	1.0	-	-	-	1.0	50.00	14.28	0.00
T57	-	2.0	-	-	-	1.0	30.00	14.28	0.00
T58	-	1.0	-	-	-	1.5	30.00	14.28	0.00
T59	-	2.0	-	-	-	1.5	30.00	14.28	0.00
T60	-	-	-	-	-	2.0	30.00	14.28	0.00
T61	-	-	-	-	-	-	0.00	0.00	0.00

When different concentration of BA was supplemented in the medium, maximum callus induction was recorded in the medium supplemented with 1 mgL^{-1} of BA (42.84 per cent). With the increase of concentration in to 1.5 and 2 mg L^{-1}, reduction in the callus induction was recorded from 28.50 per cent into zero per cent.

Shoot tip showed less response to callusing compared to leaf. Callus induction per cent was less in SH medium compared to MS medium.

Coming to the callus induction from node in SH medium, maximum callus induction frequency was recorded for treatment T52 (42.85 per cent with SH+1 mg L^{-1} picloram) (Plate 7). With increase of concentration picloram to 2 and 4 mg L^{-1}, there was a decrease in callus induction frequency in to 28.56 per cent and zero per cent respectively. With decrease of concentration to 1.0 and 0.5 mg L^{-1}, 14.28 per cent of callus induction was recorded. While trying different concentration of BA, the medium supplemented with 1 and 1.5 mg L^{-1} BA produced 12.5 per cent of callusing. The rest of the treatments did not respond positively towards callusing (Table 19).

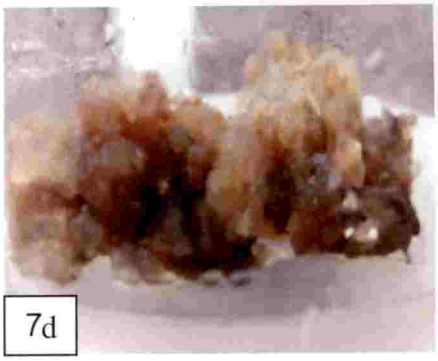
Among all the combinations tried for getting callus using node as explant, the most suitable combinations were MS and SH medium supplemented with 1 mg L^{-1} of picloram.

Plate 7. Callus initiated in SH media from *in vitro* raised node on 40 th day of inoculation with morphological characters

7a

7b

7c



7d

7a. SH+1 mg L^{-1} Picloram (Light brown intermitted with greenish bubbly calli)

7b. SH+1.5 mg L^{-1}Picloram(Light brown intermitted with greenish bubbly calli)

7c. SH+0.5 mg L^{-1} Picloram (Light brown bubbly calli)

7d. SH+2 mg L^{-1} Picloram (Light brown bubbly calli)

4.3 INDUCTION OF SOMATIC EMBRYOS FROM CALLUS

Twenty six treatments were tried in MS (Table 20) and fifteen treatments in SH (Table 21) media, with different concentrations of sucrose for somatic embryogenesis from the calli. But all the calli turned black (Plate 8) and not responded in this study.

Table20. Effect of different growth regulators on induction of somatic embryos from callus (Basal medium: MS+3 per cent sucrose)

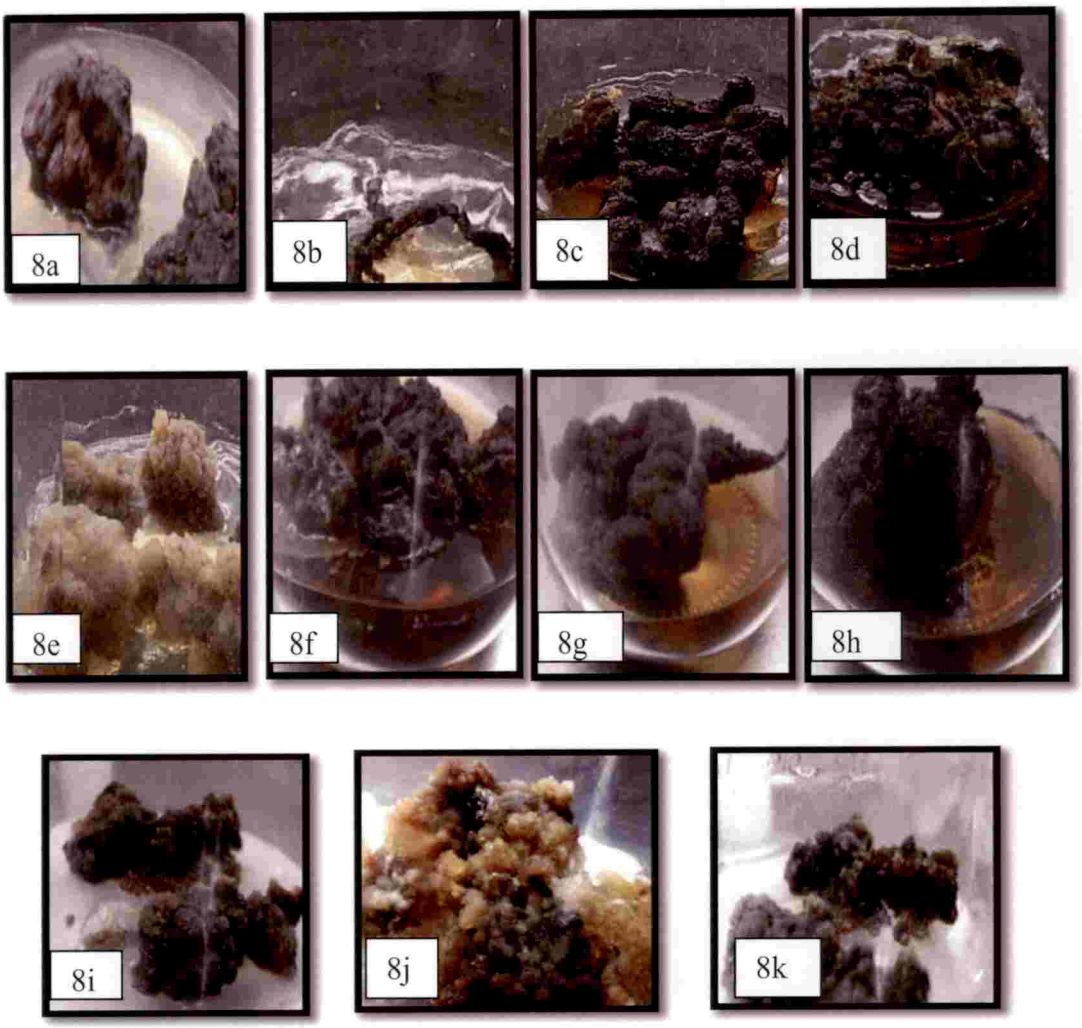
Treatments	**PGR conc. (mg L^{-1})**				**% of embryo regeneration**
	BA	IAA	2-4,D	Picloram	
T1	-	-	-	-	0
T2	1	-	-	-	0
T3	1.5	-	-	-	0
T4	2	-	-	-	0
T5	4	-	-	-	0
T6	-	1		-	0
T7	-	1.5	-	-	0
T8	-	2	-	-	0
T9	-	4	-	-	0
T10	1	1	-	-	0
T11	1	2	-	-	0
T12	2	1	-	-	0
T13	2	2	-	-	0
T14	-	-	1	-	0
T15	-	-	1.5	-	0
T16	-	-	2	-	0
T17	-	-	-	1	0
T18	-	-	-	1.5	0
T19	-	-	-	2	0
T20	-	-	-	4	0

T21	1	-	-	1	0
T22	1	-	-	1.5	0
T23	1	-	-	2	0
T24	2	-	-	1	0
T25	2	-	-	1.5	0
T26	2	-	-	2	0

Table21. Effect of different growth regulators on induction of somatic embryos from callus (Basal medium: SH+3 per cent sucrose)

Treatments	**PGR conc. (mg L^{-1})**				**% of embryo regeneration**
	BA	IAA	2-4,D	Picloram	
T1	-	-	-	-	0
T2	0.5	-	-	-	0
T3	1	-	-	-	0
T4	1.5	-	-	-	0
T5	2	-	-	-	0
T6	-	-	-	1	0
T7	-	-	-	1.5	0
T8	-	-	-	2	0
T9	-	-	-	4	0
T10	1	-	-	1	0
T11	1	-	-	1.5	0
T12	1	-	-	2	0
T13	2	-	-	1	0
T14	2	-	-	1.5	0
T15	2	-	-	2	

Plate 8. Response of calli in somatic embryo induction medium



8a. MS+BA 1mg L^{-1}

8b. MS+2,4D 1 mg L^{-1}

8c. MS+Picloram 1 mg L^{-1}

8d. MS+BA+IAA 1 mg L^{-1}

8e. SH (3.5%sucrose)

8f. SH (1.5% sucrose)

8g. MS+BA mg L^{-1}

8h. SH+Picloram1 mg L^{-1}

8i. SH+BA 1 mg L^{-1}

8j. MS+BA 1.5 mg L^{-1}

8k. SH+BA 1 mg L^{-1}

4.4 SOMATIC EMBRYOGENESIS FROM SEED

4.4.1 Initiation of somatic embryogenesis

Among the 48 treatments tried (21 treatments in SH medium and 27 treatments in MS medium), 7 treatments responded positively for the initiation of somatic embryogenesis from seeds.

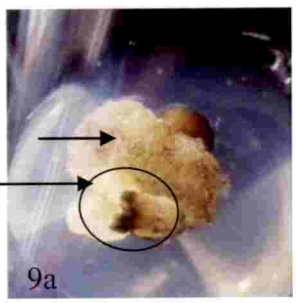
Medium supplemented with1.5 and 2 mg L^{-1} of picloram produced indirect (Plate 9) somatic embryogenesis (46per cent) from the calli initiated in the micropylar region of the seed. Full strength SH medium provided with 2 mg L^{-1} picloram induced 57.14 per cent somatic embryogenesis within 41.57 days (Table22). When the concentration of picloram was reduced to1.5 mg L^{-1}, there was reduction in the induction of somatic embryogenesis (31.42 per cent), and with the further decrease there was no embryogenic responses; however they produced callusing from the micropylar region. Further growth and development of embryos were not observed in the medium supplemented with picloram. Full strength MS medium supplemented with 1.5 and 2 mg L^{-1} of picloram also induced (28.57 per cent and 31.42 per cent) indirect somatic embryos (Table 23). The initiated embryos like structures were easily detachable from the callus. When they were transferred to medium without any growth regulators, they regenerated in to plantlets. Keeping the structures in the picloram supplemented medium led to increase in the size of the callus but later the entire callus including the embryo like structure got blackened.

Direct embryogenesis (Plate10) was observed from ring tissue of the micropylar region (full strength SH medium, SH with1.5 per cent sucrose and 1/2 strength SH).When the seeds are inoculated in full strength SH medium, 31.42 per cent of embryo induction was noticed. The embryos were able to mature and regenerate in to plantlet in the same medium. When tried on SH medium along with1.5 per cent sucrose 42.8 per cent embryos induced from the ring tissue of the micropylar region of the seed within 48.28 days of inoculation. 81.9 per cent of the embryos got matured and 90 per cent of the matured embryos regenerated in to plantlets in the same medium (Table 24). With the increase of sucrose

concentration to 4.5 per cent seeds did not show any positive response on the embryo induction.

Inoculation of seeds in half strength SH medium, led to the development of embryo like globular structures from the ring tissue that developed on the micropylar region of the seed. Somatic embryos initiated within 50.57 days of inoculation (37.14per cent). Among the initiated embryos 66.67 per cent embryos got matured and 70 per cent of the matured embryos regenerated to plantlets in the same medium (Table 24).

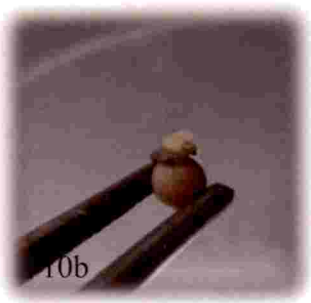
Plate 9. Somatic embryogenesis in medium containing picloram (Indirect somatic embryogenesis)

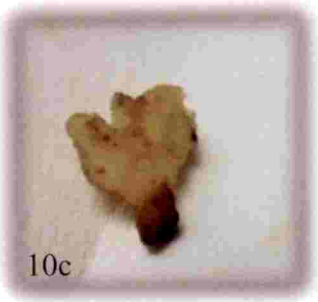


9a. Somatic embryo genesis from the callus

9b. Degeneration of the embryo

Plate 10. Somatic embryogenesis from seed and regeneration of plantlet on SH (1.5per cent sucrose)





10a. Initiation of globular stage

10b. Develops into heart shaped stage

10c. Torpedo stage

10d. Regeneration of plant

Table22. Initiation of somatic embryos in SH media with different concentration of sucrose and growth regulators

Treatments	Media	PGR conc. (mg L^{-1})				Development	% of embryo initiation
		BA	Picloram	IAA	2, 4-D		
T1	SH+3%sucrose	-	-	-	-	Direct	31.42
T2	SH+1.5%Sucrose	-	-	-	-	Direct	42.83
T3	SH+ 4.5% Sucrose	-	-	-	-	-	0
T4	½ SH+3%Sucrose	-	-	-	-	Direct	37.14
T5	SH+3%sucrose	-	1	-	-	-	-
T6	SH+3%sucrose	-	1.5	-	-	Indirect	31.42
T7	SH+3%sucrose	-	2	-	-	Indirect	57.14
T8	SH+3%sucrose	1	1	-	-	-	0
T9	SH+3%sucrose	1	1.5	-	-	-	0
T10	SH+3%sucrose	1	2	-	-	-	0
T11	SH+3%sucrose	2	1	-	-	-	0
T12	SH+3%sucrose	2	1.5	-	-	-	0
T13	SH+3%sucrose	2	2	-	-	-	0
T14	SH+3%sucrose	1	-	1	-	-	0
T15	SH+3%sucrose	1	-	1.5	-	-	0
T16	SH+3%sucrose	1	-	2	-	-	0
T17	SH+3%sucrose	2	-	1	-	-	0
T18	SH+3%sucrose	2	-	1.5	-	-	0
T19	SH+3%sucrose	2	-	2	-	-	0
T20	SH semi solid+3%sucrose	-	-	-	-	-	0
T21	Liquid SH+3%sucrose	-	-	-	-	-	0

Table23. Initiation of somatic embryos in SH media with different concentration of sucrose and growth regulators

Treatments	Media	PGR conc. (mg L^{-1})				Development	% of embryo initiation
		BA	Picloram	IAA	2, 4-D		
T1	MS+3%sucrose	-	-	-	-	-	0
T2	MS+1.5% Sucrose	-	-	-	-	-	0
T3	MS+4.5% Sucrose	-	-	-	-	-	0
T4	MS+3%sucrose	-	1	-	-	-	0
T5	MS+3%sucrose	-	1.5	-	-	Indirect	28.57
T6	MS+3%sucrose	-	2	-	-	Indirect	31.42
T7	MS+3%sucrose	1	1	-	-	-	0
T8	MS+3%sucrose	1	1.5	-	-	-	0
T9	MS+3%sucrose	1	2	-	-	-	0
T10	MS+3%sucrose	2	1	-	-	-	0
T11	MS+3%sucrose	2	1.5	-	-	-	0
T12	MS+3%sucrose	2	2	-	-	-	0
T13	MS+3%sucrose	-	-	-	0.5	-	0
T14	MS+3%sucrose	-	-	-	1	-	0
T15	MS+3%sucrose	-	-	-	1.5	-	0
T16	MS+3%sucrose	-	-	-	2	-	0
T17	MS+3%sucrose	-	-	-	4	-	0
T18	MS+3%sucrose	1	-	1	-	-	0
T19	MS+3%sucrose	1	-	1.5	-	-	0
T20	MS+3%sucrose	1	-	2	-	-	0
T21	MS+3%sucrose	2	-	1	-	-	0
T22	MS+3%sucrose	2	-	1.5	-	-	0
T23	MS+3%sucrose	2	-	2	-	-	0
T24	MS+3%sucrose	4	-	4	-	-	0
T25	MS semi solid+3%sucrose	-	-	-	-	-	0
T26	Liquid MS+3%sucrose	-	-	-	-	-	0

Among all the explants tried, seed was the most suitable explants for initiation of somatic embryogenesis in pepper. Among the various media used SH was more effective for initiation of somatic embryogenesis than MS. Full strength SH medium supplemented with 2 mg L^{-1} picloram was recorded as the best treatment for the initiation of somatic embryogenesis (Table 24). However further development and regeneration of embryos was noticed better in SH (1.5per cent sucrose).

Table24. Somatic embryogenesis from different media, when the initiated embryos are kept in the same medium

Media	**Days taken to initiate embryogenesis**	**Initiation of embryos (%)**	**Matured embryos (%)**	**Regeneration of plantlets (%)**
SH+3%sucrose	51.57	31.42	76	77
SH+1.5% Sucrose	48.28	42.8	81.9	90
½ SH+3%sucrose	50.57	37.14	66.67	70
SH+2mg L^{-1} Picloram	41.42	57.15	0	0
SH+1.5 mg L^{-1} Picloram	43.14	31.42	0	0
MS+2 mg L^{-1} Picloram	61.57	31.42	0	0
MS+1.5 mg L^{-1} Picloram	62.57	28.57	0	0

4.5 MULTIPLICATION AND FURTHER DEVELOPMENT

Among the forty two treatments tried (23 treatments in SH media and 20 traetments in MS media (table 26)), only full strength SH (Plate 11) and semi solid SH (1.5 % sucrose) responded positively for multiplication (Table 25).

The embryo initiated in full strength SH medium (Plate11a), SH (1.5 per cent sucrose) (Plate11b) and half strength SH medium (Plate 11c), when transferred to full strength SH medium, 20per cent of the primary embryos produced secondary embryos. 100 per cent of the multiplied embryos were regenerated in to plantlets in the full strength SH medium, within 80-85 days. The embryo initiated in the full strength MS or SH medium containing picloram, when transferred to full strength SH, multiplication of the primary embryos was not observed, they regenerated in to plantlets by taking 92.71 days in full strength SH+2 mg L^{-1} picloram, 93.14 days in full strength SH+1.5 mg $L^{-1}$of picloram, 104.28 days in full strength MS+2 mg L^{-1} of picloram and 106.57 days in full strength MS+1.5 mg L^{-1} of picloram.

Primary embryos initiated in full strength SH medium transferred to semi solid SH (1.5 per cent sucrose) for the multiplication, 40per cent of the primary embryos multiplied. Rate of multiplication was more than ten per primary embryo. The multiplied embryos when transferred to full strength liquid SH regenerated in to plantlets with 73.45 days of inoculation. When the multiplied embryos were transferred to liquid SH (3.5 per cent sucrose), they took only 43.26 days for regeneration.

Table25.Responses of primary embryo on secondary embryogenesis in SH media with different concentration of sucrose

Treatments	**Media**	**PGR conc. (mg L^{-1})**		**Response on secondary embryo**
		BA	Picloram	
T1	SH+1.5% Sucrose	-	-	0
T2	SH+4.5% Sucrose	-	-	0
T3	½ SH+3%sucrose	-	-	0
T4	SH+3%sucrose	-	-	+
T5	Liquid SH+3%sucrose	-	-	0
T6	Liquid SH+3.5%sucrose	-	-	0
T7	Liquid SH+4%sucrose	-	-	0
T8	Semi solid SH+3%sucrose	-	-	+
T9	SH+3%sucrose	1	1	0
T10	SH+3%sucrose	1	1.5	0
T11	SH+3%sucrose	1	2	0
T12	SH+3%sucrose	2	4	0
T13	SH+3%sucrose	1.5	1	0
T14	SH+3%sucrose	1.5	1.5	0
T15	SH+3%sucrose	1.5	2	0
T16	SH+3%sucrose	3	4	0
T17	SH+3%sucrose	2	1	0
T18	SH+3%sucrose	2	1.5	0
T19	SH+3%sucrose	2	2	0
T20	SH+3%sucrose		1	0
T21	SH+3%sucrose	-	1.5	0
T22	SH+3%sucrose	-	2	0
T23	MS+3%sucrose	-	-	0

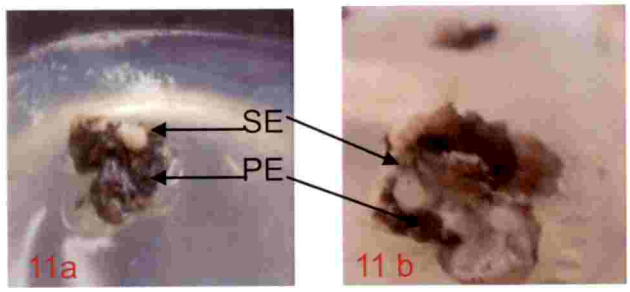
Table26.Responses of primary embryo on secondary embryogenesis in MS media with different concentration of sucrose

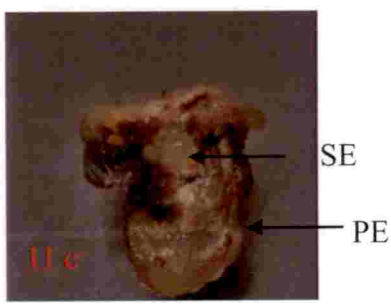
Treatments	Media	PGR conc. (mg L^{-1})		Response on secondary embryo
		BA	Picloram	
T1	MS+3%sucrose	-	-	0
T2	MS+1.5% Sucrose	-	-	0
T3	MS+4.5% Sucrose	-	-	0
T4	Liquid MS+3%sucrose	-	-	0
T5	Semi solid MS+3%sucrose	-	-	0
T6	MS+3%sucrose	1	1	0
T7	MS+3%sucrose	1	1.5	0
T8	MS+3%sucrose	1	2	0
T9	MS+3%sucrose	2	4	0
T10	MS+3%sucrose	1.5	1	0
T11	MS+3%sucrose	1.5	1.5	0
T12	MS+3%sucrose	1.5	2	0
T13	MS+3%sucrose	3	4	0
T14	MS+3%sucrose	2	1	0
T15	MS+3%sucrose	2	1.5	0
T16	MS+3%sucrose	2	2	0
T17	MS+3%sucrose	-	1	0
T18	MS+3%sucrose	-	1.5	0
T19	MS+3%sucrose	-	2	0
T20	MS+3%sucrose	-	4	0

Table27. Secondary embryogenesis from different media

Embryo initiated medium	Multiplication medium	Multiplication %	Days taken to initiate regeneration	Regeneration medium	Regeneration %
SH	SH	20	83.16	SH	100
SH+1.5% Sucrose	SH	20	81	SH	100
½ SH	SH	20	82.85	SH	100
SH+ 2 mg L^{-1} picloram	SH	0	92.71	SH	70
SH+ 1.5 mg L^{-1} picloram	SH	0	93.14	SH	80
MS+2 mg L^{-1} picloram	SH	0	104.28	SH	80
MS+1.5 mg L^{-1} Picloram	SH	0	106.57	SH	70
SH+3%sucrose	Semi solid SH+1.5% sucrose	40	73.45	Liquid SH	51
SH+1.5% Sucrose	Semi solid SH+1.5% sucrose	50	62.14	Liquid SH	50
½ SH+3%sucrose	Semi solid SH+1.5% sucrose	40	72.71	Liquid SH	50
SH+3%sucrose	Semi solid SH+1.5% sucrose	40	53.26	Liquid SH +3.5%Sucrose	50
SH+1.5% Sucrose	Semi solid SH+1.5% sucrose	50	48.73	Liquid SH +3.5%Sucrose	54
½ SH+3%sucrose	Semi solid SH+1.5% sucrose	40	54.9	Liquid SH +3.5%Sucrose	50

Plate 11. Secondary embryogenesis in full strength SH medium



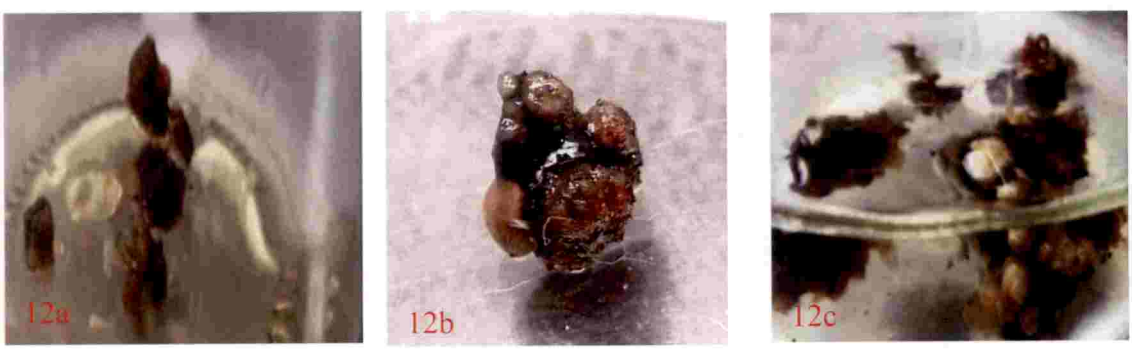


11a. Primary embryo (PE) from SH+3per cent sucrose

11b. PE from SH+1.5per cent sucrose

11c.PE from ½ SH+3per cent sucrose

Plate 12. Multiplication in semi solid SH (1.5per cent sucrose medium)



12a.Initiation of multiplication in semisolid SH (1.5per cent sucrose) medium

12b. Multiplied embryos

12c. Maximum multiplication in semi solid SH+1.5per cent sucrose

Fifty per cent of the primary embryos initiated in SH (1.5 per cent sucrose) were multiplied (Plate12) in semisolid SH medium with1.5per cent sucrose (maximum of 13 no). The multiplied embryos regenerated to plantlets within 62.17 days in full strength liquid SH and in 48.73 days in (Plate13) liquid SH (3.5 per cent sucrose).

Forty per cent of the primary embryos initiated in the1/2 strength SH medium were multiplied in semi solid SH (1.5 per cent sucrose) and regenerated in to plantlets within 72.71 days. In liquid SH (3.5per cent of sucrose), they regenerated within 54.9 days.

More number of multiplied somatic embryos were obtained with limited time, when the primary embryos induced on SH (1.5 per cent) sucrose were transferred to semi solid SH (1.5per cent sucrose). Quick regeneration was accessed when the multiplied embryos were transferred to liquid SH (3.5 per cent sucrose) (Table 26).

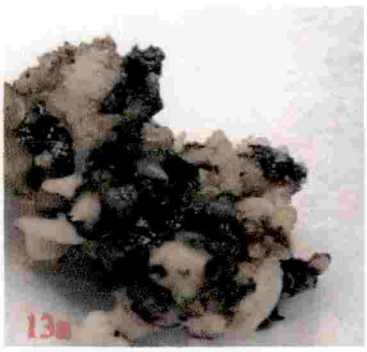
4.5 HISTOLOGY

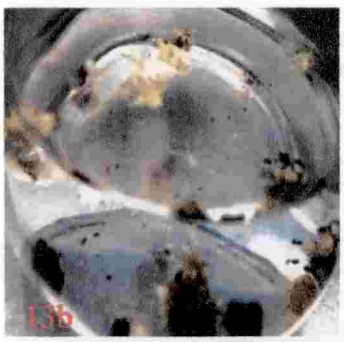
Different developmental stages of the somatic embryos *viz.* globular, heart shape, torpedo and cotyledanory (Plate14) were visible under stereomicroscope.

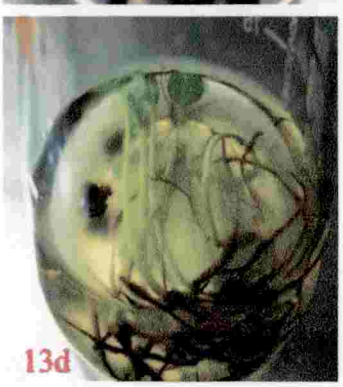
Microscopic view of the sections prepared showed emergence of globular structure from the callus (Plate 15a), cross sectional view of heart shaped embryos (Plate15b) with shoot and root pole (Plate15c), actively dividing of cells inside somatic embryos (Plate16b), and also the development of secondary embryos from the root pole of primary embryo (Plate16a).

Difference between the development of plants from zygotic embryo (Plate 17) and somatic embryo were confirmed by the cross sectional observation of the seeds having zygotic embryo as well as somatic embryo.

Plate 13. Detachment of individual embryos in Liquid SH and regeneration of plantlets







13a. Multiplied embryo

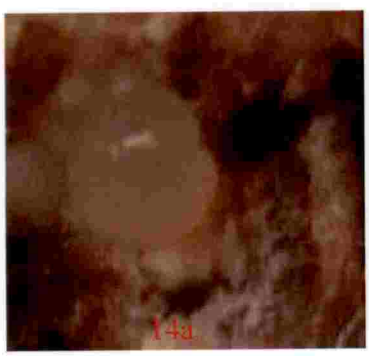
13b. Detachment of secondary embryos in liquid SH medium

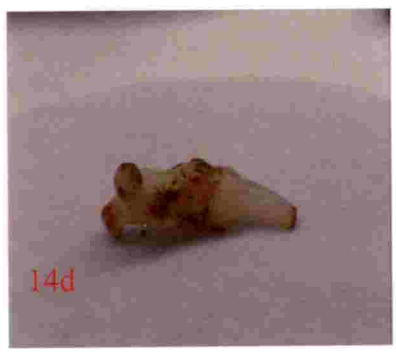
13c. Detached embryos (13 nos from single seed)

13d. Development of regenerated plantlets in liquid SH medium

13e. well developed plant transferred to plain medium

Plate 14. Developmental stages of somatic embryos





14a. Globular

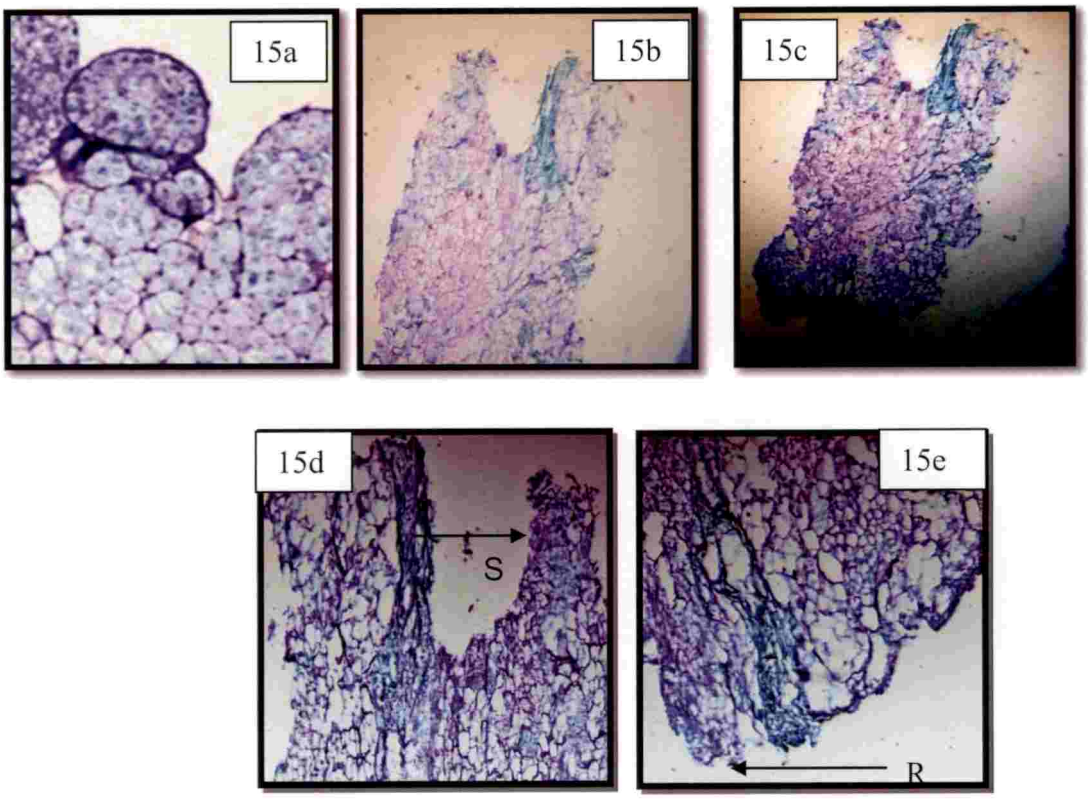
14b. Heart shaped

14c. Torpedo

14d. Cotyledanory

14e. Regenerated plant

Plate 15. Histology of the developmental stages of somatic embryo



15a. Globular structure arising from callus

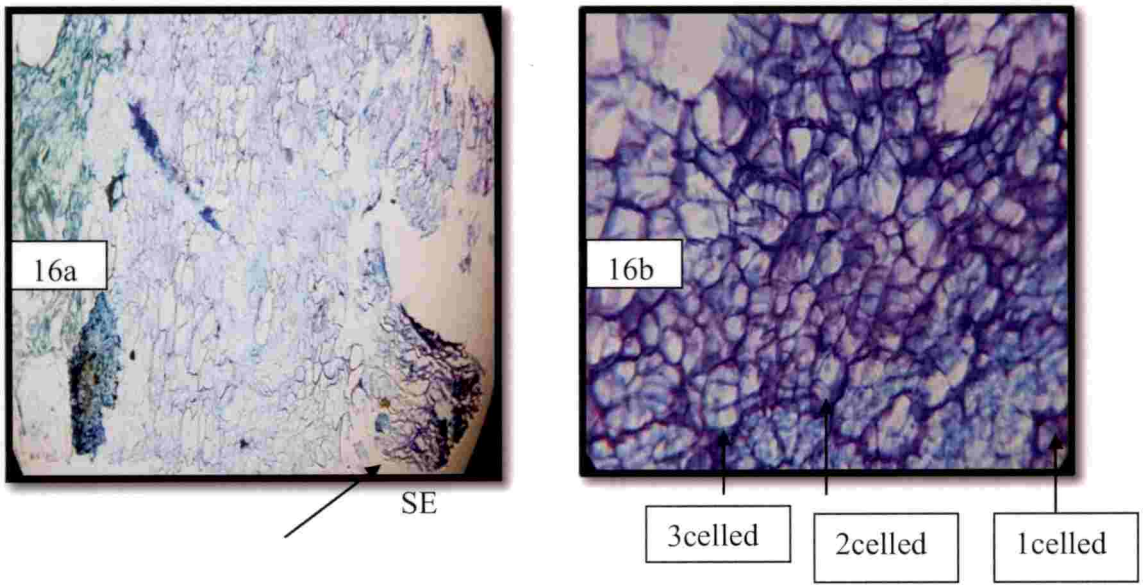
15b. Heart shaped embryo

15c. Torpedo

15d. Shoot pole(S)

15e. Root pole(R)

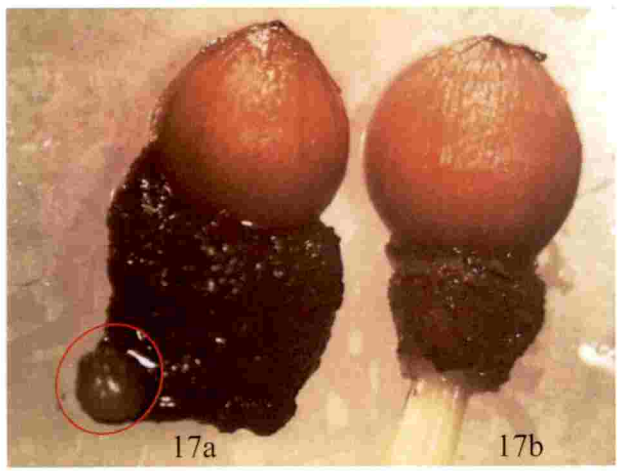
Plate 16. Histology of multiplication of somatic embryo



16a. Secondary embryo (SE) raising from primary embryo

16b. Active division inside secondary embryo

Plate17. Development of zygotic and somatic embryo



17a. Globular embryo arising from the ring tissue of the seed

17b. Plant development from zygotic embryo

Cross sectional view

4.6 *EX VITRO* ESTABLISHMENT OF PLANTS

The rooted plants at 2-3 leaf stage were taken and transferred to pots containing coir pith compost and sand media in 1:1 ratio (Plate 18) in greenhouse and acclimatized for 4 weeks. Hundred percent survival was noticed.

Plate 18. Hardened plants

Discussion

DISCUSSION

Black pepper is an important spice crop grown for its berries. Globally, India ranks 3rd for the production of black pepper after Vietnam and Indonesia. Black pepper production in India in continuously showing a declining trend. Biotic and abiotic stresses are considered as the major factors, but non availability of healthy, elite, and genetically uniform planting materials also contribute to the decrease in production. In black pepper vegetative propagation through stem cuttings is commonly followed. The conventional techniques are inadequate to meet the demand for planting material. As seeds yield heterogeneous progenies, propagation through seed is not advisable for stable production. In vitro propagation techniques are reported in black pepper, but with less efficiency. Future crop improvement programmes will be based on in vitro techniques and genetic manipulations, for which a single cell mediated regeneration protocol is highly essential. Somatic embryogenesis is the best option for that.

Somatic embryogenesis has been reported only in few varieties of black pepper. No reports are there on somatic embryogenesis in black pepper var.Panniyur-5. All the available reports on the somatic embryogenesis in black pepper is from the maternal tissue of the zygotic embryos. In this study other explants viz. shoot tip, node and leaf ere also tried for the induction of somatic embryogenesis.

The present study was under taken at the Department of Plant Biotechnology, College of Agriculture, Vellayani under the title "Somatic embryogenesis in black pepper (*Piper nigrum* L.) var. Panniyur 5" for developing a reliable protocol for somatic embryogenesis in this variety.

5.1 ESTABLISHMENT OF INVITRO CULTURE TECHNIQUE

5.1.1 Surface sterilization and pre treatments

Various types of explants were used for the induction of embryogenic callus and somatic embryos from the black pepper. Major constraints in the *in vitro* propagation of black pepper are the presence of systemic endophytes and the high polyphenolic content which oozes out into the medium. So, different pre treatment methods were tried to minimize the rate of contamination.

For checking the interference of polyphenolics, ascorbic acid 250 mg L^{-1} for 50 min and PVP 50 mg L^{-1} for 1 hour were found to be effective and for systemic contaminants 300 mg L^{-1} of cefotaxime was effective among the all the treatments tried. Also in the present study, addition of $CuSO_4$ 15 mg L^{-1} in to the culture media reduced the rate of contamination. Previous studies in black pepper showed that culture medium containing 100 mg L^{-1} of $CuSO_4$ supported the maximum growth of explants, with minimum bacterial contamination in black pepper and 15 mg $L^{-1}$of $CuSO_4$ in the medium increased the proliferation of shoot (Rajmohan *et al.*, 2010).

Dipping the explants in mercuric chloride 0.08 per cent for 7 minutes followed by rinsing with double distilled water for 3-4 times was found to be effective for reducing the microbial load. For seeds 0.1per cent mercuric chloride was found to be effective. It was followed based on earlier reports (Sasi and Bhat, 2015).

5.2 CALLUS INDUCTION FROM DIFFERENT EXPLANTS OF BLACK PEPPER

The selection of explant has an important role in determining the efficiency of *in vitro* propagation (Abbasi *et al.*, 2007). Morphogenetic potential of root, leaf, and node and inter node explant of *P. nigrum* was studied by Bhat *et al.* (1995). There are also other reports on *in vitro* techniques by using mature shoot-tip explants (Nazeem *et al.*, 1992; Philip *et al.*, 1992; Babu *et al*; Joseph *et*

al., 1996), Leaf explants (Sujatha *et al.*, 2003), node explants (Bhat *et al.*, 1992), and seeds (Nair and Gupta, 2006). In the present study, leaf, shoot tip and stem node taken from field grown plants *in vitro* raised seedling were taken. Seeds were also used.

5.2.1 Callus initiation from field grown explants

In the present study, callus induction was tried in MS and SH media with different PGRs using explants taken from field grown plants. Maximum callus induction was recorded in the MS medium supplemented with 1 mg L^{-1} of picloram (37per cent) from leaf tissue, followed by MS+ 1 mg L^{-1} picloram from shoot tip and node, MS+1.5 mg L^{-1} picloram from shoot tip, leaf and node, SH+ mg L^{-1} picloram from leaf and shoot tip and 1.5 mg L^{-1} BA in MS and SH medium from leaf and shoot tip (28.5per cent). Even though callus initiation was obtained from *ex vitro* raised explants, in later stages of subculturing they got blackened due to polyphenolic oxidation and finally died.

5.2.2 Callus initiation from *in vitro* raised explants

In the present investigation, callus induction was tried in MS and SH media with different combination and concentration of plant growth regulators (2, 4-D, BAA, IAA, NAA, TDZ and picloram) from *in vitro* raised explants.

Callus was induced from all the explants tried with most of the PGRs. MS medium supplemented with 1.5 mg L^{-1} of picloram produced highest callus induction from leaf tissues taken from *in vitro* raised explants (68.75per cent), followed by 1 and 0.5 mg L^{-1} of picloram, Picloram+TDZ (1, 0.5 mg L^{-1}) (62.5per cent) in MS medium and 0.5 mg L^{-1} of picloram in SH medium (62.5per cent). In case of shoot tip and node maximum callus induction was recorded in MS medium supplemented with 1 mg L^{-1} of picloram (52.17 and 42.87 per cent respectively).

Callus induction was higher in both the MS and SH medium while using picloram at range of 0.5,1 and 1.5 mg L^{-1} in all the three explants and did not show callusing in any of the treatment tried with 4 mg L^{-1} of picloram. Addition

of picloram along with other PGRs like TDZ and BA also produced callusing at lower concentrations from leaf and shoot tip.

Hussain *et al.*, (2011) reported that 1 mg L^{-1} BA supplemented MS medium produced callus from the leaves of black pepper. In this study leaf tissues of the panniyur 5 were the most responsive explants for callus induction compared to shoot tip and node. 1 and 1.5 mg L^{-1} of BA produced 57.14 per cent of callus induction from leaf tissue on MS medium. The same concentration of BA produced 42.84per cent of callus induction in SH medium.

Shaylaja and Nair (2000) reported callus induction from stem and leaf segments of black pepper variety Panniyur 1 and 4 cultivars on MS medium supplemented with IAA and BA 1 mg L^{-1} each. They also reported that callus induction, proliferation and regeneration are significantly differed in according to the genotypes of the variety. In this study various combinations of BA and IAA showed callus induction. Similar results were obtained in this study also, where maximum callus induction was recorded with the addition of 1 mg L^{-1} each of BA and IAA from leaf tissues both in MS and SH medium.

Combination of BA and 2, 4-D also produced callusing in a few treatments tried in leaf. Callogenic response was more when 2,4-D alone was used. Hussain *et al.*, (2011) have also reported the callogenic responses of different concentration of 2, 4-D from shoot tip, stem and leaf in MS medium.

In the present study while trying with different concentration (0.5,1,1.5 and 2 mg L^{-1}) of BA, highest callusing was induced on MS medium supplemented with 1 and 1.5 mg L^{-1} of BA from leaf tissue (57.14per cent), followed by shoot tip on MS medium with 1.5 mg L^{-1} of BA. The results of the study conducted by Hussain *et al.* (2011) are supportive to this, where they used different explants from mature vine for callus induction. Best callus was produced on MS medium with 1.5 mg L^{-1} BA by shoot tip explant.

Calli initiated from shoot tip, node, and leaf tissues were did not show any embryogenic response in any of the treatments tried. They were blackened and degenerated.

5.3 SOMATIC EMBRYOGENESIS FROM SEEDS

Fully ripened seeds of panniyur 5 were selected as explants for the induction of somatic embryogenesis.

There are a few reports on somatic embryo induction from the ring tissue on the micropylar region of the zygotic embryo of black pepper (Joseph *et al.* 1996; Nair and Gupta,2003; Manju and Soniya, 2012) of other varieties.

5.3.1 Direct Somatic embryogenesis

Among the 48 treatments tried, only 7 treatments responded positively for the initiation of somatic embryogenesis from seeds. Direct embryogenesis was observed from the micropylar region of the seeds in SH with 1.5sucrose and 3 per cent per cent sucrose and half strength SH with 3 per cent sucrose. Maximum embryogenesis (42.8 per cent) was obtained in SH medium with 1.5 per cent sucrose in 48.28 days. In SH with 3 per cent sucrose, 31.42 per cent somatic embryogenesis was observed out of which, 5 per cent of the seeds produced multiple embryos to a maximum of 4 numbers per seed. All the embryos formed were easily detachable and got matured and regenerated into plantlets in the same media. The results are in agreement with the reports of Nair and Gupta (2003), where they observed the direct somatic embryogenesis from the sporophytic tissues of germinating seeds of black pepper (*Piper nigrum*). Their ontogeny from single cells was confirmed. A report is also available on callus mediated somatic embryogenesis from zygotic embryos (Joseph *et al.* 1996).

Sucrose is reported to have an important role in somatic embryogenesis. As an osmotic regulator and as a major carbon source, it stimulates proliferation and regulates the developmental stages of embryos (Nair and Gupta, 2006). So the optimum concentration for somatic embryogenesis may vary with crops. Biju *et al.* (1996) reported somatic embryogenesis from zygotic embryos cultured on solid and liquid basal SH medium. In their study also maximum number of

embryos were obtained when the sucrose concentration was reduced to 1.5 per cent.

5.3.2 Indirect somatic embryogenesis from seeds

MS and SH media supplemented with1.5 and 2 mgl^{-1} of Picloram showed indirect somatic embryogenesis. SH medium with $2mgl^{-1}$ Picloram induced 57.14 per cent somatic embryogenesis in 41.42 days. These embryos were not able to mature and form plantlets in the same medium. But when transferred to MS or SH with 3 per cent sucrose without any growth regulators, all the embryos got matured and converted into plantlets.

Auxin in the proembryogenic mass is believed to play an important role in the globular stage of embryogenesis (Zimmerman, 1993). However, exposure to very high auxin content during development may prevent accumulation of storage protein leading to lower germination frequency (Stuart *et al.*, 1985). In the present study also increasing the concentration of picloram above 2 mg L^{-1}, prevented somatic embryogenesis.

Picloram has been reported to induce callus in many crops like banana, oil palm etc. (Leksmi *et al*; 2016; Madhavan *et al.*, 2015). Since Picloram acts as an auxin, the results obtained in the present study are in accordance with the reports of Zimmerman (1993). He reported that most of the protocols for somatic embryogenesis require a strong auxin. He has described the role of auxin present in the pro embryogenic mass for the induction of globular stages of the embryo.
The callus formed turned brown during subculturing and none of the fifty treatments produced somatic embryogenesis. In an earlier report, the callus induced by picloram 0.5 and 1.0 mg L^{-1} from seeds showed necrosis and failed to produce embryogenis response (Manju and Sonia, 2012). The embryos induced from the calli were not able to mature and regenerate in the same medium of induction containing picloram. They were matured and regenerated in to plantlets when transferred plain SH medium with3per cent sucrose. Zimmerman., (1993) also reported that if embryos are too much exposed to auxin during the stages of

development, they fail to accumulate storage protein and germinate at lower frequency.

Occurrence of direct secondary somatic embryogenesis in PGR-free media is well established in several crop plants (Smith and Krikorian 1988, Merkle *et al.*1990; Parra and Amo-Marco 1998; das Neves *et al.* 1999; Vasic *et al.* 2001). In the present study, direct somatic embryogenesis was occurred in SH+3 per cent sucrose (31.42 per cent), SH+1.5per cent sucrose (42.8 per cent) and half strength SH (37.14 per cent). Somatic embryos were induced from the micropylar region of the zygotic embryo. All the induced embryos were able to mature and regenerate in to plantlets in the same medium of induction.

In a similar work conducted by Nair and Gupta (2003), the explants cultured on full-strength SH medium containing 0.8 per cent (w/v) agar and sucrose 30 gL^{-1}without any plant growth regulators produced primary somatic embryos derived from micropylar tissues of germinating seeds after 90 days of culture.

In the present study embryos were induced in full strength SH medium with cent sucrose without any plant growth regulators within 51.57 days. And in addition to this embryos were induced in full strength SH medium +1.5 per cent sucrose within 48.28 and in half strength SH within 50.57 days.

5.3.3 Secondary embryogenesis

Secondary embryos were produced from the root pole of the primary embryos in full strength SH medium (20 per cent) and semi solid SH medium +1.5 per cent sucrose (50 per cent). Secondary embryos were visible within 10 days of inoculation as yellowish tissue and multiplied in to a mass of secondary embryos

Secondary embryos were visible from the root pole of the PEs within 10 days of culture. The brownish yellow tissue at the root pole of the PE proliferated

into a small mass of tissue from which several secondary embryos emerged (Nair and Gupta., 2005).

Proliferation and development of secondary embryos were strongly influenced by the strength of medium and sucrose concentration. Optimum secondary embryogenesis was observed in full-strength SH medium containing 1.5 per cent sucrose (Nair and Gupta., 2005). In contrast with this in the present study only primary embryos were induced in the SH medium supplemented with 1.5 per cent sucrose .Instead of this, semi solid SH medium with 1.5 per cent sucrose produced maximum number of secondary embryos.

Secondary somatic embryogenesis also been reported from several plants such as *Glycine max* (Finer 1988), *Juglans regia* (Tuleke *et al.* 1988), *Manihot esculenta*(Raemakers *et al.* 1993), *Trifolium repens* (Weissinger II and Parrott 1993), *Dalbergia sisso* (Das *et al.* 1997), *Medicago* sp. (das Neves *et al.* 1999), *Arachis hypogaea* (Little *et al.* 2000), *Phalaenopsis amabilis* (Chen and Chang 2004) and *Coffea* sp. (Giridhar *et al.* 2004). In most of the above examples, Secondary embryos originated from the regions such as cotyledons and hypocotyls of PE.

The secondary embryos formed in semi solid SH medium with 1.5per cent sucrose were not able to regenerate to plantlets in the same medium. They were detached from the mass of secondary embryos, only when transferred to liquid SH with different concentration of sucrose (1.5, 3 and 3.5 per cent sucrose). In this study, the best medium for maximum conversion of embryos in to plantlets was liquid SH+3.5 per cent sucrose. Similar observations were made by Sasi and Bhat (2016) in as study conducted on different varieties of black pepper. They reported that optimum sucrose concentration for conversion of cyclic secondary embryos to plantlets was 3.5per cent for all the varieties tried. Nair and Gupta (2005) reported the use of 3per cent sucrose for conversion of cyclic somatic embryo to plantlets

5.4 HISTOLOGY

Various developmental stages of the embryo *viz.* globular, heart, torpedo and cotyledonary were clearly visible under stereo microscope. In the present study histological analysis confirmed the origin of secondary embryos from the primary embryos. The development of globular structure was from the callus and not from the zygotic embryos.

Nair and Gupta (2003) reported that histological evidence indicates the origin of secondary embryos from the root pole of primary embryos and secondary embryogenesis occurred more frequently from PEs which were detached from the original explant than the ones remaining attached to it.

Summary

SUMMARY

The study entitled "Somatic embryogenesis in black pepper (*Piper nigrum* L.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2017, with an objective to standardise a protocol for somatic embryogenesis in black pepper (*Piper nigrum* L.) var. Panniyur 5.

In the present study, explants (shoot tip, node and leaf) collected from mature field grown plants and *in vitro* raised seedlings were used. Since black pepper contains systemic contaminants and high polyphenolics, the explants collected from field grown plants were pretreated. Keeping the explants in 250 mg L^{-1} ascorbic acid for 50 minutes and 50 mg L^{-1} of PVP for 1 hour, reduced the phenolic exudation. Treatment with bavistin 0.3 per cent for 30 min, followed by surface sterilization with mercuric chloride (0.08 per cent) for 7 minutes and 300 mg L^{-1} of cefotaxime for 1 hour was found effective in reducing the systemic contaminants.

Different combinations of 2, 4-D, BA, IAA, NAA, TDZ and picloram in MS and SH media were tried to study their effect on callus induction from the explants. Percentage of callus induction varied among type of explants as well as the medium used. Leaf tissue from *in vitro* raised plants was found to be most responsive to the callus induction (68.75per cent in 40 days) in MS medium supplemented with 1.5 mg L^{-1} of picloram. The same concentration of picloram in SH medium produced 57.4per cent callus induction from leaf. Shoot tip and node taken from *in vitro* raised seedling showed the highest callus induction in MS medium supplemented with 1 mg L^{-1} of picloram (57.14per cent and 42.85per cent respectively). Among the explants collected from the field grown plants, the maximum callus induction occurred in leaves (37per cent). Calli obtained from

leaf, shoot tip and node did not show embryogenesis in any of the 50 treatments and the calli turned black and dried.

As there was no response on the somatic embryo induction from shoot tip , stem node and leaf, somatic embryogenesis was tried in fully ripened seeds of Panniyur-5. Out of 48 treatments tried (MS and SH media, with different combination of 2,4-D,BA,IAA,NAA,TDZ and picloram), only seven treatments responded. In 46per cent of the seeds, somatic embryos emerged indirectly from the micropylar region of the seed and direct somatic induction was occurred in the rest (SH with 1.5 or 3 per cent sucrose and half strength SH with 3 per cent sucrose). The calli initiated from seeds inoculated in full strength MS and SH media supplemented with 1.5 and 2 mg L^{-1} picloram produced somatic embryos in the same medium. Somatic embryogenesis was early in SH medium (40 days) compared to MS medium (60 days). Five per cent of the directly induced embryos produced multiple embryos in full strength SH medium.

The embryo initiated in full strength SH medium, SH (1.5 per cent sucrose) half strength SH medium, when transferred to full strength SH medium, 20per cent of the primary embryos produced secondary embryos. 100 per cent of the multiplied embryos were regenerated in to plantlets in full strength SH medium, within 80-85 days. The embryo initiated in the full strength MS or SH medium containing picloram, when transferred to full strength SH, multiplication of the primary embryos was not observed, however, they regenerated in to plantlets in 92.71 days in full strength SH+2 mg L^{-1} picloram, 93.14 days in full strength SH+1.5 mg $L^{-1}$of picloram, 104.28 days in full strength MS+2 mg L^{-1} of picloram and 106.57 days in full strength MS+1.5 mg L^{-1} of picloram.

Fifty per cent of the primary embryos initiated in SH (1.5 per cent sucrose) were multiplied in semisolid SH medium (1.5per cent sucrose) (maximum of 13 no). The multiplied embryos regenerated to plantlets within 62.17 days in full stregth liquid SH and in 48.73 days in liquid SH (3.5 per cent sucrose).

Somatic embryogenesis was confirmed by visualizing the different developmental stages of the somatic embryos *viz.* globular, heart, torpedo and

cotyledonary stages under the stereo microscope. Microscopic view of the sections prepared showed emergence of globular structure from the callus, cross sectional view of heart shaped embryos with shoot and root pole, actively dividing of cells inside somatic embryos, and also the development of secondary embryos from the root pole of primary embryo.

Difference between the development of plants from zygotic embryo and somatic embryo were confirmed by the cross sectional observation of the seeds having zygotic embryo as well as somatic embryo.

The present study was successful in developing a somatic embryogenesis mediated regeneration protocol (induction of somatic embryo in SH+1.5per cent sucrose followed by multiplication and regeneration in semi solid SH +1.5per cent sucrose and liquid SH+3.5per centsucrose respectively) in black pepper var. Panniyur 5 which can be used for the i*n vitro* propagation and genetic modification based crop improvement programmes

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Somatic embryogenesis in black pepper (*Piper nigrum* L.)

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ABSTRACT

The study entitled "Somatic embryogenesis in black pepper (*Piper nigrum* L.)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2015-2017, with an objective to standardise a protocol for somatic embryogenesis in Black pepper (*Piper nigrum* L. *)* var. Panniyur 5.

Black pepper is one of the important spices in the world and is often referred as the 'King of Spices'. Among the many hybrids grown around the world, Panniyur-5 is the popular and demanded hybrid grown in Kerala (India). Research efforts in black pepper have been focused on hybridization, polyploid breeding and micropropagation to achieve the global demand for sustainable yield and high-quality produce.

In vitro propagation of black pepper has been reported using shoot tips, nodal segments, apical meristems (Philip *et al.*, 1992; Bhat *et al.*, 1995; Ahmad *et al.*, 2010). Regeneration through somatic embryogenesis is the most appealing technology in *in vitro* propagation since it produces large number of somatic embryos from single explant. Protocols for somatic embryogenesis in black pepper have also been reported (Joseph *et al.*, 1996; Nair and Gupta, 2006). In this context the present study was taken up to standardise a protocol for somatic embryogenesis in Panniyur-5 hybrid.

Systemic contaminants and polyphenols are the major check in *in vitro* culture techniques of black pepper. Pretreatments of *ex vitro* explant like shoot tip node and leaf tissue with 250 mg/l ascorbic acid for 50 minutes and 50 mg/l of PVP for 1 hour reduced the phenolic exudation. 20 mg/l of $CuSO_4$for 10 minutes and 300 mg/l of cefotaxime for 1 hour were found to be effective in controlling the systemic contaminants.

Callus was induced from different *ex vitro* and *in vitro* explants viz; shoot tip, stem node, leaf and seeds. Murashige and Skoog's (MS) medium and Schenk and Hilderbrandt (SH) medium containing different combinations and concentrations of plant growth regulators were used for callus induction. Percentage of callus induction varied among type of explants (*ex vitro* or *in vitro*) as well as the medium used. Callus induction was more in explants used from *in vitro* plants while it was less in explant used from *ex vitro* raised plants due to high interference of phenolics and microorganisms.

Leaf tissue from *in vitro* raised plants inoculated in MS medium was found to be most responsive to the callus induction than other explants and SH medium. Among the sixty one treatments tried in MS medium for the induction of callus using leaf, 1.5 mg L^{-1} of picloram (Pic) recorded the highest callus induction of 68.75%s in a period of 40 days. The same concentration of Pic in SH medium produced 57.4% callus induction from leaf. Callus obtained from leaf, shoot tip and node failed to induce somatic embryos.

Fully ripened seeds of panniyur-5 was used as explant after sterilization and it was inoculated on both MS and SH media with different combinations of PGR. Somatic embryos were induced directly from the micropylar region on the seed in plane SH, SH+1.5% sucrose, and ½ SH. Somatic embryos were also initiated indirectly through the callus produced on the micropylar region of the seed in media such as SH+2 mgL^{-1} Pic,SH+1.5 mgL^{-1}Pic,MS+2mgL^{-1}Pic and MS+1.5 mgl^{-1} Pic. Maximum embryo initiation was noticed in SH+2mgL^{-1}(57.17%) within 40-45 days of inoculation. In SH+1.5% sucrose, 42.8% of initiation also noticed. More number of the embryos got matured in SH+1.5% of sucrose (81.9%). Regeneration of plantlets was maximum in SH+1.5% of sucrose medium (90%). Maturation and regeneration of plants was not seen in callus obtained from SH and MS medium supplemented with Pic.

Secondary embryos were produced from the root pole of the primary embryo when kept for multiplication. In SH, 20% of the primary embryos

produced secondary embryos but the rate of multiplication in each embryo was very less. In semi solid SH+1.5% sucrose media, more than forty percent of embryos produced secondary embryos and the rate of multiplication was high. Fifty percent of the multiplied embryo could regenerate into plants in liquid SH+3.5% sucrose within 48.73 days.

Different developmental stages of the somatic embryos like globular, heart, torpedo and matured embryos were observed under a stereomicroscope. Histological studies of the embryos were done by the method developed by Sharma and Sharma, 1980.

The present findings says maximum number of somatic embryos can be initiated in SH medium supplemented with 2 mgL^{-1} Pic with less time. The somatic embryos initiated in SH+1.5% sucrose and ½ SH medium can be multiplied in semi solid SH+1.5% sucrose. The multiplied embryos from SH+1.5% sucrose can be regenerated in plants in liquid SH+3.5% sucrose with continuous shaking and this can be employed as a protocol for producing somatic embryogenesis in Panniyur-5.

Appendices

APPENDIX I

Stock solutions of Murashige and Skoog's medium

Constituent	Quantity (mg L^{-1})	Quantity required for preparing the stock (g)	Volume of stock (ml)	Concentration of stock	Volume required for 1L of medium (ml)
Stock A					
NH_4NO_3	1650	16.5	250 ml	40 x	25 ml
KNO_3	1900	19.0			
$MgSo_4.7\ H_2O$	370	3.7			
KH_2PO_4	170	1.7			
Stock B					
$CaCl_2.2H_2O$	440	8.8	100 ml	200 x	5 ml
Stock C					
H_3BO_3	6.2	0.62	100 ml	1000 x	1 ml
$MnSo_4.4\ H_2O$	22.3	1.69			
$Na_2MoO_4.2\ H_2O$	0.25	0.25			
KI	0.83	0.083			
$ZnSo_4.7\ H_2O$	8.6	0.860			
Stock D					
$Na_2EDTA2HO$	37.3	0.745	100 ml	200 x	5 ml
$FeSo_4.7H_2O$	27.8	0.556			
Stock E					
$CuSo_4.5H_2O$	0.025	0.125	250 ml	2000 x	0.5 ml
$COCl_2.6H_2O$	0.025	0.125			
Stock F					
Glycine	2	0.2			
Nicotinic acid	0.5	0.05	100 ml	1000 x	1 ml
Pryridoxine-HCl	0.5	0.05			
Thiamine-HCl	1	0.01			

APPENDIX II

Stock solutions of Schenk and Haberland medium

Constituent	Quantity (mg L^{-1})	Quantity required for preparing the stock (g)	Volume of stock (ml)	Concentration of stock	Volume required for 1L of medium (ml)
Stock A					
NH_4NO_3	300	0.12	250 ml	40 x	25 ml
KNO_3	2500	1.00			
$MgSo_4.7\ H_2O$	400	0.16			
$NH_4\ H_2PO_4$	300	0.12			
Stock B					
$CaCl_2.2H_2O$	200	0.4	100 ml	200 x	5 ml
Stock C					
H_3BO_3	5.00	0.05	100 ml	1000 x	1 ml
$MnSo_4.4\ H_2O$	10.00	0.10			
$Na_2MoO_4.2\ H_2O$	0.1	0.001			
$ZnSo_4.7\ H_2O$	1.00	0.01			
Stock D					
$Na_2EDTA2H_2O$	20.00	0.03	100 ml	200 x	5 ml
$FeSo_4.7H_2O$	15.00	0.04			
Stock E					
$CuSo_4.5H_2O$	139	69.5	250 ml	2000 x	0.5 ml
$COCl_2.6H_2O$	186.5	93.25			
Stock F					
Nicotinic acid	5	0.05	100 ml	1000 x	1 ml
Pryridoxine-HCl	5	0.05			
Thiamine-HCl	50	0.5			