GENETIC TRANSFORMATION OF BLACK PEPPER (*Piper nigrum* L.) FOR *PHYTOPHTHORA* FOOT ROT RESISTANCE / TOLERANCE

By
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THESIS

Submitted in partial fulfilment of the
requirement for the degree of

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DECLARATION

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

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CERTIFICATE

Certified that the thesis, entitled **"Genetic transformation of black pepper (*Piper nigrum* L.) for *Phytophthora* foot rot resistance/tolerance"** is a record of research work done independently by **Smt. Lissamma Joseph** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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EXTERNAL EXAMINER

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

BA	-	benzyl adenine
2,4-D	-	2,4-dichlorophenoxy acetic acid
CPPU	-	phenyl N' (2-chloro, 4-pyridial) urea
NAA	-	naphthalene acetic acid
BAP	-	benzylaminopurine
GA_3	-	gibberellic acid
TDZ	-	thidiazuron
min.	-	minute
h	-	hour
cm	-	centimetre
MS	-	Murashige and Skoog's medium
SH	-	Schenk and Hildebrandt's medium
O.D.	-	optical density
µM	-	micro molar
mM	-	milli molar
mg l^{-1}	-	milligram per litre
ppm	-	parts per million
GUS	-	beta glucuronidase
npt II	-	neomycin phospho transferase
hpt IV	-	hygromycin phospho transferase
PCR	-	polymerase chain reaction
Ti	-	tumour inducing
YEM	-	yeast extract mannitol
YEP	-	yeast extract peptone
LB	-	luria broth

Introduction

INTRODUCTION

Black pepper (*Piper nigrum* L.) popularly known as 'black gold' and 'king of spices' is the most important export oriented commodity and foreign exchange earner among the Indian spices. It is widely used in culinary preparations and food processing as a spice throughout the world. It also finds application in perfumery and medicines. India is a major producer and exporter of black pepper, the annual export being over Rs. 4000 million.

The productivity of black pepper in its native land, Kerala is very low (280 kg ha^{-1}) due to various contributory factors (Nybe *et al.*, 2007). Of these, crop loss due to diseases and pests is considered to be a major factor and foot rot disease caused by *Phytophthora capsici* is the most critical among them. The annual crop loss due to *Phytophthora* foot rot of black pepper on global scale is estimated to be around 4.5-7.5 million US dollars (Sarma and Kalloo, 2004).

Conventional breeding programmes to develop black pepper varieties resistant to foot rot disease have not been successful so far since high degree resistance is lacking in the available germplasm. The immunity exhibited by the distantly related wild species, *Piper colubrinum* to the foot rot disease could also not been exploited due to inherent hybridization barriers.

The advent of plant genetic transformation has lead to the possibility of genetically improving crops for enhanced resistance to insects and diseases. Several crops like tomato (Heath, 1985, Jongedijk *et al.*, 1995), tobacco (Howie *et al.*, 1994), rice (Lin *et al.*, 1995) and cucumber (Tabei *et al.*, 1998) were transformed with defense genes from different sources like rice, barley, tomato, petunia, tobacco and bean. The transgenics so developed were found to show enhanced disease resistance.

The possibility of exploiting the potential of transformation in black pepper for development of resistance to *Phytophthora capsici* by introducing defense genes like osmotin, glucanase etc. as reported in other crops is a novel idea. Among the various methods employed in transferring the foreign genes into crop plants,

Agrobacterium mediated method is the most popular one because of the high frequency of transformation, broad host range and high rate of expression of inserted genes.

The development of suitable plant regeneration protocols is one of the main prerequisites for the genetic improvement of crop plants using biotechnological methods. In genetic transformation, regeneration through somatic embryogenesis is preferred over organogenesis as in most cases somatic embryos are of single cell origin and chimaeric plants are less likely to develop. Hence, the present study was taken up with the following objectives,

1) To develop an *in vitro* regeneration protocol for somatic embryogenesis in black pepper.

2) To develop an efficient vector mediated genetic transformation system in black pepper for *Phytophthora* foot rot resistance / tolerance.

Review of Literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.) belongs to the family of Piperaceae and is one of the most important spices. A major constraint in the cultivation of the crop is the incidence of pests and diseases. Among these, foot rot disease of black pepper caused by *Phytophthora capsici* is the biggest constraint. Development of foot rot resistant cultures of black pepper through conventional breeding strategies have not yielded fruitful results.

Natural resistance to *Phytophthora* is not available in any known cultivars of black pepper or related species of *Piper* (Holliday and Mowat, 1963; Turner, 1973; Sarma *et al.*, 1982 and Vilasini, 1982). However, related wild species like *Piper colubrinum* and *Piper obliqum* showed resistance (Purseglove *et al.*, 1991). Natural hybridization barriers prevent the transfer of these resistance to black pepper. *In vitro* culture techniques have been applied to develop foot rot tolerant lines. Somaclonal variants and variants induced by gamma radiation have been screened against toxic metabolities of the pathogen for identifying cell lines showing tolerance to the pathogen (Shylaja, 1996 and Nazeem *et al.*, 1997).

Genetic transformation of black pepper with genes which can impart resistance to *Phytophthora capsici* is a promising alternative to conventional breeding. The natural ability of the phytopathogenic *Agrobacterium* for gene transfer to plants has been exploited for the genetic engineering of several woody species including rubber (Arokiaraj *et al.*, 1996) and Almond (Miguel and Oliveira, 1999). A successful molecular breeding approach through *Agrobacterium*-mediated genetic transformation would represent a significant step in overcoming the existing constraints in pepper improvement programmes. There are three basic requirements for the introduction of foreign genes in to plants. They are the availability of agronomically useful genes, a method for transferring those genes into plant cells and a protocol for regenerating whole plants from transgenic cells.

2.1 APPLICATION OF *IN VITRO* CULTURE TECHNIQUES IN TRANSFORMATION

The development of suitable protocols for plant regeneration is one of the main pre requisites for the genetic improvement of crop plants using biotechnological methods. Direct organogenesis, indirect organogenesis and somatic embryogenesis have been effectively utilized in production of genetically transformed plants.

2.2 SOMATIC EMBRYOGENESIS

Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without the fusion of gametes. Somatic embryogenesis provides a suitable system for efficient micropropagation and for production of transgenic plants. It also provides a model system for studying the early events of plant embryo development (Zimmerman, 1993).

Two general types of somatic embryogenesis may occur, direct embryogenesis in which embryos originate directly from tissues in absence of conspicuous callus proliferation and indirect embryogenesis in which callus proliferation and embryogenic tissue precede embryo development. Direct embryogenesis reduces the time required for plant regeneration, which may be beneficial to minimize culture-induced genetic changes and helps to retain clonal fidelity.

The induction and regeneration of somatic embryos is very sensitive to culture conditions such as the composition of the medium, physical environment of the culture, the genotype and the explant source.

2.2.1 Carbohydrate source

It is well documented that specific carbohydrates may have differential effects on morphogenesis *in vitro*. The concentration and type of exogenous carbohydrate has been found to promote embryo formation in various crop species.

Sucrose has been the most tested carbon source and osmoticum for somatic embryogenesis in angiosperm species. The effective concentration for initiation of embryogenic callus ranged among species from one per cent to 12 per cent (Lu *et al.*, 1982). Nevertheless, a variety of carbohydrates were found superior to sucrose in somatic embryogenesis of *Daucus carota* (Verma and Dougall, 1977) and *Medicago sativa* (Strickland *et al.*, 1987).

In alfalfa, maltose stimulated embryo yield and improved embryo morphology at low concentrations and was superior to sucrose when used at equal osmolarity (Strickland *et al.*, 1987). The authors concluded that the effect of maltose is primarily nutritional and not osmotically mediated. It is also suggested that maltose is broken down more slowly than sucrose providing a metabolizable carbon source over a longer period of *in vitro* culture (Orshinsky *et al.*, 1990).

Monosaccharides, especially glucose increased pollen-derived somatic embryos and plant production in *Triticum aestivum* (Chu *et al.*, 1990). The use of maltose in wheat anther culture resulted in greater microspore callus induction and green shoot regeneration than use of sucrose containing medium (Orshinsky *et al.*, 1990).

Sandra *et al.* (2000) reported significant differences for embryogenic response among *Coffea canephora* clones with respect to different carbohydrate sources added in the media. The number of somatic embryos per explant increased in genotypes N_{91} and N_{128} when fructose was substituted for sucrose as the sole carbohydrate source. N_{75} exhibited a high embryogenic response to maltose with a three fold increase over sucrose containing medium.

2.2.2 Growth regulators

Exogenously supplied growth regulators are essential in the process of somatic embryogenesis (Ammirato, 1983). Induction and development of somatic embryos were regulated by endogenous and exogenous growth regulators.

In general, the presence of auxins or substances with auxin activity is necessary for the induction and proliferation of cells that later differentiate into somatic embryos (Michalczuk *et al.*, 1992; Dejong *et al.*, 1993). The frequency of somatic embryo induction in peanut was dependent on the type and concentration of auxin used, 2,4-D was found to be the best auxin producing the highest frequency of responding culture and highest average number of somatic embryos per culture (Eapen and George, 1993). The effects of 11 different auxins and cytokinin like compounds were tested at four concentrations for their ability to induce primary and repetitive somatic embryos from mature, dry peanut epicotyls of genotype AT 120. Treatment with picloram and centrophenoxine at 83.0 and 124.4 µm resulted in the greatest number of embryos per explant and the highest percentage of responding explants (Little *et al.*, 2000).

Griga (1998) reported direct somatic embryogenesis from shoot apical meristems of pea. Somatic embryos were induced directly from meristematic tissues grown on a medium supplemented with 2.5 µm picloram.

Thidiazuron (TDZ) is a urea derivative with cytokinin like activity (Mok *et al.*, 1982) and stimulates shoot regeneration in a number of plant species. Within a species TDZ can stimulate the production of both somatic embryos and adventitious shoots (Fiola *et al.*, 1990).

Junyan *et al.* (1994) cultured unpollinated ovary explants of *Cayratia japonica* on MS medium supplemented with different concentrations of 2,4-D alone or in combination with 0.009 µM thidiazuron or 0.23 µM kinetin for the induction of embryogenic callus and somatic embryogenesis. They found that both cytokinin and auxin are required in the medium and the cytokinin activity of TDZ is much stronger than that of kinetin.

Griga (1998) observed improvement of germination of pea somatic embryos by application of 10 µM thidiazuron.

The influence of various growth regulators on induction of somatic embryogenesis of *Rosa hybrida* cultivars was evaluated (Li *et al.*, 2002). Thidiazuron (TDZ) was more effective than BA in inducing somatic embryogenesis and addition of 2.9 μM l^{-1} GA_3 to either TDZ or BA containing media at any level has improved the frequency of somatic embryogenesis. Combining BA and TDZ in the same medium was less effective in inducing somatic embryogenesis than either growth regulators used alone.

Ipekci and Gozukirmizi (2003) obtained highest induction frequencies of somatic embryos from *Paulownia elongata* in MS medium supplemented with 3 per cent sucrose, 0.61 per cent phytagel, 500 mg l^{-1} casein hydrolysate and 10 mg l^{-1} TDZ. Subsequent withdrawal of TDZ from the induction medium resulted in the maturation and growth of embryos into plantlets on MS basal media.

Abscisic acid (ABA) has been used to increase embryogenic response in tissue cultures of various monocot, dicot and conifer species. It is also used to promote and synchronise maturation of somatic embryos. ABA in the culture medium can promote the normal development of somatic embryos (Ammirato, 1974), arrest precocious germination of somatic embryos and promote the accumulation of storage reserves.

Desiccation affected the endogenous ABA level. Brown *et al.* (1989) reported that ABA and Mannitol promoted somatic embryogenesis in wheat. In *Corydalis yanhusao* direct somatic embryogenesis was observed at the junction of cotyledonary leaf and root when cultured on MS basal medium supplemented with 0-16 mg l^{-1} ABA and with 2.0 mg l^{-1} being optimum for further development (Kuo *et al.*, 2002).

2.2.3 Explants

The use of immature embryos as explants for the regeneration of white clover via direct somatic embryogenesis has been reported by Maheswaren and Williams (1984) and commented that in addition to predetermined potential of direct

somatic embryogenesis, embryogenic tissues frequently show enhanced potential for direct regeneration via organogenesis.

Somatic embryogenesis was strongly influenced by developmental stage of zygotic embryos. In *Prunus avium* (Ghislaine *et al.*, 1993) cotyledons from size class 2.5-3.5 mm was reported to be more embryogenic than those from size class (3.6-4.5 mm). Culture of immature zygotic embryos at various stages of development could lead to unique culture responses in comparison with mature zygotic embryos. Teixeira *et al.* (1993) used immature zygotic embryos of oil palm at different developmental stages for callus induction and regeneration studies. Compact embryogenic tissue began differentiating directly from embryo explants after two weeks of culture. The percentage of embryos forming compact embryogenic tissue ranged from 28.6 per cent for 91 day old embryos to 0 per cent for 140 day old embryos. Friable embryogenic tissue was observed in callus cultures derived from 100 day old embryos.

Park and Facchini (1999) reported a rapid protocol for high efficiency somatic embryogenesis and plant regeneration from seed derived embryogenic callus cultures of California poppy.

The effects of tissue culture condition and explant characteristics on direct somatic embryogenesis were studied on *Oncidium*. Embryo formation was significantly affected by explant position. Leaf tip segments had a significantly higher embryogenic response than other segments of leaves. Adaxial-side-up orientation significantly promoted embryogenesis in comparison with abaxial-side-up orientation (Chen and Chang, 2002).

Ipekei and Gozukirmizi (2003) developed a reproducible system for efficient, direct somatic embryogenesis from leaf and internodal explants of *Paulownia elongata*. Somatic embryos were induced from leaf (69.8%) and internode (58.5%) explants on MS medium after seven days.

Somatic embryos formed directly at cut edges or on the surface of leaf explants, around cut ends or along side surfaces of petiole and stem explants of

Golden *pothos* on MS medium supplemented with CPPU or TDZ with NAA (Zhang *et al.*, 2005).

2.3 STATUS OF *IN VITRO* TECHNIQUES IN BLACK PEPPER

Good callus growth was reported from explants of axenic seedlings (Mathews and Rao, 1984) as well as from mature vine of Black Pepper (Rajmohan, 1985). Variation in callus induction was observed among different cultivars of black pepper by Philip *et al.* (1995). Variation in callus induction and callus growth among different genotypes of black pepper were also observed by Shylaja (1996).

Successful callus mediated organogenesis from *in vitro* seedling explants of black pepper was reported by Nazeem *et al.* (1990). Indirect organogenesis from explants of mature vines of black pepper was further reported by Nazeem *et al.* (1993) and Philip *et al.* (1995), but with different hormonal combination. Philip *et al.* (1995) reported significant variation in callus mediated organogenesis among different cultivars of black pepper with Malamundi, Narayakodi and Karimunda showing the best response. Similarly, Shylaja (1996) found significant variation in regeneration from callus among different cultivars of black pepper with Panniyur-I showing the least response. Cheriyan (2000) reported callus regeneration from cotyledonary leaf explants to the extent of three per cent in Panniyur 1.

Micropropagation through direct organogenesis from bud culture has also been standardized in black pepper. Philip *et al.* (1992) developed a technique for micropropagation of black pepper from shoot tip explants taken from field grown plants. Later, Joseph *et al.* (1996) standardized a protocol for mass multiplication of planting material through *in vitro* micropropagation using nodal explants.

Plant regeneration through somatic embryogenesis has also been reported in black pepper. Joseph *et al.* (1996) were successful in inducing somatic embryos from embryogenic callus derived from zygotic embryos of black pepper. As per the report up to 1500 number of plants were produced from 5.0 mg of embryogenic callus obtained by culturing zygotic embryos in Schenk and Hildebrandt medium. Culturing

zygotic embryos in liquid SH medium containing 2,4-D (0.5-5 mg l^{-1}) did not yield embryogenic callus. Nair and Gupta (2003) developed a protocol for induction, maturation and germination of somatic embryos from the germinating seeds of black pepper in growth regulator free SH medium. Somatic embryos originated from a ring like tissue on the micropylar region of the seeds. Sucrose concentration of the medium was found to be crucial for the induction of somatic embryos and 30 g l^{-1} was found to be optimum. Suspension culture enhanced the process of maturation and germination.

Influence of various fruit derived explants and different genotypes on induction of somatic embryogenesis in black pepper was studied by Nair and Dutta (2005). Among the various explants cultured on plant regulator free solid SH medium, abortively germinated seeds *in vitro* produced the highest per cent response as well as number of somatic embryos per responded explant. Of the different genotypes tested, cultivar karimunda was found to be highly embryogenic.

2.4 *AGROBACTERIUM TUMEFACIENS* - THE NATURAL PLANT GENETIC ENGINEER

Agrobacterium tumefaciens is a soil bacterium which can genetically transform plant cells with a segment of DNA from the tumour inducing plasmid (Ti plasmid) to produce crown gall, a plant tumour. A number of sophisticated plant transformation vector systems, based on this naturally occurring gene-transfer mechanism have been developed and widely employed in plant molecular biology and genetic engineering.

Agrobacterium tumefaciens infection involves a complex series of processes that is initiated by a bacteria - host cell interaction and results in T-DNA transfer from the microbe into the plant cell and stable insertion into the host genome (Zupan and Zambryski, 1997). The most characterized genetic factors, which facilitate infection and T-DNA integration into the host genome are the Ti plasmid virulence (*vir*) genes.

Virulent strains of *Agrobacterium tumefaciens* are characterized by the presence of a large (200-250 kb) plasmid known as Ti plasmid (Scott *et al.*, 1988). These bacteria induce neoplastic growth on most dicots and gymnosperms (De Cleene and De Leney, 1976) but also on a few monocots (Bytebier *et al.*, 1987; Eady *et al.*, 2000). After attachment to cell walls of wound activated plant cells, *Agrobacterium* transfer part of their Ti plasmid (T-region) into the nucleus of the host plant where it becomes stably integrated (T-DNA).

The genes bracketed by the border sequence in a T-DNA, eventhough they are of prokaryotic origin, contain eukaryotic promoters. The genes encoded by the T-DNA can be replaced without interfering with the transfer of the T-DNA to the plant cell, as long as the 24 bp border sequences are maintained. The removal of oncogenes from T-DNA of the Ti plasmid and replacement with the desired gene permits the use of this bacterium for genetic transformation of plant tissue.

Foreign genes can be placed within the boundaries of the T-DNA either on the original virulent but disarmed plasmid (cis) or on an additional, separate non-virulent plasmid (trans).

2.4.1 Promoters and other regulatory DNA sequences

Plants are usually transformed with relatively simple constructs in which the gene of interest is coupled with an appropriate promoter, 5' leader and 3' terminator sequences to ensure efficient transcription, stability and translation of mRNA. The promoter can be of plant, viral or bacterial origin. Some promoters confer constitutive expression, whereas others may be selected to permit tissue specific expression or environmentally inducible expression.

Plant viruses which depend upon plant transcription and translation factors have been used as sources of regulatory elements. Most commonly used are the promoters of the 35S RNA of the cauliflower mosaic virus (CaMV). It directs high levels of expression in most species. Others such as maize ubiquitin 1 promoter, *rbees* (ribulose biphosphate carboxylase small sub unit), *Adh 1* (alcohol dehydrogenase) nos

(nopaline synthase) and the rice actin promoter / intron sequences are often preferred for expression in monocots.

The promoter derived from maize alcohol dehydrogenase / (Adh 1) 5' flanking sequence showed the level of expression in monocot cells equivalent to or higher than CaMV 35S promoters. The presence of the Adh 1 intron / between promoter and coding sequence has also been shown to increase expression of the *npt II* gene in maize (Callis *et al.*, 1987). When this intron was used with the CaMV 35S promoter, higher levels of expression were found than without the intron region. Similar results were obtained with the first intron of the maize Shrunken-1 locus (Vasil *et al.*, 1989).

2.4.2 Reporter genes

A reporter gene is a test gene whose expression results in a quantifiable phenotype. A reporter system is useful in the analysis of plant gene expression and standardization of parameters for successful gene transfer.

The bacterial genes chloramphenicol acetyl transferase (*CAT*) and neomycin phospho transferase (*npt II*) that encodes enzymes with specificities not normally found in plant tissues (Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983; Bevan *et al.*, 1983b) have been the most commonly useful reporter genes.

Neomycin phosphotransferase II (*npt II*) gene from transposon Tn5, detoxify neomycin, kanamycin and G 418 by phosphorylation. It is widely used in dicotyledon systems, including tobacco, potato and tomato (An *et al.*, 1986), legumes, such as clover (White and Greenwood, 1987) and pea (Puonti-Kaerlas *et al.*, 1989). But this marker gene proved unsuitable for some dicotyledonous species such as Arabidopsis, in which large number of non-transformed cells survive and for many monocotyledonous species whose growth is not significantly inhibited by the antibiotic (Potrykus *et al.*, 1985; Dekeyser *et al.*, 1989).

Hygromycin phosphotransferase (*hpt*) gene was originally derived from *E. coli*. This inactivates the antibiotic hygromycin. This gene was successfully used as a selectable marker in straw berry (Nehra *et al.*, 1990) and *Solanum* sp. (Kumar *et al.*, 1995).

The firefly luciferase gene has been used as a marker in transgenic plants (Ow *et al.*, 1986), but the enzyme is labile and difficult to assay with accuracy.

Jefferson *et al.* (1987) developed the *E. coli* β-glucuronidase gene as a reporter gene system for transformation of plants. It can be assayed histochemically to localize GUS activity in cells and tissues.

β-glucuronidase which is encoded by *uid A* locus (Novel and Novel, 1973) is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronides, many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates. The β-glucuronidase gene has been cloned and sequenced. It encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions (Jefferson *et al.*, 1987).

Stiekema *et al.* (1988) used GUS reporter gene for transforming potato plant. Eapen and George (1994) in their *Agrobacterium* mediated gene transfer in peanut found that the expression of GUS activity in the regenerated shoots was not directly correlated with kanamycin resistance. The lack of expression of the gus A gene in kanamycin resistant shoots may be due to the alteration or loss of gus A gene. The copy number and localization of insertion and subsequent rearrangements can significantly effect expression level of the gene. Methylation of GUS reporter gene is also known to alter gene expression in potato (Ottavisni *et al.*, 1993).

Genes conferring resistance to herbicide are also used as selectable marker. The Bar gene isolated from *Streptomyces hygroscopicus* confers resistance to the herbicide phosphinothricin (PPT), bialophose and gluphosinate. The bar gene codes for the enzyme phosphinothricin acetyl transferase (PAT), which converts

PPT/bialophose into non-herbicidal acetylated form. This gene has been inserted and expressed in tomato, tobacco and potato (De Block *et al.*, 1989).

Modified version of EPSP isolated from *E. coli* confers resistance to glyphosate in transformed plants. Such transformants have been produced in tomato (Filatti, 1987), tobacco (Comai *et al.*, 1985) and soybean (Hinchee *et al.*, 1988).

2.4.3 *Agrobacterium* mediated plant genetic transformation

The first report of plant genetic transformation using *Agrobacterium tumefaciens* was made in 1983, when a foreign gene was introduced and expressed in tobacco cells (Bevan *et al.*, 1983; Herrera-Estrella *et al.*, 1983; Fralely *et al.*, 1983). Since then this gene delivery system has been used widely in a number of crops and transgenic plants of commercial importance were produced (Lindsey, 1992).

2.4.3.1 Explant for genetic transformation

A basic requirement for successful genetic transformation is the receptivity of the cells to the T-DNA and the ability of the transformed cells to develop into whole plants.

Diverse plant tissues were found successfully transformed using *Agrobacterium tumefaciens*. Leaf (De Block *et al.*, 1989), callus (Komari, 1989), petiole (Pawlicki *et al.*, 1992), cotyledons (Park and Facchini, 2000), somatic embryo (Mondal *et al.*, 2001), hypocotyl (Zaldivar-Cruz *et al.*, 2003), root segments (Franklin and Lakshmi Sita, 2003), cell suspension (Fuentes *et al.*, 2004), embryo axes (Sujatha and Sailaja, 2005) etc. have been used for genetic transformation.

The choice of explant should depend upon the efficiency of the regeneration protocol developed. In an experiment in carrot, Pawlicki *et al.* (1992) found that of the different seedling tissues taken for transformation, petiole was found best compared to cotyledon, hypocotyl or roots. Similarly, four weeks old seedling responded better than one week old seedlings.

Wounded tissues have been used as explants in most of the transformation experiments involving inoculation of *Agrobacterium*. One hypothesis is that the efficiency of *Agrobacterium* infection is effected by the ethylene from wounded tissues. Ethylene is a factor involved in plant microbe interactions (Spanu and Boller, 1989) and is released from wounded tissues in plants.

An efficient *Agrobacterium* mediated protocol has been developed for the stable genetic transformation of intact opium poppy, plants via shoot organogenesis from excised cotyledons (Park and Facchini, 2000).

Franklin and Lakshmi Sita (2003) demonstrated for the first time an efficient genotype-independent (*Agrobacterium tumefaciens*) mediated transformation of egg plant using root explants.

Sanyal *et al.* (2003) evaluated mature embryo axes and cotyledonary nodes of chick pea for genetic transformation and the production of stable transgenic plants expressing the reporter gene *gus* was documented. The major limitation in delivering the T-DNA in these explants has been the locus frequency and inconsistency.

Sujatha and Sailaja (2005) achieved stable genetic transformation of castor using embryo axes from mature seeds.

2.4.3.2 Agrobacterium infectivity

Agrobacterium infectivity is a result of the interaction between the plant cell and the bacterial cell. The infectivity is improved by the use of right strain of the bacteria, varying host genotype, manipulating explant physiology, manipulating inoculating and co-cultivation conditions (Godwin *et al.*, 1992).

2.4.3.2.1 Strain specificity

Dicotyledonous plants were shown to be better responding to *Agrobacterium* mediated transformation than monocots. Even among dicots, there are differences in susceptibility between species and even between cultivars and

genotypes of the species. On the same genotype, different bacterial strains show differences in infectivity.

Various studies indicate that monocotyledonous and other recalcitrant species can be transformed using *Agrobacterium tumefaciens* by manipulating various factors such as explant tissues, inoculation and co-culture conditions. The systematic analysis and application of several factors, such as *Agrobacterium* strains, pre-induction of the bacteria, bacterial concentration, days of co-culture and wounding procedure can significantly improve the transformation efficiency of recalcitrant species.

Best bacterial strain and plant genotype compatible system can be selected based on the tumour producing ability of the native strains. This approach was used successfully in soybean where compatible systems were found (Owens and Cress, 1985; Byrne *et al.*, 1987) and subsequently used to obtain transgenic plants (Hinchee *et al.*, 1988). This method may not be useful for all species since tumour function is a function of both the degree to which the *vir* genes direct T-DNA transfer and the degree to which the hormones produced by T-DNA genes stimulates tumorigenic growth in a particular species. Hence, other alternative is to try transformation with different strains harbouring a good selectable marker, till we get the genotype/strain combination.

A 281 is a super virulent strain of *Agrobacterium tumefaciens* and its host range is wider and transformation efficiency is higher than those of other strains. Komari (1989) opined after conducting experiments with four strains of *Agrobacterium* that A 281 should be chosen as one of the strains when unfamiliar plant species or plant materials are to be transformed.

In genetic transformation experiments with carrot, Pawlicki *et al.* (1992) found that *Agrobacterium* strain C58 C_1 was more active than the LBA4404 strain in the infection and transformation of carrot explants. Likewise, Orlikowska (1995) reported that *Agrobacterium* strain EHA105/p35SGUSINT was more infective than LBA4404 with safflower cultivar 'Centennial'.

In a study with *Robinia pseudoacacia,* when the same binary vector PSMAH 704 was put in three disarmed strains of *Agrobacterium tumefaciens*, namely LBA4404 (pAL 4404); EHA101 (pEHA 100) and GV3101 (pMP 90), tremendous differences were seen in the rate of transformation. Strain GV3101 (pMP 90) gave the highest frequency of transformation of *Robinia pseudoacacia* (Igasaki *et al.*, 2000).

Strain specificity has also been reported in black pepper (Sasikumar and Veluthambi, 1996). Here binary vector (LBA4404 and EHA105 harbouring pGA 472) was found to successfully transform black pepper whereas cointegrate vector (pGV 2260-pCOSFR 280) did not produce transformation. However, these results were based only on phenotypic observation.

2.4.3.2.2 Pre-culture

This is the culture of the explants on the inoculation medium prior to co-cultivation.

Venkatachalam *et al.* (2000) pre-cultured the cotyledonary explants of ground nut for 0, 1, 2, 3, 4 and 5 days and transformation frequency was calculated by their embryo forming ability in the selection medium. When cotyledon explants were pre-cultured on the regeneration medium for two days, the hypersensitivity response was reduced compared to co-cultivation without pre-culture. Pre-culture of explants in the regeneration medium for 2 days prior to co-cultivation was found to be optimum for efficient transformation. However, in tea, Mondal *et al.* (2001) reported that somatic embryos directly infected with *Agrobacterium* without pre-culture showed a higher transformation competence than the ones cultured prior to bacterial treatment.

Li *et al.* (2003) studied the effect of the length of differentiation rate of cotyledon explants of *Capsicum annuum* and found that the explants pre-conditioned for a relatively long time can not be infected well by *A. tumefaciens.* In their study the 2-day period gave the best result with differentiation rate of 85%.

2.4.3.2.3 Inoculation/Infection time and media

The explant, in their most receptive stage is exposed to the *Agrobacterium* culture in the inoculation medium at an optimum bacterial density. Both the composition of the inoculation media and time of inoculation may have a role in the efficiency of transformation.

For inoculation, regeneration medium of the explant that can support bacterial growth is used. Murashige and Skoog medium can support both in most of the cases. The pH of the medium usually maintained range from 5.5 to 5.7. For infection the explants are mostly immersed in the inoculation medium for a time varying 30 seconds to 1 hour.

Archiletti *et al.* (1995) tried various inoculation times ranging from 10 minutes to 180 minutes during their transformation studies in almond. Using MS medium for inoculation they found that 30 min was sufficient for infection to occur, while longer time did not increase the transformation frequency and caused problems in eliminating bacteria.

Le *et al.* (1996) reported inoculation time of one h for successful transformation of *Casuarina glauca*. Transformation works in *Pinus pinea* revealed that the inoculation time of five min gave better transformation frequency compared to 30 minutes of inoculation time (Humara *et al.*, 1999).

Fatima *et al.* (2005) studied the effect of different infection times on survival rate of explants of capsicum and found survival rate of transformed tissues was highest (85%) in 10 min when compared to 50 per cent and 45 per cent in 5 and 15 min respectively.

Jayasree *et al.* (2005) evaluated the effect of co-cultivation period on transformation frequency of rubber tree by culturing *Agrobacterium* infected calli for 1, 2, 3, 4 and 5 days in the co-cultivation medium. The highest transformation frequency (4%) was observed following a 3 day co-cultivation. No transformation was

observed when the callus was transferred to selection medium immediately after infection with *Agrobacterium* (without co-cultivation).

2.4.3.2.4 Elimination of bacteria after co-cultivation

After co-cultivation, the explants are transferred to media containing bacteriostatic agent to kill the bacteria. Complete elimination of the bacteria from the explant after co-cultivation is very essential otherwise it will interfere with the growth and organogenesis of the explant. Elimination of the bacteria from the explant is done by use of antibiotics. The most commonly used antibiotics for this purpose are carbencillin and cefotaxime.

Cefotaxime has been used at different strengths for elimination of *Agrobacterium* during transformation works. It was used at a strength of 200 mg l^{-1} in white clover (Voisey *et al.*, 1994) and 300 mg l^{-1} in phalaenopsis orchid (Belarmino and Mii, 2000).

2.4.3.3 Screening

Screening of untransformed cells or selection of transformed cells is an important aspect of transformation work. The explants are transferred to a selection medium containing appropriate selection agent depending on the plant selectable marker employed for transformation.

2.4.3.3.1 Pre-selection

Selection pressure immediately after co-cultivation leads to reduced rate of transformation in many crops. Here selection pressure applied a couple of days after co-cultivation gives better recovery of transformed shoots.

Leaf disc transformation in almond done by Archilletti *et al.* (1995) revealed that the most critical factor in successful transformation was a pre-selection of six days. This may be because the death of untransformed cells may inhibit the

transformed cells. However, a pre-selection of ten days had deleterious effect on transformation efficiency.

Based on the experiments with *Moricandia arvensis*, Rashid *et al.* (1996) reported that a pre-selection period after co-cultivation was essential for successful transformation. When selection using 200 mg l^{-1} kanamycin was done immediately after co-cultivation no callus growth was observed .Similar result was obtained when selection on kanamycin was started after five days from co-cultivation. However, shoot regeneration from calli preceded best when kanamycin selection was done seven days after co-cultivation.

2.4.3.3.2 Selection of transformed cells

Genes conferring resistance to a variety of toxin compounds, such as antibiotics and broad range herbicides, have been fused to suitable promoters and used to select and identify transformed cells. Thus a range of selectable marker genes are now available for *Agrobacterium* mediated transformation.

Neomycin phosphotransferase II (*npt II*) conferring resistance to kanamycin has been used most widely as a selectable marker in transformation works primarily because resistance to kanamycin is generally not found in plant tissue.

Sasikumar and Veluthambi (1994) have reported that 50 mg l^{-1} of kanamycin completely arrests callus induction on leaf segments of black pepper. Sin *et al.* (1998) had used 75 mg l^{-1} of kanamycin for selection of transformed cells in black pepper. Cheriyan (2000) reported that callus induction on black pepper leaf was completely inhibited at 50 mg l^{-1} kanamycin and callus growth at 100 mg l^{-1}. Hygromycin at 10 mg l^{-1} completely suppressed callus induction on leaf and callus growth at 30 mg l^{-1}.

There are instances in alfalfa where *npt II* gene even after integration into host genome failed to produce enough of the enzyme to give adequate kanamycin resistance to the transformed cells (Pezzotti *et al.*, 1991) and in cultivar, Adriana,

kanamycin had to be withdrawn from culture medium for regeneration of plantlets during transformation. Nagaraju *et al.* (1998) in trying to find an appropriate selection condition for *Gerbera hybrida*, found that though the transformed calli grew at 40 and 60 mg l^{-1} of kanamycin, the explant turned brown. Hence kanamycin at a minimal concentration of 20 mg l^{-1} was chosen for transformation experiments.

In crops where regeneration through embryogenesis is resorted to for transformation, kanamycin seems to have a detrimental effect on embryogenesis. Hadfi and Batschauer (1994) reported that they had to transfer explants to kanamycin free media as soon as embryonic nodules started appearing on the explant, otherwise it dedifferentiated into callus again. Similarly, Jun *et al.* (1995) during their transformation work in Chineese cabbage had to transfer the explants soon after induction of shoots from screening media to rooting medium without kanamycin, as kanamycin inhibited root formation.

Joersbo *et al.* (1998) utilized phosphomannose isomerase (PMI) gene as selectable marker and mannose as selective agent in the successful transformation of sugarbeet. The selection pressure was about 1-1.5 g l^{-1} of mannose. They reported 10 fold increase in transformation efficiency compared to *npt II* kanamycin selection system.

2.5 PATHOGENESIS RELATED (PR) PROTEINS

Plants have defense system which involve pathogen-related proteins like chitinase (Legrand *et al.*, 1987; Shinshi *et al.*, 1990; Nishizawa and Hibi, 1991) and ß 1,3-glucanase (Kombrink *et al.*, 1988). Genes encoding hydrolytic enzymes such as chitinase, which can degrade fungal cell wall components are attractive candidates for improving disease resistance. It has been reported that transgenic tobacco plants that constitutively express a bean chitinase gene showed increased resistance to the fungal pathogen, *Rhizoctonia solani* (Broglie *et al.*, 1991). In addition, several studies have been made on transgenic plants integrated with chitinase genes.

The transgenic rice expressing rice endochitinase exhibited resistance to blast (Nishizawa *et al.*, 1999). While transgenic cucumber (Tabei *et al.*, 1998) and strawberry (Asao *et al.*, 1997) harbouring rice chitinase possessed increased resistance to various fungal diseases.

Yamamoto *et al.* (2000) evolved transgenic grape vine plant harbouring a rice chitinase gene from somatic embryos by *Agrobacterium* infection. The transformants showed enhanced disease resistance to powdery mildew and anthracnose.

Transformation of *Brassica juncea* with bacterial *cod A* gene which imparts tolerance to salt stress has been reported by Prasad *et al.* (2000). The stable insertion of the cod A gene in the shoots of the variety Pusa Jaikisan obtained on medium with kanamycin and hygromycin was confirmed by PCR analysis of *npt II* gene.

Osmotin is a basic 24 KD protein that was originally identified in tobacco cells adapted to NaCl and dessication. Osmotin has been implicated in conferring tolerance to drought and salt stress in plants. Barthakur *et al.* (2001) were able to over express the osmotin gene under the control of constitutive CaMv 35 S promoter in transgenic tobacco and studied involvement of the protein in imparting tolerance to salinity and drought stress. The transgenic plants exhibited retarded leaf senescene and improved germination on a medium containing 200 mM NaCl.

A pea chitinase and ß-1,3 glucanase genes in two independent plasmids (P^{chit} and P^{Gluc}) multiplied in separate *Agrobacterium tumefaciens* (LBA 4404) clones were simultaneously introduced into internode explants of *in vitro* grown potato (Chang *et al.*, 2002).

LEA proteins are classified into 3 major groups and LEA 3 proteins play a protective role in plant cells under various stress conditions. Wang *et al.* (2004) transformed strawberry with late embryogenesis abundant protein gene from barley. In comparison with control plants, the salt tolerance of transgenic strawberry was increased.

The Grapevine Fan Leaf Virus-Coat Protein (GFLVCP) gene was inserted through *Agrobacterium* mediated transformation in *Vitis vinifera* (Gambino *et al.*, 2005). Forty three transgenic lines were regenerated.

Takahashi *et al.* (2005) introduced the rice chitinase (cht-2; RCC2) gene into calli of Italian rye grass with *hpt* gene as a selectable marker by particle bombardment. PCR analysis revealed regenerants containing the *hpt* gene and RCC 2 gene was detected in 65.5 per cent of those regenerants.

2.6 GENETIC TRANSFORMATION BY PARTICLE BOMBARDMENT

The method of gene transfer using the particle bombardment is a versatile one and has proved successful in introducing foreign genes not only to the nucleus but also to the chloroplast in plants. It avoids bacterial co-cultivation and its elimination. DNA transfer by particle bombardment makes use of physical process to achieve the transformation of crops plants. Suitability of particle bombardment for the precision engineering of a variety of plants producing genetically enhanced varieties of crops with stable and high level transgene expression has been reported (Taylor and Fauquet, 2002).

Stable transgenic plants with bar and gus genes were produced in 3 wheat cultivars following micro projectile bombardment and the transformation frequencies ranged from 0.14 to 0.83% (Gopalakrishnan *et al.*, 2000).

Micro projectile bombardment mediated genetic transformation of *Sorghum bicolor* and transient expression of the gus gene was reported by Devi and Sticklen (2001). The expression vector with Gus gene driven by the rice actin 1 promoter was delivered by tungsten micro projectiles to the apical meristems of shoot tip clumps of sorghum. They observed higher degree of Gus expression with smaller particle size (0.9 µm), lower particle density (75 µg/shot), longer bombardment distance (9.8 cm), lesser acceleration pressure (1550 psi) and with higher osmoticum.

2.7 GENETIC TRANSFORMATION IN BLACK PEPPER

Literature on genetic transformation of black pepper is limited. Sin *et al.* (1998) reported about transformation studies on black pepper leaf and cotyledonary nodal explants with strain LBA 4404 and the presence of transient gus expression on infected explants.

Agrobacterium mediated transformation was also attempted by Cheriyan (2000) using strain AGL.1.1303, leaf explants and leaf derived calli. Effective elimination of bacteria after co-cultivation was the main hurdle reported by him. Black pepper tissues, both leaf and callus, were found to be susceptible to kanamycin and hygromycin when freshly prepared antibiotic stocks were used and tissues subcultured at two weeks interval. Callus induction on leaf was completely inhibited at 50 mg l^{-1} kanamycin and callus growth at 100 mg l^{-1}. Hygromycin at 10 mg l^{-1} completely suppressed callus induction on leaf and callus growth at 30 mg l^{-1}. Leaf transformation was carried out by varying different factors affecting transformations. None of the leaf explants showed callus induction in screening media. Cefotaxime at concentrations of 500 and 1000 mg l^{-1} could not eliminate *Agrobacterium* effectively from the leaf tissue.

Putative transgenics harboring osmotin were successfully regenerated from hypocotyl segments of *in vitro* grown seedlings through *Agrobacterium* mediated transformation. Presence of osmotin and *npt II* genes in the genome of the transformants were confirmed by PCR analysis (IISR, 2004). *Agrobacterium* strain GV 2260 with the gene Osmotin and pBZ100 with glucanase and chitinase genes were used for transformation studies in black pepper. Leaf explants from both juvenile as well as mature tissue, immature zygotic embryos and *in vitro* germinated seedlings were used as the tissue source of explants for transformation experiments. Following preculture on MS medium in the dark at 28°C, the excised explants were immersed in the bacterial suspension and after 15 minutes of incubation explants were blot dried and cultured under dark for 48 hours. After co-culturing, leaf explants, germinating embryos and hypocotyls were washed twice with sterile distilled water and once with

liquid MS medium supplemented with cefotoxime 250 mg l^{-1}. Explants were blot dried on filter paper and transferred to shoot regeneration medium with cefotaxime 250 mg l^{-1} for two weeks and then transferred to selection medium - Woody plants medium BAP 3 mg l^{-1}, kinetin 1 mg l^{-1} + 3% sucrose with 'Cefotaxime 250 mg l^{-1} and kanamycin 50 mg l^{-1}. Small adventitious shoot that survived on selection medium were detached from explants and transferred to hormone free WPM medium for inducing rooting. Shoot regeneration frequencies were drastically reduced after successive transfer through selection medium. However a few putative transgenics were successfully regenerated from germinating embryos and hypocotyls of *in vitro* grown seedlings treated with *Agrobacterium* containing osmotin.

From the review it could be concluded that the advent of genetic transformation has lead to the possibility of genetically improving several crops for enhanced resistance to insects and disease. Since successful genetic transformation reported in black pepper is limited, it is desirable to evolve a successful protocol for production of black pepper transformants with defense genes like osmotin, glucanase, chitinase etc.

Materials and Methods

3. MATERIALS AND METHODS

The present investigations were carried out at the Department of Plantation Crops and Spices and the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur during 2001 to 2006. The study was aimed at developing an *in vitro* regeneration protocol for somatic embryogenesis and an efficient *Agrobacterium tumefaciens* mediated genetic transformation system in black pepper for induction/better expression of defense genes for *Phytophthora* foot rot resistance/ tolerance. Details regarding the experimental materials used and methodology adopted for various experiments under the study are presented in this chapter.

3.1 SOURCE OF EXPLANT

Three varieties of black pepper namely, Panniyur 1, Panniyur 4 and Panniyur 6 were selected for the study.

Panniyur 1 is the first hybrid in black pepper with very good yield potential. It is the hybrid of the cross between Uthirankotta x Cheriyakaniakadan. It performs better under open conditions and is highly susceptible to foot rot.

Panniyur 4 is a selection from Kuthiravally. It is a good yielder with better adaptability to adverse conditions and it responds well to *in vitro* culture.

Panniyur 6 is a selection from the popular cultivar Karimunda having good yield potential.

Explants for the study were derived from the seeds and mature plants of these varieties.

3.2 CULTURE MEDIUM

3.2.1 Chemicals

The major and minor elements required for the preparation of media were of analytical grade and procured from M/s BDH Laboratories, Sisco Research

Laboratories (SRL) and Merck, India. The amino acids, vitamins and plant growth regulators were obtained from M/s Merck India and Sigma chemicals, USA.

3.2.2 Glassware

Borosilicate glassware of Corning/Borosil brand were used for the experiments.

3.2.3 Composition of media

MS medium (Murashige and Skoog, 1962), SH medium (Schenk and Hildebrandt, 1972), B_5 medium (Gamborg *et al.*, 1968) and modifications of the three media supplemented with various plant growth regulators were used for plant tissue culture in the present study. Yeast Extract Peptone (YEP) medium (An *et al.*, 1988) and Yeast Extract Manitol (YEM) medium were used for bacterial culture. The basal composition of these media are given in Appendix-I and II.

3.2.4 Preparation of the medium

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the plant tissue culture media. After mixing appropriate quantities of the stock solutions and making up the volume to the required quantity by distilled water, pH of the medium was adjusted between 5.6 and 5.8 using 0.1N NaOH/HCl. After adding agar at the rate of 7 g l^{-1}, the medium was heated to melt the agar. The medium was then dispensed to test tubes (15 x 2.5 cm) at the rate of 15 ml each or to conical flask of required size at the rate of 50 ml in 100 ml conical flask, 100 ml in 200 ml conical flask and 250 ml in 500 ml conical flask. Test tubes and conical flasks were plugged with non-absorbent cotton. Autoclaving was done at 121°C at 15 psi (1.06 kg/cm^2) for 20 min. (Dodds and Roberts, 1982) to sterilize the medium. The medium was allowed to cool to room temperature and stored in culture room until use.

For bacterial culture, the pH of the YEP and YEM medium was adjusted to 7.0. Solidification of the YEP medium was done using agar at the rate of 20 g l^{-1}.

3.3 TRANSFER AREA AND ASEPTIC MANIPULATIONS

All the aseptic manipulations were carried out under the hood of a clean laminar air flow cabinet (Klenzaids) fitted with UV lamp.

3.4 CULTURE CONDITIONS

The cultures were incubated at 26 ± 2°C in air conditioned culture room with 16 h photoperiod (1000 lux) supplied by fluorescent tubes unless otherwise mentioned. Humidity in the culture room varied between 60 and 80 per cent.

3.5 PRODUCTION OF AXENIC SEEDLINGS

Ripe berries were collected from the mature vines of the varieties Panniyur-1 and Panniyur-4 maintained by the Department of Plantation Crops and Spices, College of Horticulture. Ripe berries of the variety Panniyur-6 were collected from Pepper Research Station, Panniyur. Seeds were extracted after removing the pulp, washed thoroughly in water and dried in shade. The seeds were washed in water containing a few drops of teepol. The seeds were taken to the laminar air flow and surface sterilized with $HgCl_2$ (0.1%) for ten minutes. These were then washed free of $HgCl_2$ by rinsing with five changes of sterile distilled water and air dried on sterile blotting paper. Surface sterilized seeds were inoculated in double sterilized moist sand in test tubes. The percentage of seeds germinated and days taken for germination were noted. The germinated seedlings served as the explant source for standardizing regeneration protocols and genetic transformation studies.

3.6 PRODUCTION OF *In vitro* REGENERANTS FROM MATURE TISSUE

Shoots were collected from potted plants of varieties Panniyur-1, Panniyur-4 and Panniyur-6 maintained in the green house. Leaves were removed, nodal segments of 1.0 cm were taken and washed thoroughly. These were treated by immersing in fungicidal solution (Dithiocarbamate 0.2%) for 10 min. The fungicide was removed with distilled water. Further, surface sterilization was done in the laminar flow. The explants were dipped in 50 per cent alcohol for 2 min. and then

treated with $HgCl_2$ (0.1%) for 12 min. Explants were then washed free of $HgCl_2$ by rinsing with five changes of sterile distilled water, blotted dry on sterile blotting paper and inoculated in ½ MS medium with 1.0 mg l^{-1} each of BA and IAA. Cultures were incubated at 26 ± 2°C with 16 h photoperiod and light intensity of 1000 lux. Observations were taken on the percentage of establishment, percentage induction of multiple shoots, percentage of elongated shoots and average number of multiple shoots/explant. The generated plantlets served as source of explant for somatic embryogenesis and transformation studies.

3.7 STANDARDISATION OF SOMATIC EMBRYOGENESIS

Different explant sources and media combinations as detailed below were tried to evolve a suitable protocol for somatic embryogenesis.

3.7.1 Seeds from ripe berries as explant

Seeds of the selected varieties were extracted and surface sterilized as stated before (3.3). Seeds were then inoculated in two different media, SH basal and ½ MS basal with inositol 1.0 g l^{-1}. All the cultures were incubated under 24 h dark condition. Seeds were subcultured at one month interval to fresh medium. Response of the cultures with respect to germination/initiation of pro-embryos was recorded. Cultures with pro-embryo initiation was sub cultured in the same medium at monthly intervals for further development and germination.

3.7.2 Zygotic embryo as explant

Induction of somatic embryogenesis was attempted from zygotic embryo as well as zygotic embryo with endosperm. Zygotic embryos and embryo along with endosperm were dissected out aseptically from surface sterilized ripe and mature berries of the three selected varieties (P_1, P_4 and P_6). The media and hormonal combinations tried for somatic embryogenesis are listed in Table 1. Cultures were maintained in dark.

Table 1. Media and growth regulator combinations used for somatic embryogenesis from zygotic embryo/embryo with endosperm

Sl. No.	Media
1	MS basal
2	½ MS basal
3	½ MS + TDZ 0.1 mg l^{-1}
4	½ MS + TDZ 0.2 mg l^{-1}
5	½ MS + Dicamba 0.5 mg l^{-1}
6	½ MS + Dicamba 1.0 mg l^{-1}
7	½ MS + 2,4-D - 1.0 mg l^{-1}
8	SH basal (semi solid)
9	SH basal (liquid)
10	SH + Dicamba 0.5 mg l^{-1}
11	SH + Dicamba 1.0 mg l^{-1}
12	SH + 2,4-D - 1.0 mg l^{-1}
13	½ MS + BA 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}

Response of the cultures with respect to embryo germination, callus induction and somatic embryogenesis were recorded at biweekly intervals.

3.7.3 *In vitro* germinated seedlings for somatic embryogenesis

Cotyledonary node, hypocotyl and cotyledonary leaf segments from the *in vitro* germinated seedlings of variety P_1 served as the explants. These were cultured in the following media (Table 2) under light for induction of somatic embryogenesis.

Table 2. Growth regulator combinations used for somatic embryogenesis from axenic seedlings explants

Sl. No.	Media
1	½ MS + BA 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}
2	½ MS + TDZ 0.2 mg l^{-1}
3	½ MS + TDZ 0.5 mg l^{-1}
4	½ MS + Dicamba 2.0 mg l^{-1}

Response of the cultures with respect to callus and somatic embryo induction were recorded.

3.8 *IN VITRO* REGENERATION FROM LEAF SEGMENTS

Cotyledonary leaves of *in vitro* raised seedlings as well as leaves from *in vitro* plantlets raised from nodal segments were used as explants for callus induction and regeneration. The medium reported by Nazeem *et al.* (1990) which consisted of ½ MS supplemented with 1.0 mg l^{-1} each of IAA and BA was used for culturing the leaf exlants. Explants were subcultured as and when the phenolic exudates appeared in the medium. Observations with respect to callus induction and regeneration were recorded.

3.9 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

3.9.1 Evaluation of the sensitivity of black pepper tissues to various antibiotics

Explants viz., leaf segments, nodal segments and zygotic embryo of the three selected varieties of black pepper viz., P_1, P_4 and P_6 were tested for their sensitivity to various antibiotics. Leaves and nodal segments were excised from micropropagated plants, cut into segments of size 1 cm^3 and precultured for 4 to 5 days. Zygotic embryo scooped from surface sterilized ripe black pepper seeds were also used for antibiotic sensitivity studies. The antibiotics used for sensitivity testing were kanamycin, hygromycin, cefotaxime and carbenicillin. The concentration of the antibiotics tried are given in Table 3.

Table 3. Concentration of antibiotics used for evaluating the antibiotic sensitivity of black pepper tissues

Sl. No.	Antibiotics	Concentrations (mg l^{-1})
1	Kanamycin	0, 25, 50, 100, 200, 400
2	Hygromycin	0, 10, 20, 30, 40, 50
3	Carbenicillin	0, 100, 200, 250, 300, 400
4	Cefotaxime	0, 25, 50, 100, 200, 400

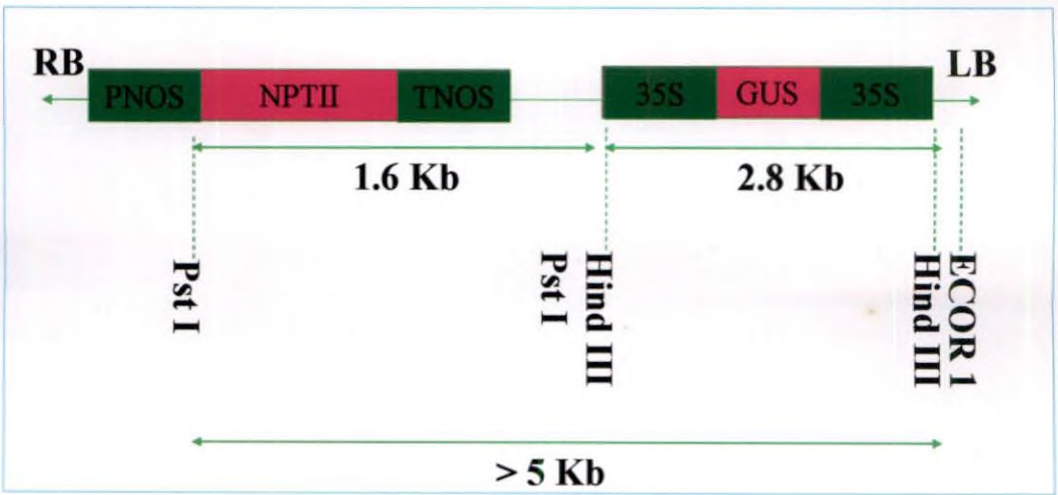
Callus induction/proliferation medium, viz., half MS with 1.0 mg l^{-1} each of BA and IAA was supplemented with different concentration of each antibiotic separately. A control having no antibiotic was also maintained. Observations on culture response viz., callus induction/regeneration were recorded.

3.9.2 *Agrobacterium tumefaciens* strains used for the study

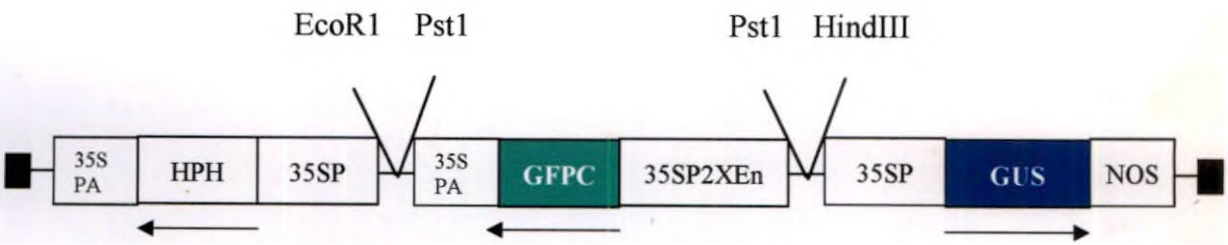
Agrobacterium tumefaciens strains EHA105, AGL-1 .1303, GV2260, and LBA4404 were used for the study (Table 4). The EHA105 contains the plasmid p35SGUSINT. This plasmid contains *gusA* gene fused to the CaMV35S promoter and the *npt II* gene controlled by the nos promoter (Fig.1a). The AGL.1.1303 contains the plasmid harbouring antibiotic resistant selectable marker genes (*npt II* and *hpt IV*) and GUS and GFP reporter genes. These genes were under the control of CaMV35S promoter (Fig. 1b). The *gusA* gene has an intron preventing its expression in *Agrobacterium*. The GV2260 contains the plasmid pGV2260. This plasmid contains osmotin gene and *npt II* gene tagged to the CaMV35S promoter (Fig. 1c). The LBA4404 contains the plasmid pBZ100 containing alfalfa glucanase gene, rice chitinase gene and *npt II* gene tagged to the CaMV35S promoter (Fig. 1d).

Table 4. Details of constructs used for transformation studies in black pepper

Sl. No.	Name of construct	Strain of *Agrobacterium*	Plant selection marker	Gene	Source of construct
1	p35SGUSINT	EHA105	Kanamycin	GUS	National Research Centre for Plant Biotechnology, Indian Agricultural Research Institute, New Delhi
2	pSK53	AGL.I.1303	Kanamycin Hygromycin	GUS GFP	SPIC Science Foundation, Chennai
3	pGV2260	GV2260	Kanamycin	Osmotin	National Research Centre for Plant Biotechnology, Indian Agricultural Research Institute, New Delhi
4	pBZ100	LBA4404	Kanamycin	Glucanase Chitinase	Rajiv Gandhi Centre for Biotechnology, Trivandrum



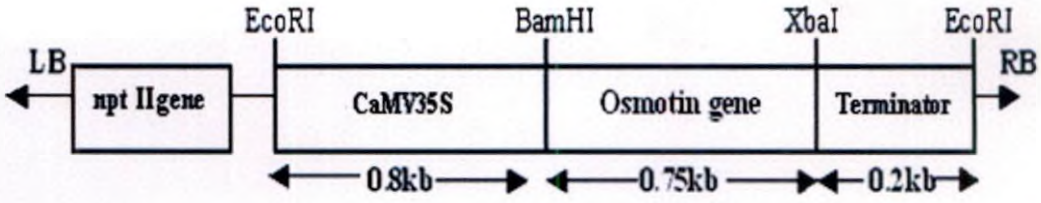
a) p35SGUSINT



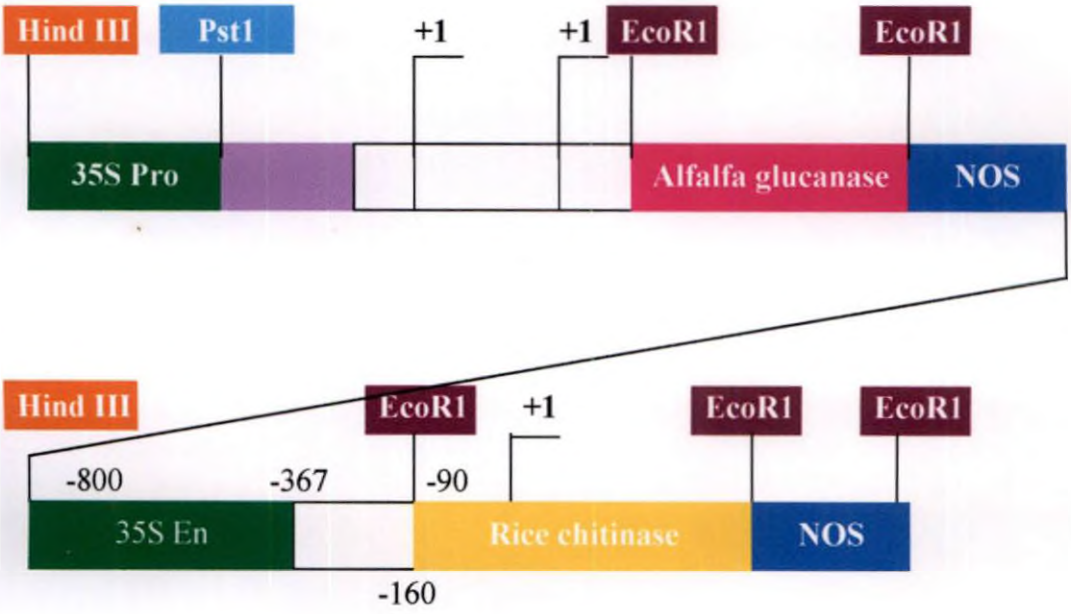
b) pSK53

NPT II - Neomycin phosphotransferase
GUS - β glucuronidase
NOS - Nopaline synthase promoter
RB - Right border
LB - Left border
35S - 35S Promoter

Fig. 1. Schematic representation of T-DNA region of binary plasmids



c) pGV2260



d) pBZ100

NPT II - Neomycin phosphotransferase
GUS - β glucuronidase
NOS - Nopaline synthase promoter
RB - Right border
LB - Left border
35S - 35S Promoter

3.9.2.1 Production of Agrobacterium transformants

3.9.2.1.1 *Agrobacterium* transformation by triparental mating

The binary plasmid pBZ100 from *E. coli* was transferred to *Agrobacterium* by triparental mating (Ditta *et al.*, 1980) through conjugation process.

Bacterial strains for triparental mating include,

1. *Agrobacterium* strain LBA 4404: Harbours a derivative of Ti plasmid that lacks T-DNA region but contain the vir region, grows at 30°C on AB minimal medium + rifampicin 20 µg/ml.
2. *E. coli* (1) with binary plasmid pBZ100 that contains the T-DNA borders and multiplies in *E. coli* and *Agrobacterium*. *E. coli* harbouring this plasmid grows on LB medium with 50 µg/ml kanamycin. pBZ100 is not self transmissible from *E. coli* to *Agrobacterium*. It is possible with the help of pRK2013 (helper plasmid).
3. *E.* coli (2) with plasmid pRK2013. This plasmid when introduced into *E. coli* strain harbouring pBZ100 mobilises pBZ100 into *Agrobacterium*. *E. coli* harbouring pRK2013 grows on LB medium with 50 µg/ml kanamycin.

Procedure for triparental mating

Day (1) LBA4404 was streaked to get single colonies on AB minimal medium with 20 µg/ml rifampicin at 28-30°C.

Day (2) The two *E. coli* strains were streaked on LB plates with 50 µg/ml kanamycin.

Day (3) One colony from each *Agrobacterium* and *E. coli* was inoculated in 100 µl of LB broth in a sterile Eppendorf tube and incubated overnight.

Day (4) The above suspension was plated on antibiotic free LB plate in 2/3 portion and the remaining 1/3 was streaked with Agro LBA4404, *E. coli* (1) and *E. coli* (2).

Day (5) The bacterial growth was scraped out and streaked on LB plate with kanamycin 50 + rifampicin 20 on 2/3 portion. 1/3 portion was split in to three and streaked with LBA4404, pBZ100 and pRK2013.

Day (6) The recombinants alone will grow and single loop streaked on YEP/AB medium with kanamycin 50 + rifampicin 20 was kept at 30°C under dark.

Isolation of plasmid and verification

Isolation of plasmid from *Agrobacterium* by Alkaline lysis mini preparation method (Sambrook *et al.*, 1989) was used to confirm structure of vector sequences within transformed *Agrobacterium* colonies.

3.9.3 Culturing of *A. tumefaciens* strains

The bacteria were cultured in YEM/YEP medium containing suitable antibiotics as follows.

1. EHA105 → YEM + kanamycin 50 mg l^{-1} + rifampicin 20 mg l^{-1}
2. GV2260 → YEM + carbenicillin 100 mg l^{-1} + kanamycin 50 mg l^{-1} + rifampicin 20 mg l^{-1}
3. AGL-1: 1303 → YEP + carbenicillin 100 mg l^{-1} + kanamycin 50 mg l^{-1}
4. LBA 4404 → YEM + kanamycin 50 mg l^{-1} + rifampicin 20 mg l^{-1}

The YEM/YEP medium for the study was prepared in required volume in conical flask and kept in culture room until use. On the day of use, the media was melted and then cooled to 40 to 50°C. Inside a laminar flow cabinet the antibiotics were added to the required medium and mixed thoroughly by vigorous shaking. The medium was then distributed in sterile petriplates and allowed to solidify and attain room temperature. Using sterile bacterial loop a single colony was scooped from the previous bacterial culture plate and streaked on to the freshly prepared solidified medium. The newly streaked bacterial plate was sealed with parafilm and incubated in inverted position at room temperature (28-30°C). The bacterial strains were subcultured once in a month.

3.9.4 Maintenance of strains

Agrobacterium strains were maintained as stabs and glycerol stocks.

3.9.4.1 Preparation of stabs

YEM medium containing marker antibiotic for each strain was poured in sterilized culture tubes and allowed to solidify. The transfer loop was flamed, cooled and plunged into a single bacterial colony. The loop loaded with bacteria was used to stab the solid medium in the culture tube. Similarly stabs for all bacterial strains were prepared and incubated at 27°C. The stabs showing good growth of bacteria were stored in refrigerator at 4-6°C till further use.

3.9.4.2 Preparation of glycerol stock

Liquid YEM medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use, the required antibiotics were added under aseptic conditions to the medium and shaken well. Using a sterile bacterial loop a single colony of the bacteria from the culture was inoculated into the YEM liquid medium and shaken well to form a uniform bacterial suspension. The conical flask was plugged and kept in a shaker (120 rpm) at 28°C for 18 to 24 h according to required density of the bacteria.

Inside the laminar air flow, 800 µl of the bacterial suspension was added to a sterile Eppendorf tube. To this, 200 µl autoclaved glycerol was added and the tubes stored at -20°C.

3.10 SCREENING OF *A. TUMEFACIENS* STRAINS FOR ANTIBIOTIC SENSITIVITY

A. tumefaciens strains used for the study were tested for their sensitivity to various antibiotics. The antibiotics used for testing sensitivity were kanamycin, rifampicin, carbenicillin and cefotaxime.

YEM medium was supplemented with 50 mg l^{-1}, 100 mg l^{-1}, 200 mg l^{-1}, 300 mg l^{-1}, 400 mg l^{-1} of each antibiotics separately. Bacteria from a single cell colony were spotted gently on the antibiotic medium in petriplates. A control having no antibiotic was also maintained. Observations regarding the growth of bacteria were documented.

3.11 STANDARDISATION OF *AGROBACTERIUM* MEDIATED TRANSFORMATION

Agrobacterium mediated genetic transformation of *Piper nigrum* L. was standardized with *Agrobacterium* strain EHA105 with p35GUSINT. Leaf disc and zygotic embryo were used as the explants for transformation. Pepper plant regeneration medium, ½ MS with BA and IAA at 1.0 mg l^{-1} was used for the study.

3.11.1 Preparation of explants

Leaf segments and zygotic embryo were the explants. Leaf segments were excised from micropropagated plants inside the laminar flow cabinet. Leaf segments were placed in regeneration media in sterile petri plates. The petri plates were sealed with parafilm and incubated in culture room for 2 days. Zygotic embryo scooped from surface sterilized ripe seeds were precultured in regeneration medium for one week.

3.11.2 Preparation of *Agrobacterium* culture

Liquid YEM medium was prepared in required volume in conical flasks and kept in culture room until use. On the day of use, kanamycin (50 mg l^{-1}) and rifampicin (20 mg l^{-1}) were added to the medium and shaken well in a laminar flow. Using a sterile bacterial loop, a single colony of the bacteria from the culture was taken and inoculated in the YEM liquid medium. The conical flask was plugged and kept in shaker (120 rpm) at 28°C for 18 to 24 h. according to the required density of the bacteria. The optical density was measured in Spectronic 20 spectrophotometer at a wave length of 600 nm.

3.11.3 Preparation of *Agrobacterium* inoculum

Agrobacterium suspension in conical flask after overnight incubation was centrifuged at 5000 rpm for 10 min. at 4°C. The supernatant was discarded and the bacterial pellet was washed thrice with liquid MS. The bacterial pellet was resuspended in MS liquid medium to bring the required bacterial density.

3.11.4 Standardisation of inoculum density

Agrobacterim strain EHA105 was made to infect pepper explants viz., leaf segments and zygotic embryos for an infection time of 10 min. at varying bacterial density of 0.1, 0.25, 0.5, 0.7, 0.9 and 1.0 at OD_{600nm}. All treatments were given co-cultivation period of 48 h and then bacteria were eliminated with antibiotic cefotaxime 250 mg l^{-1} containing liquid MS. After blotting the explants on sterile blotting paper they were transferred to regeneration medium with kanamycin 100 mg l^{-1}. Subculturing was done at three weeks interval. For co-cultivation, cultures were maintained under dark. Transient gus expression was monitored after three days of co-cultivation. A control of non infected explants were also maintained.

3.11.5 Standardisation of infection time

Leaf segments and zygotic embryo were immersed in *Agrobacterium* suspension for different periods of time viz., 2, 5, 10, 15 and 30 min. Explants were then blotted dry with sterile blotting paper and co-cultivated with the bacteria for a period of 48 h. They were then washed with cefotaxime (250 mg l^{-1}) containing liquid MS and transferred to regeneration medium with kanamycin 100 mg l^{-1}. Transient gus expression was monitored after 3 days of co-cultivation.

3.11.6 Standardisation of co-cultivation period

Two days precultured leaf explants and one week precultured zygotic embryo were infected for 10 min. with *Agrobacterium* strain EHA105 at a bacterium density of 0.9 (OD_{600nm}). Infected explants were co-cultivated in regeneration medium for various time intervals of 24 h, 48 h, 72 h and 96 h and maintained under dark. After co-cultivation, *Agrobacterium* was eliminated by washing three times with liquid MS containing cefotaxime at 250 mg l^{-1}. Explants free of *Agrobacterium* were cultured in regeneration medium with kanamycin 100 mg l^{-1} as selectable marker. Non infected control explants in the regeneration media with kanamycin served as the control. Transient expression of gus in the infected and non infected explants were observed.

3.11.7 Standardisation of acetosyringone concentration for increasing the efficiency of transformation

Effect of acetosyringone in increasing the efficiency of transformation was studied. Acetosyringeone was added at a concentration of 0, 50 and 100 µM to the *Agrobacterium* inoculum and explants were incubated for 0, 30 and 60 min.

3.11.8 Pre-selection

After co-cultivation, the explants were washed with liquid MS containing 250 mg l^{-1} cefotaxime. The explants were then blotted dry and inoculated in pre selection medium containing bacteriostatic agent to kill the bacteria (regeneration medium + 250 mg l^{-1} cefotaxime).

3.11.9 Screening of transformants

After pre-selection, the explants were transferred to screening media in petri plates or culture tubes. The screening media consisted of regeneration media containing antibiotics used as selecting agent and the bactericidal agent. Control explants (without infection) were also maintained in the screening medium.

Cultures in the screening media were subcultured every 3 weeks. All the cultures were maintained in the culture room under light.

3.11.10 Transient expression of GUS reporter gene (histochemical assay)

Histochemical GUS assay was carried out as described by Jefferson *et al.* (1987). The transient expression of GUS was monitored after 3 days of transfer to the pre-selection medium. The composition and preparation of X-Gluc stain is given in Appendix-III.

The explants were incubated in X-Gluc stain for 24-48 h at 37°C in dark. Both transformed and control explants were subjected to X-Gluc assay. After 24-48 h the explants were washed and kept in 100 per cent ethanol for preservation. Development of blue spots/specks was monitored.

3.12 TRANSFORMATION EXPERIMENTS WITH *AGROBACTERIUM TUMEFACIENS* STRAINS VIZ., AGL 1-1303, GV2260 and LBA4404

After confirming the feasibility of *Agrobacterium* mediated transformation of black pepper by observing the transient gus expression on infected leaf segments, further transformation experiments were done with the other *Agrobacterium* strains.

3.12.1 Transformation with *Agrobacterium* strain AGL.1.1303

Explants used for transformation with this strain were the following

1. Leaf segments from micropropagated plants
2. Cotyledonary node from axenic seedlings
3. Zygotic embryo.

Based on the initial standardization experiment with the *Agrobacterium* strain EHA105 with p35GUSINT, the following parameters were kept constant

Infection time	- 10 min
Co-cultivation period	- 2 days
Bacterial density	- 0.9 (OD_{600})
Acetosyringone in the induction media	- 100 µM

After two days of co-cultivation, the explants were washed with liquid MS medium containing 250 mg l^{-1} cefotaxime. Explants were blotted dry and inoculated in pre selection medium containing 250 mg l^{-1} cefotaxime for one week. Subsequently, explants were transferred to screening medium with cefotaxime 250 mg l^{-1}, kanamycin 100 mg l^{-1} and hygromycin 30 mg l^{-1}. Subculturing of the explants to fresh screening medium were done at biweekly intervals. Response of the explants with respect to survival percentage and regeneration/callus induction were recorded for a period of three months.

3.12.2 Transformation with *Agrobacterium* strain GV2260

The *Agrobacterium* strain GV2260 carrying the plasmid PGV2260 was used as vector system for transformation. The plasmid contains osmotin gene and *npt II* gene as plant selection marker.

Explants used for transformation were the following

1. Leaf segments from micropropagated plants
2. Cotyledonary node from axenic seedlings
3. Zygotic embryo.

The best parameters obtained from the previous experiments with the *Agrobacterium* strain EHA105 as mentioned in section 3.12.1 were used for transformation using osmotin gene.

The screening medium used for this *Agrobacterium* strain was regeneration medium with cefotaxime 250 mg l^{-1} and kanamycin 100 mg l^{-1}. Subculturing of the explants to fresh screening medium were done at biweekly intervals. Response of the explants with respect to survival percentage and callus induction/regeneration were recorded.

3.12.3 Transformation with *Agrobacterium* strain LBA4404

The *Agrobacterium* strain LBA4404 carrying the plasmid PBZ100 was used as vector system for transformation. The plasmid contains alfalfa glucanase gene and rice chitinase gene and *npt II* gene as plant selection marker.

Explants used for transformation included

1. Leaf segments from micropropagated plants
2. Cotyledonary node from axenic seedlings
3. Zygotic embryo.

The best parameters obtained from the previous experiments with *Agrobacterium* strain EHA105 as mentioned in section 3.12.1 were used for transformation.

The screening medium used for this *Agrobacterium* strain was regeneration medium with cefotaxime 250 mg l^{-1} and kanamycin 100 mg l^{-1}. Subculturing of the explants to fresh screening medium were done at biweekly intervals. Response of the explants with respect to survival percentage and callus induction/regeneration were recorded.

3.13 PARTICLE BOMBARDMENT - MEDIATED TRANSFORMATION

Direct gene transfer method using Particle Delivery System (PDS/1000 He, Biorad) was also attempted using cotyledonary nodal segments and adventitious buds of the variety Panniyur 4. The protocol followed is given below.

I. Preparation of Microprojectiles

Accurately weighed 50 mg of tungsten powder and transferred to an eppendorf tube. Added one ml of freshly opened absolute ethanol to the tungsten powder and centrifuged for 10 seconds in a microfuge. Replaced the alcohol and centrifuged once more for 10 seconds. Alcohol was removed and added one ml water and centrifuged. This step was repeated three times. Tungsten particles were finally resuspended in one ml water. Sample preparation for six shots included adding in order 50 µl tungsten suspension, 10 µl DNA in TE buffer, 50 µl 2.5 M $CaCl_2$, 20µm 0.1 M spermidine and 200 µl ethanol. Centrifuged for 10 seconds in a microfuge. Supernatant was removed, added fresh ethanol and centrifuged. Repeated three times. Resuspended final pellet in 30 µl ethanol. Transferred 5 µl on to each disc for shooting.

II. Preparation of plant sample for shooting

Using sterile forceps and scalpels cotyledonary node explants were excised from axenic seedlings and kept at the centre of the petriplate plated with regeneration

medium. Nodal segments with adventitious buds were also placed similarly in another petriplate.

III. Shooting plant samples

1. The disposable shooting components like rupture disc, flying disc, stopping screen and gun parts were surface sterilized by wiping with absolute ethanol.
2. The biolistic particle acceleration device (PDS 1000/He, Bio-Rad) was used for bombardment of samples under a chamber pressure of 26 mm of Mercury. Petriplate with plant sample were placed on the sample platform with lid off. After bombardment petriplates were taken out of the chamber and transferred the cotyledonary nodal segments and adventitious buds to fresh petriplates containing regeneration medium (½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA) with kanamycin 100 mg l^{-1}.

3.14 MOLECULAR CHARACTERIZATION OF TRANSGENIC PLANTS

The presence of the transgene in four putative transgenic plantlets was analysed by PCR.

3.14.1 Isolation of plant DNA

DNA was isolated from leaf tissues of the putative transgenic plants, following the method by Doyle and Doyle (1987).

Procedure

Pre warmed the lysis buffer in 30 ml centrifuge tube. Ground 0.5 g plant tissue with liquid nitrogen and 3 ml extraction buffer using a mortar and pestle. Poured the homogenate to a fresh 30 ml tube containing 3 ml lysis buffer, pre warmed and maintained at 65°C and added 1 ml sarcosine. Mixed the content by slight inversion. Kept the centrifuge tube at 50-65°C for 15 min. Added equal volume (7 ml) of chloroform : Isoamyl alcohol mixture (24:1). Mixed gently by slight inversion (5 times). Centrifuged at 10,000 rpm for 15 min at 4°C. Transferred the upper aqueous phase to new centrifuge tube. Added 0.6 volume of chilled isopropanol. Mixed gently

and kept at -20°C for half an hour. Collected DNA pellet by centrifugation at 10,000 rpm for 15 min at 4°C. Discarded isopropanol, washed the pellet with 70 per cent absolute ethanol centrifuged, collected pellet after each wash. Finally air dried pellet and dissolved in very minimum quantity TE buffer. Subjected to RNAse treatment. Electrophoresed on 1 per cent agarose.

3.14.2 Polymerase chain reaction (PCR)

PCR was performed to identify the presence of the marker gene (*npt*-II) in the transgenic plants. DNA isolated from regenerants from the transformants was used as template. Primers of *npt*-II gene were used to amplify the fragment of interest with the reaction conditions as indicated below:

Primer *npt*-II (Bangalore Genei)

Forward primer 5' CAA TCG GCT GCT CTG ATG CCG 3'

Reverse primer 5' AGG CGA TAG AAG GCA ATG CGC 3'

Reaction mixture for PCR

Components	**Volume**
1. Template DNA	1 µl
2. Forward primer	1 µl
3. Reverse primer	1 µl
4. d NTP mix	1 µl (10 mM)
5. Taq buffer	2.5 µl
6. Taq DNA polymerase	2.0 µl (0.6 units)
7. Sterile double distilled water	16.5 µl
Total volume	25 µl

PCR programme (30 cycles)

DNA amplification was carried out on PCR machine (MJ Research) with the following cycles.

Condition	Temperature in °C	Time (min)
1. Initial denaturation	94	2
2. Denaturation	94	1
3. Primer annealing	54	1
4. Primer extension	72	2
5. Hold go to '2' (repeat 29 cycles)		

Final extension 72°C for 10 min

A control was also run with non-transformed plantlet.

The PCR products were visualised in 0.8 per cent ethidium bromide stained agarose gel along with 1 kb DNA ladder mix as marker.

Results

4. RESULTS

The results of the investigations on "Genetic transformation of black pepper for *Phytophthora* foot rot resistance/tolerance" carried out at the Department of Plantation Crops and Spices and the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture from January 2001 to April 2006 are presented in this chapter.

4.1 SOURCE OF EXPLANTS FOR GENETIC TRANSFORMATION

4.1.1 Production of axenic seedlings

Axenic seedlings of the three selected varieties P_1, P_4 and P_6 were raised in moist sterile sand under *in vitro* conditions (Plate 1a). The data on percentage of seeds germinated, days taken for germination and recovery of seedlings of the three selected varieties of black pepper in sterilized moist sand is given in Table 5. All the varieties gave 100 per cent germination within a period of 15-18 days. The recovery of healthy seedlings in each variety was reduced to 50 per cent due to fungal contamination.

Table 5. Seed germination of black pepper under *in vitro* conditions

Sl. No.	Varieties	Days taken for germination	Percentage of germination	Percentage recovery of seedlings
1	P_1	18	100	50
2	P_4	15	100	50
3	P_6	16	100	50

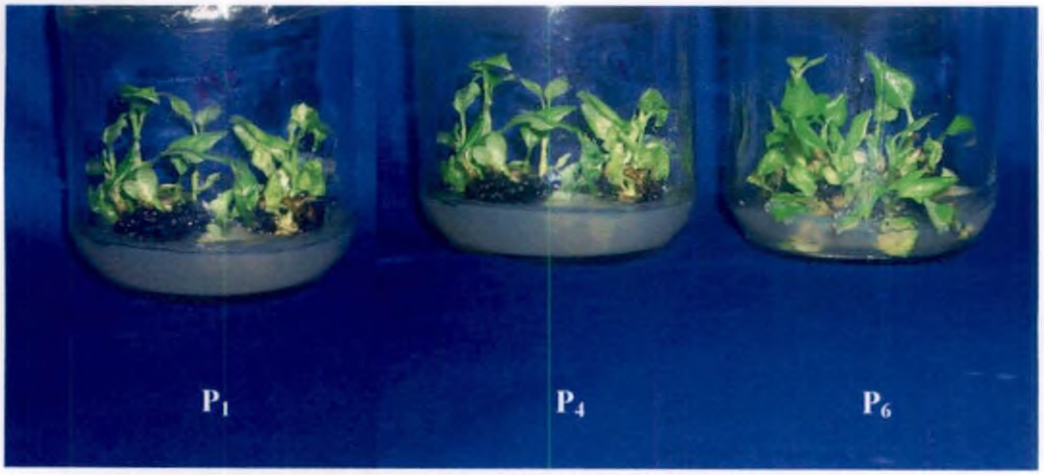
Medium - Sterilized moist sand; Number of seeds - 200
Culture condition – dark

4.1.2 Production of *in vitro* regenerants from mature tissue

To provide sufficient source of explants for transformation studies, tissue culture plants of the three selected varieties were raised (Plate 1c). Response of nodal segments of the selected black pepper varieties viz., P_1, P_4 and P_6 in the media identified for *in vitro* regeneration of black pepper is presented in Table 6. The

a) Axenic seedlings

b) Regenerants from zygotic embryo



c) Regenerants from nodal segments

Plate 1. Explant source for regeneration and transformation

medium for culture establishment and multiple shoot production is the same, that is ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA. Among the varieties, P_6 gave the maximum culture establishment of 90 per cent closely followed by P_4 (85%). Percentage of cultures with induction of multiple shoots was same for P_4 and P_6 (80%). Average number of multiple shoots/explant was also the highest for variety P_6 (20) followed by P_4 (15). Variety P_1 showed least response for culture establishment (60%), multiple shoot induction (40%) and multiple shoot production (5). Multiple shoot induction was observed within two months of culture establishment. Cultures were maintained by periodical sub culturing in the same media and served as explant source for somatic embryogenesis and genetic transformation studies.

Table 6. Response of nodal segments of selected varieties of black pepper to culture establishment and multiple shoot production

Sl. No.	Response	Varietal response (%)		
		P_1	P_4	P_6
1	Establishment of cultures	60	85	90
2	Induction of multiple shoots	40	80	80
3	Average number of multiple shoots/explant	5	15	20

Medium - ½ MS + BA 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}
Culture condition - light; Number of cultures - 50

4.2 STANDARDISATION OF SOMATIC EMBRYOGENESIS

Somatic embryogenesis of black pepper was attempted using different explants.

4.2.1 Response of seeds from ripe berries as explant for somatic embryogenesis

The data on response of ripe seeds with respect to germination and pro-embryo initiation on two basal media, SH and ½ MS with Inositol 1.0 g l^{-1} are presented in Table 7. The response of seeds from different varieties was different in

the two basal media combinations tested. The varieties P_1, P_4 and P_6 germinated in SH basal whereas in modified ½ MS none germinated. In SH seeds of variety P_1 recorded the least seed germination (1%) while P_4 and P_6 recorded better germination of 16.36 and 16.40 per cent respectively. Seeds of P_4 and P_6 showed proembryo formation at the micropylar end in SH medium. However none of the cultures developed further on subculturing to solid SH medium. P_1 did not show any proembryo formation. In ½ MS basal media with 1.0 g l^{-1} Inositol, 2.5 per cent of cultures of P_6 developed proembryos after third subculture to fresh medium (Plate 2). Somatic embryos initiated from the proembryos were transferred to liquid SH medium in conical flask and maintained in orbital incubating shaker at 110 rpm. Within two weeks, elongation of the somatic embryos with well developed cotyledonary leaves was visible (Plate 3). These were subcultured to culture tubes for development of leaves. Plantlets with well developed green leaves were transferred to sterile potting mixture within a period of one month. Plantlets were hardened under shade.

Table 7. Response of ripe seeds of selected varieties P_1, P_4 and P_6 in somatic embryogenesis with different basal media

Sl. No.	Varieties	SH basal			½ MS + Inositol 1.0 g l^{-1}		
		Response percentage			Response percentage		
		Seed germinated	Initiation of proembryos	Viable somatic embryos	Seed germinated	Initiation of proembryos	Viable somatic embryos
1	P_1	1.0	0	0	0	0	0
2	P_4	16.36	16.36	0	0	0	0
3	P_6	16.40	16.40	0	0	2.50	2.50

Culture condition - dark; Culture period - one year
No. of seeds per treatment - 100

4.2.2 Zygotic embryo and zygotic embryo with endosperm as explant

Zygotic embryo and zygotic embryo with endosperm scooped from sterilized ripe seeds were the explants for induction of somatic embryogenesis. The data on response of zygotic embryo and zygotic embryo with endosperm in various media and growth regulator combinations are given in Table 8.

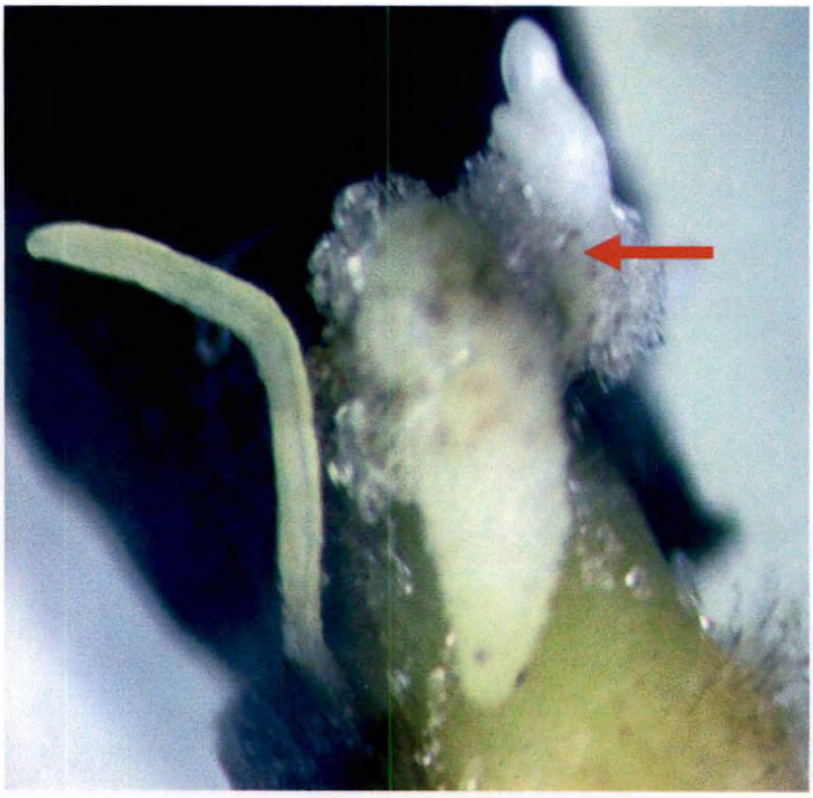


Plate 2. Somatic embryogenesis in black pepper variety Panniyur 6

Plate 3. Plants regenerated through somatic embryogenesis in black pepper variety Panniyur 6

Table 8. Response of zygotic embryo and zygotic embryo with endosperm of three varieties in media and growth regulator combinations to somatic embryogenesis

Explant	Media (Growth regulator in mg l^{-1})	Per cent of cultures germinated			Per cent of cultures callus induction			Per cent of cultures with somatic embryos		
		P_1	P_4	P_6	P_1	P_4	P_6	P_1	P_4	P_6
E	MS basal	-	-	-	21.0	-	-	-	-	-
E + E	„	-	-	-	-	8.33	-	-	-	-
E	½ MS + TDZ (0.1)	-	-	-	-	-	-	-	-	-
E + E	„	-	-	-	-	-	-	-	-	-
E	½ MS + TDZ (0.2)	-	-	-	-	-	-	-	-	-
E + E	„	-	-	-	-	-	-	-	-	-
E	½ MS + Dicamba (0.5)	-	-	-	62.5	-	-	-	-	-
E + E	„	-	-	44.4	-	-	44.4	-	-	-
E	½ MS + Dicamba (1.0)	-	-	-	25.0	-	-	-	-	-
E + E	„	-	-	18.75	-	-	18.75	-	-	-
E	½ MS + 2,4-D (0.1)	-	-	-	-	-	-	-	-	-
E + E	„	-	-	37.5	-	-	37.5	-	-	-
E	SH basal (semi solid)	4.2	-	-	7.04	-	-	-	-	-
E + E	„	10.2	-	50	0.0	-	-	-	-	-
E	SH basal (liquid)	-	-	-	-	-	-	-	-	-
E + E	„	-	-	-	-	-	-	-	-	-
E	SH + Dicamba (0.5)	-	-	-	-	-	-	-	-	-
E + E	„	-	-	66.66	-	-	66.6	-	-	-
E	SH + Dicamba (1.0)	-	-	-	-	-	-	-	-	-
E + E	„	-	-	-	-	-	72.2	-	-	-
E	SH + 2,4-D (0.1)	-	-	-	-	-	-	-	-	-
E + E	„	-	-	-	-	-	33.33	-	-	-
E	½ MS + BA (1.0) + IAA (1.0)	13.5	15.78	-	8.1	35.71	-	-	-	-
E + E	„	-	-	-	34.2	-	5.5	-	-	-

E – Embryo alone; E + E - Embryo + Endosperm; (-) no response
Culture condition – Dark
Number of cultures per treatment - 100
Culture period - 3 months

Culturing of embryo and embryo with endosperm resulted in embryo germination as well as callus induction in suitable media combinations. None of the explants induced somatic embryogenesis in various media combinations tested. Zygotic embryo of Panniyur 4 gave the highest germination (15.78%) in pepper regeneration medium, ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA. Embryo of P_1 also germinated in this medium in 13.5 per cent of cultures. SH basal also supported germination of P_1 embryos.

With respect to embryo callusing, embryo of P_1 and P_4 callused in suitable media combination and percentage of cultures showing callusing ranged from 7.04 to 62.5 per cent in a culture period of three months. P_6 embryo did not callus in any of the media tested. P_1 embryo callused in 62.5 per cent of cultures in the medium of ½ MS + dicamba 0.5 mg l^{-1}. An increase of dicamba concentration from 0.5 to 1.0 mg l^{-1} reduced the callusing efficiency of cultures to 25.0 per cent. In ½ MS (21%) and SH basal (9.04%) of P_1 embryo callused. In pepper regeneration medium P_4 embryo callused in 35.71 per cent of cultures and P_1 embryo callused in 8.1 per cent of cultures. In this medium callus showed organogenesis under culture condition of dark.

Supplementation of TDZ 0.1 to 2.0 mg l^{-1} or 2,4-D o.1 mg l^{-1} in ½ MS medium did not support embryo callusing. 2,4-D (0.1 mg l^{-1}) in SH also did not support embryo callusing.

Pepper regeneration medium supported both embryo germination and callusing in P_1 and P_4 (Plate 4a).

Explant, embryo along with endosperm of all the three varieties responded in specific media combinations by embryo germination or callusing at varying intensities. Embryo in presence of endosperm germinated in five media combination and responding cultures ranged from 10.2 to 66.6 per cent. Maximum germination was recorded in SH + Dicamba 0.5 mg l^{-1} by P_6 cultures (66.66%). SH basal also supported germination in 50 per cent of P_6 cultures. In ½ MS medium also dicamba 0.5 to 1.0 mg l^{-1} supported embryo germination in 44.46 per cent and 18.75 per cent of cultures.

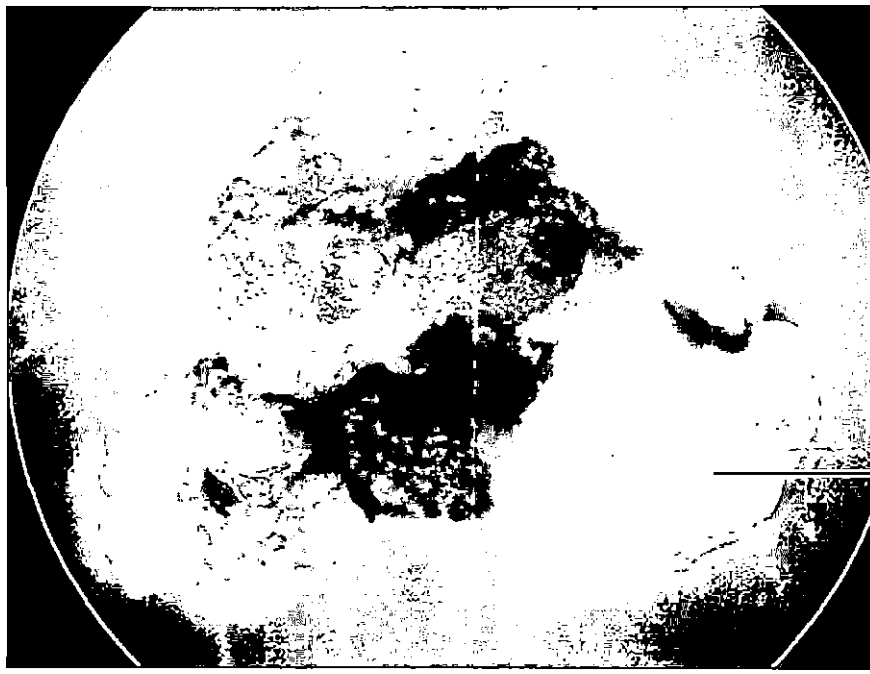
P_1 embryo in presence of endosperm also germinated in SH basal. Response was observed in 10.2 per cent of cultures. The data with respect to callusing of embryo along with endosperm revealed that the explant of P_6 was the most regenerative followed by P_4 and P_1. The P_6 embryo along with endosperm callused in seven media combinations and the maximum was observed in SH + dicamba 1.0 mg l^{-1} (92.2%) followed by SH + dicamba 0.5 (66.6%). Dicamba 1.0 mg l^{-1} supported embryo and endosperm callusing in ½ MS also in 18.75 per cent of cultures.

2,4-D 0.1 mg l^{-1} in ½ MS as well as SH basal supported embryo + endosperm callusing in 37.5 and 33.33 per cent of cultures.

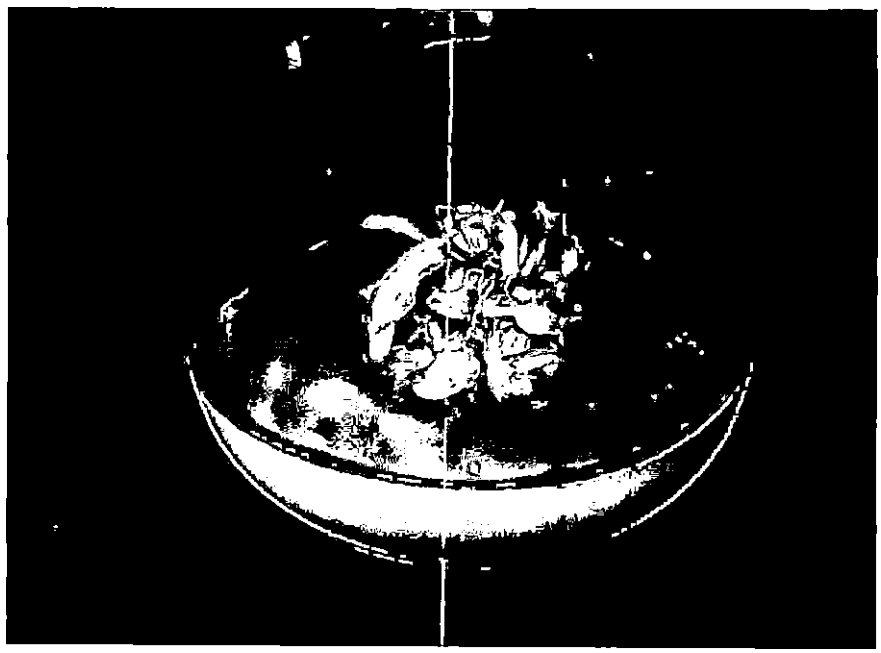
Response of embryo germination and callusing was found to be influenced by media and genotype. Pepper regeneration medium ½ MS + 1.0 mg l^{-1} each of BA and IAA was the best for embryo germination of the explant embryo alone. The medium ½ MS + dicamba (0.5 mg l^{-1}) produced the maximum callusing. For explant, embryo and endosperm maximum germination was observed in the medium of SH with dicamba (0.5 mg l^{-1}). The genotype P_6 has the greatest regeneration potential followed by P_1.

4.2.3 *In vitro* germinated seedling derived explants

The response of different seedling derived explants cotyledonary node, hypocotyl and cotyledonary leaf segments on different growth regulator regimes with respect to somatic embryogenesis are presented in Table 9. No somatic embryogenesis was observed in any of the treatments. Explant cotyledonary node sprouted with basal calli in ½ MS medium with 1.0 mg l^{-1} of BA and IAA. Hypocotyl segments exhibited greenish compact calli at the cut ends in the same medium but failed to regenerate further. In the case of cotyledonary leaf segments, callus induction was observed in all the media tried except in ½ MS with TDZ 0.5 mg l^{-1}. Here also further regeneration was not obtained. So the best regenerative explant was cotyledonary nodal segment and that was in the medium ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA (Plate 4b).



a) Zygotic embryo (Variety - Panniyur 4)



b) Cotyledonary node (Variety - Panniyur 1)

Plate 4. Organogenesis from zygotic embryo and cotyledonary node

Table 9. Response of *in vitro* seedling derived explants of variety P_1 in growth regulator combinations to somatic embryogenesis

Explant	Growth regulators	Response
Cotyledonary node	BA 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}	Sprouting with basal calli
Hypocotyl	BA 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}	Greenish compact calli at cut ends
Cotyledonary leaf segment	BA 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}	Bulging of veinal area and callus induction
Cotyledonary leaf segment	TDZ 0.2 mg l^{-1}	Whitish callus at petiole end
Cotyledonary leaf segment	TDZ 0.5 mg l^{-1}	No response
Cotyledonary leaf segment	Dicamba 2.0 mg l^{-1}	Globular friable callus

Basal medium - ½ MS
Culture condition – Light
Number of cultures per treatment - 20

4.3 *IN VITRO* REGENERATION FROM LEAF SEGMENTS

Cotyledonary leaf segments and leaf segments from *in vitro* regenerants of the three varieties were cultured in ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA. The data on percentage of callusing and callus regeneration are given in Table 10 and 11. For all the three varieties callus induction was observed in both cotyledonary leaf segments and leaf segments from *in vitro* regenerants. Leaf segments from *in vitro* regenerants from nodal segments of variety P_4 exhibited, direct shoot regeneration in 17.5 per cent of cultures (Plate 5). In the case of cotyledonary leaf segments, callus regeneration was not observed in any of the varieties (Table 11).

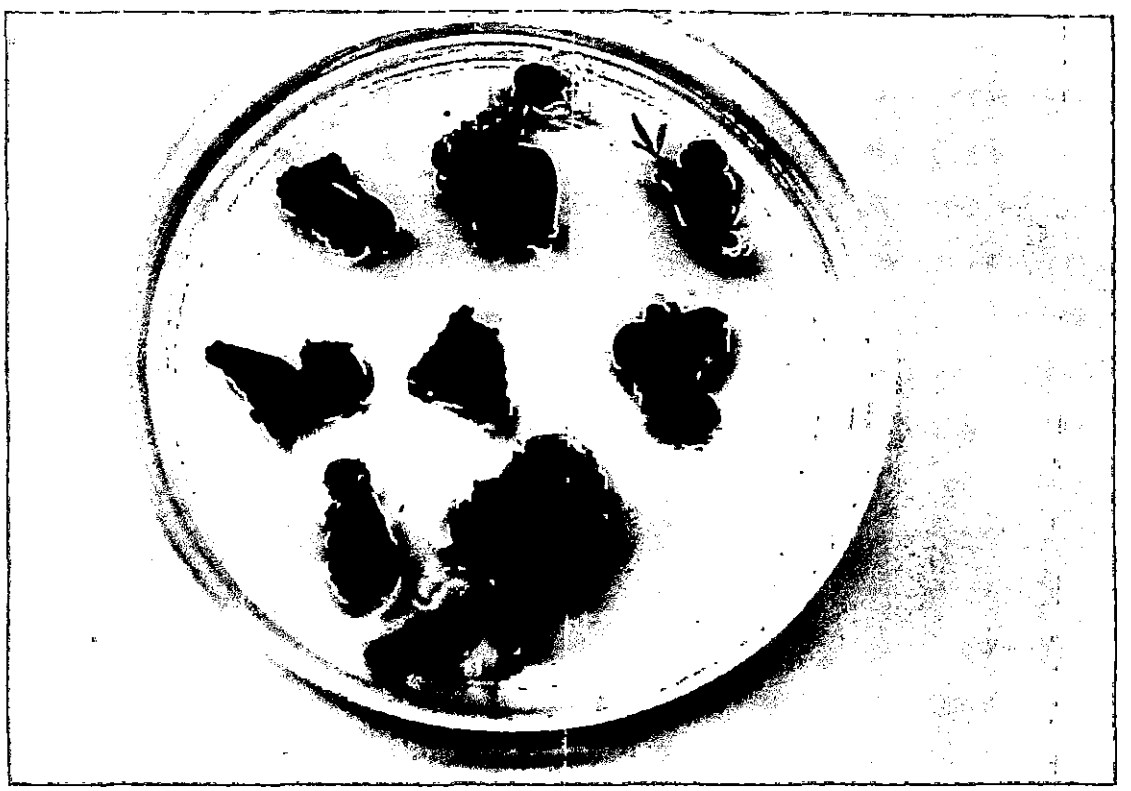


Plate 5. Regeneration from leaf segments (Variety - Panniyur 4)

Table 10. Callus induction and regeneration of leaf segments from micropropagated plantlets of varieties P_1, P_4 and P_6

Varieties	Callus induction (%)	Callus regeneration (%)	Direct regeneration (%)
P_1	100	0	0
P_4	100	0	17.5
P_6	100	0	0

Medium - ½ MS + 1 mg l^{-1} BA + 1 mg l^{-1} IAA
Culture duration - 4 months; Culture condition - light; No. of cultures - 40

Table 11. Callus induction and regeneration of cotyledonary leaf segments from axenic seedlings of varieties P_1, P_4 and P_6

Varieties	Callus induction (%)	Callus regeneration (%)	Direct regeneration (%)
P_1	100	0	0
P_4	100	0	0
P_6	100	0	0

Medium - ½ MS + 1 mg l^{-1} BA + 1 mg l^{-1} IAA
Culture duration - 4 months; Culture condition - light; No. of cultures - 40

4.4 *AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION

4.4.1 Sensitivity of explants to antibiotics

Sensitivity of leaf segments, zygotic embryo and nodal segments to four different antibiotics i.e. kanamycin, hygromycin, carbenicillin and cefotaxime at five different concentrations as mentioned in Table 3 was tested. Kanamycin and hygromycin are the commonly used antibiotics for selection of transformed tissues. Cefotaxime and carbenicillin are the antibiotics generally used for eliminating *Agrobacterium* after infection.

4.4.1.1 Sensitivity of explants to kanamycin

Response of explants to kanamycin at 25, 50, 100 and 200 mg l^{-1} were tested. The explants included were leaf segment, zygotic embryo and nodal segments. The explants in regeneration medium without kanamycin served as the control. The data are given in Table 12.

In sensitivity studies, kanamycin 50 mg l^{-1} onwards inhibited callusing of leaf segments (Plate 6b). In zygotic embryo, kanamycin 25 mg l^{-1} onwards inhibited growth causing blackening of the freshly scooped embryos. Nodal segments were found sensitive to 100 mg l^{-1} kanamycin and turned whitish without sprouting. In regeneration medium with 25 mg l^{-1} kanamycin proliferation of shoots from nodal segments was observed. Shoot growth was slow and pale coloured in 50 mg l^{-1} kanamycin. So kanamycin at 100 mg l^{-1} was selected for screening the transformants of nodal segments. For zygotic embryo explants, kanamycin 25 mg l^{-1} was the optimum level for selecting transformants. For leaf explants 50 mg l^{-1} of kanamycin was the optimum threshold level for screening the transformants.

In control, leaf segments exhibited callus induction, zygotic embryo sprouted and nodal segments proliferated.

Table 12. Response of explants to different concentrations of kanamycin

Concentrations (mg l^{-1})	Leaf segment		Zygotic embryo		Nodal segments	
	Response	Remarks	Response	Remarks	Response	Remarks
25	Green & callusing	Resistant	Blackened	Sensitive	Sprouting & green	Resistant
50	Green & no callusing	Sensitive	Blackened	Sensitive	Slow sprouting & green	Resistant
100	Green & no callusing	Sensitive	Blackened	Sensitive	Not sprouting & pale	Sensitive
200	Green & no callusing	Sensitive	Blackened	Sensitive	Not sprouting & pale	Sensitive
Control	Green & callusing	-	Sprouting	-	Sprouting	-

Culture duration - 8 weeks; Culture condition - Light
Subculture interval - 3 weeks; Number of cultures - 30
Regeneration medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA

4.4.1.2 Sensitivity of explants to hygromycin

The response of leaf segments, zygotic embryo and nodal segments to hygromycin at 10, 15, 25 and 50 mg l^{-1} were tested. The data is given in Table 13. Hygromycin at concentration of 25 mg l^{-1} onwards prevented callusing of leaf segments (Plate 6a). For zygotic embryo 10 mg l^{-1} onwards blackening of the freshly scooped embryo was noticed. Nodal segments were sensitive to 25 mg l^{-1} of hygromycin. So hygromycin at 25 mg l^{-1} was selected for screening the transformants from nodal segments and leaf segments and 10 mg l^{-1} for zygotic embryo explants.

Table 13. Response of explants to different concentrations of hygromycin

Concentrations (mg l^{-1})	Leaf segment		Zygotic embryo		Nodal segment	
	Response	Remarks	Response	Remarks	Response	Remarks
10	Green & callusing	Resistant	Blackened	Sensitive	Sprouting & green	Resistant
15	Green & callusing	Resistant	Blackened	Sensitive	Sprouting & green	Resistant
25	Green & no callusing	Sensitive	Blackened	Sensitive	Not sprouting & pale	Sensitive
50	Green & no callusing	Sensitive	Blackened	Sensitive	Not sprouting & pale	Sensitive
Control	Green & callusing	-	Sprouting	-	Sprouting	-

Culture duration - 8 weeks Culture condition - Light
Subculture interval - 3 weeks Number of cultures - 30
Regeneration medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA

4.4.1.3 Sensitivity of explants to carbenicillin

The effect of various concentrations of carbenicillin (50, 100, 200, 400 mg l^{-1}) on leaf segments, zygotic embryo and nodal segments is shown in Table 14.

Table 14. Response of explants to different concentrations of carbenicillin

Concentrations (mg l^{-1})	Leaf segment		Zygotic embryo		Nodal segment	
	Response	Remarks	Response	Remarks	Response	Remarks
50	Green & callusing	Resistant	Sprouting	Resistant	Sprouting & green	Resistant
100	Green & callusing	Resistant	Sprouting	Resistant	Sprouting & green	Resistant
200	Green & callusing	Resistant	Sprouting	Resistant	Sprouting & green	Resistant
400	Green & callusing	Resistant	Sprouting	Resistant	Sprouting & green	Resistant
Control	Green & callusing	-	Sprouting	-	Sprouting & green	-

Culture duration - 8 weeks Culture condition - Light
Subculture interval - 3 weeks Number of cultures - 30
Regeneration medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA

Callusing of the leaf segments, sprouting of zygotic embryo and nodal segments were observed at all concentrations (50, 100, 200, 400 mg l^{-1}) tested in the regeneration medium. In control i.e., medium without carbenicillin the same response was observed.

4.4.1.4 Sensitivity of explants to cefotaxime

Leaf segments, zygotic embryo and nodal segments were tested for their sensitivity to various concentrations (50, 100, 200, 400 mg l^{-1}) of cefotaxime in the regeneration medium ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA. The data is given in Table 15.

Table 15. Response of explants to different concentrations of Cefotaxime

Concentrations (mg l^{-1})	Leaf segments		Zygotic embryo		Nodal segment	
	Response	Remarks	Response	Remarks	Response	Remarks
50	Green & callusing	Resistant	Sprouting	Resistant	Sprouting & green	Resistant
100	Green & callusing	Resistant	Sprouted	Resistant	Sprouting & green	Resistant
200	Green & callusing	Resistant	Sprouted	Resistant	Sprouting & green	Resistant
400	Green & callusing	Resistant	Sprouting	Resistant	Sprouting & green	Resistant
Control	Green & callusing	-	Sprouting	-	Sprouting & green	-

Culture duration - 8 weeks Culture condition - Light
Subculture interval - 3 weeks Number of cultures - 30
Regeneration medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA

Leaf segments callused in all tested cefotaxime concentrations ranging from 50 to 400 mg l^{-1}. Zygotic embryo and nodal segments also sprouted in all the tested concentrations of cefotaxime ranging from 50 to 400 mg l^{-1}. In the control also the explants showed similar response.

4.4.2 Sensitivity of *Agrobacterium tumefaciens* strains to different antibiotics

Agrobacterium has to be eliminated from the infected explants with suitable antibiotics at appropriate concentrations. Sensitivity of *Agrobacterium* strains EHA105, AGL.1.1303, GV2260 and LBA4404 to different antibiotics at varying concentrations of 50, 100, 200, 300 and 400 mg l^{-1} is shown in Table 16.

The strain EHA105 was sensitive to cefotaxime and carbenicillin at all concentrations ranging from 50-500 mg l^{-1} and resistant to kanamycin and rifampicin from 50 mg l^{-1} onwards. Strain GV2260 found resistant to kanamycin, rifampicin and carbenicillin at all concentrations tested and was sensitive to cefotaxime from 50 mg l^{-1} onwards. Strain AGL.1.1303 was resistant to carbenicillin and kanamycin from 50 mg l^{-1} onwards and sensitive to cefotaxime and rifampicin from 50 mg l^{-1} onwards. Strain LBA4404 was resistant to kanamycin and rifampicin from 50 mg l^{-1} onwards and sensitive to cefotaxime and carbenicillin from 50 mg l^{-1} onwards.

Based on these observations cefotaxime at 250 mg l^{-1} was selected as the concentration for eliminating various strains of bacteria used in the study after infection.

4.4.3 Establishment of bacterial culture

4.4.3.1 Collection and Maintenance of Agrobacterium strains

Agrobacterium strains were collected and maintained in the respective medium for further transformation studies.

4.4.3.2 Production of Agrobacterium transformants by triparental mating

Recombinants of *Agrobacterium* tumefaciens LBA4404 containing the binary plasmid pBZ100 were produced using the method of triparental mating. The binary plasmid pBZ100 containing the genes chitinase + glucanase was mobilized into the native *Agrobacterium* strain LBA4404 using the helper plasmid pRK2013.

Table 16. Effect of antibiotics on growth of *Agrobacterium tumefaciens* strains

Agrobacterium tumefaciens strains	Response																			
	Kanamycin (mg l^{-1})					Rifampicin (mg l^{-1})					Cefotaxime (mg l^{-1})					Carbenicillin (mg l^{-1})				
	50	100	200	300	400	50	100	200	300	400	50	100	200	300	400	50	100	200	300	400
EHA 105	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
GV 2260	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+
AGL 1.1303	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
LBA 4404	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-

+ Resistant
- Sensitive

Presence of the binary plasmid in the recombinants was verified using *Agrobacterium* mini plasmid preparation method (Plate 7).

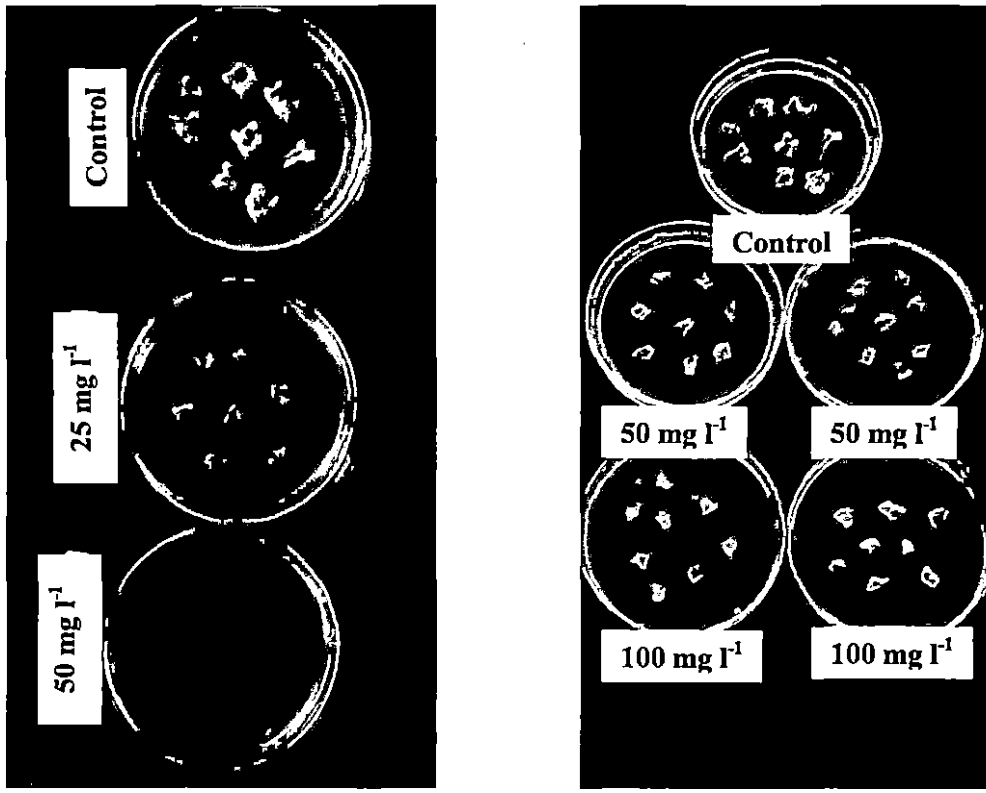
4.4.4 Standardisation of *Agrobacterium* mediated genetic transformation

Agrobacterium mediated genetic transformation of black pepper with leaf segments and zygotic embryo was standardized with strain EHA 105 having gus reporter gene and selectable marker *npt II*. The coding region of the gus reporter gene contains an intron that prevents translation by *Agrobacterium tumefaciens*. The required infection time, density of bacterial population and co-cultivation period for effective gene delivery were standardized with the aforesaid *Agrobacterium* strain. Transformants were screened in selection medium (½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA) containing 50 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime for leaf explants and 25 mg l^{-1} kanamycin for zygotic embryo. Uninfected leaf segments and zygotic embryo served as control. Transformants were subjected to X-gluc assay for confirmation of transformation.

4.4.4.1 Standardisation of inoculum density

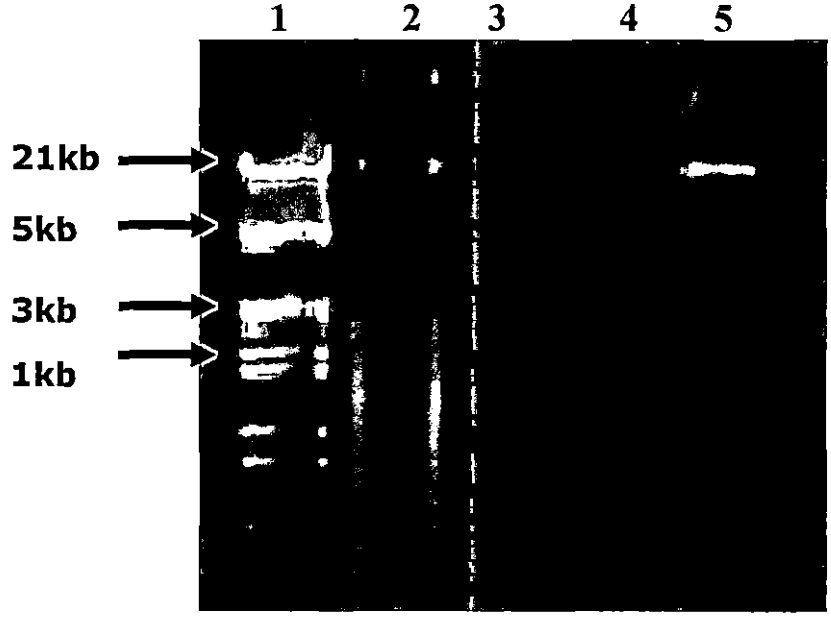
Transformation was tried with strain EHA 105 at different inoculum densities (0.1, 0.25, 0.5, 0.7, 0.9 and 1.0 OD_{600} nm). The leaf segments and zygotic embryo were used for the experiment. An infection period of 10 min and co-cultivation period of two days were fixed for the study. Survival percentage of explants monitored up to 45 days after infection is presented in Table 17 and 18.

The histochemical gus assay was performed three days after co-cultivation. Both control and infected plants were assayed. The results showed blue patches on the surface of the transformed leaf explants. When these blue coloured areas were examined under microscope a diffuse blue staining was observed. The control plants did not show any blue coloured areas (Plate 8).



a) Hygromycin b) Kanamycin

Plate 6. Sensitivity of leaf segments to antibiotics



Lane 1 : Marker DNA (λ*EcoRI*/*Hind*III)
Lane 2,3,4,5 : plasmid DNA of pBZ100

Plate 7. Confirmation of plasmid DNA (pBZ100) in LBA 4404 (Recombinant) after triparental mating

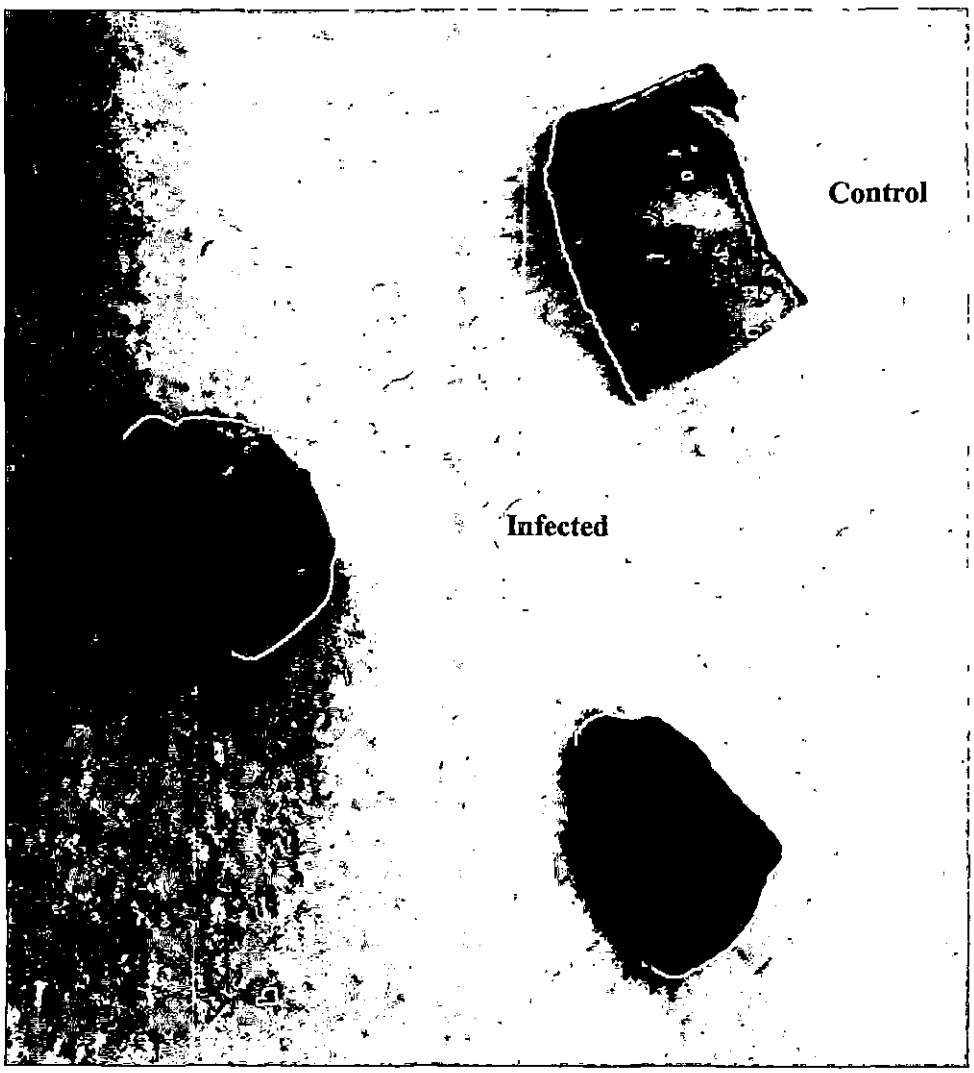


Plate 8. GUS expression in transformed leaf segments

Table 17. Standardisation of inoculum density using *Agrobacterium* strain EHA105 for leaf explants

Pre-culture (days)	Bacterial density (OD_{600} nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Callusing cultures (%)
2	0.1	10	48	+	10.0
2	0.25	10	48	+	40.0
2	0.5	10	48	+	45.0
2	0.7	10	48	+	50.0
2	0.9	10	48	+	80.0
2	1.0	10	48	+	50.0
Control	-	-	-	-	0.0

Culture duration - 45 days; Number of explants per treatment - 30
Regeneration medium - ½ MS + 1 mg l^{-1} BA + 1 mg l^{-1} IAA + Kanamycin 50 mg l^{-1} + Cefotaxime 250 mg l^{-1}

Infecting the leaf explants with EHA105 at bacterial density 0.1 to 1.0 has produced gus expression. Survival and callus induction was observed in 10 per cent of cultures at OD 0.1 and percentage of callusing cultures steadily increased with increase in the bacterial density, reaching the maximum of 80 per cent at OD 0.9 (Fig. 2). Further increase in bacterial density decreased the percentage of callusing cultures to 50. Control plants i.e., uninfected leaf segments in regeneration medium with kanamycin and cefotaxime did not show any callus induction (Table 17).

In the case of zygotic embryo no blue staining was visible in any of the treatments as the zygotic embryo explants turned completely black in X-gluc. However, the zygotic embryo explants gave survival percentage of 5-50 at varying bacterial density from 0.1 to 1.0 (Fig. 3). The maximum callusing cultures were observed at a bacterial density of 0.9 (Table 18).

Based on the survival and callusing of cultures in antibiotic medium the bacterial density selected as optimum for transformation is 0.9 (OD_{600} nm).

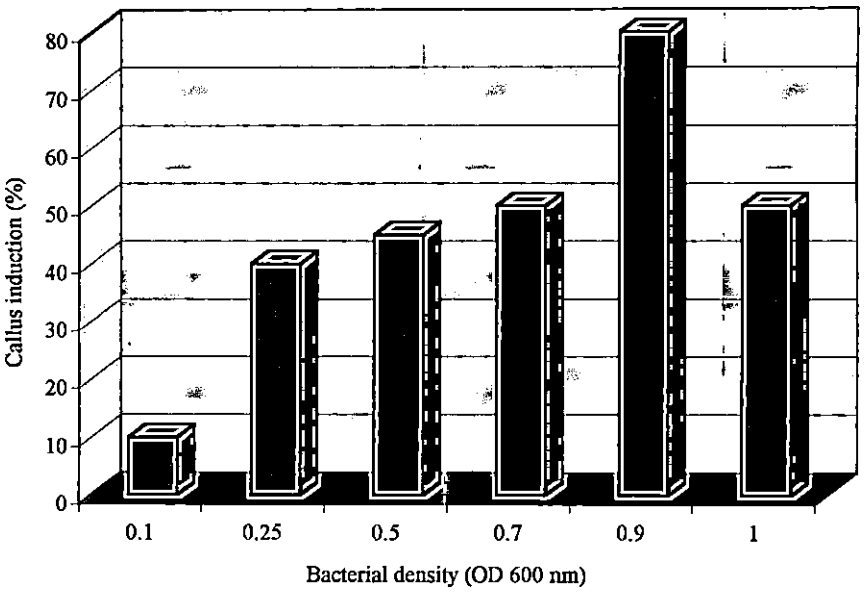


Fig. 2. Effect of inoculum density on callus induction for leaf explants

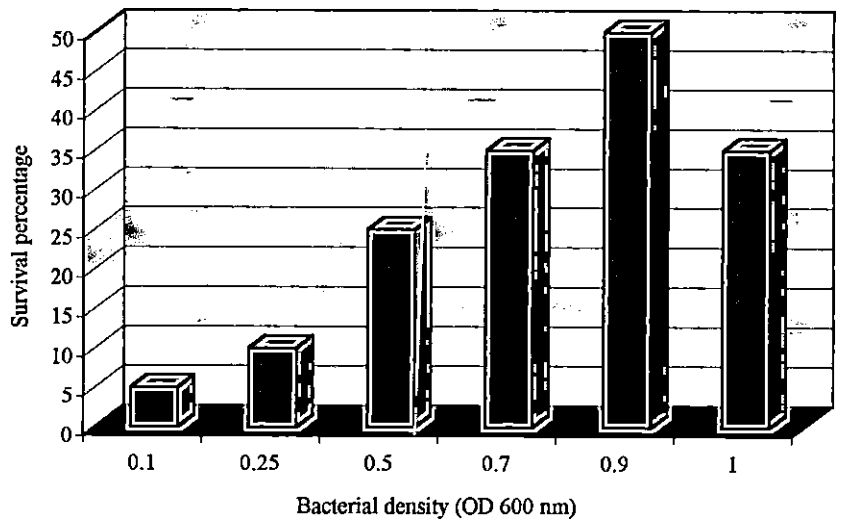


Fig. 3. Effect of inoculum density on survival percentage for zygotic embryo

Table 18. Standardisation of inoculum density using *Agrobacterium* strain EHA105 for zygotic embryo

Pre-culture (days)	Bacterial density (OD_{600} nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Callus induction (%)
7	0.1	10	48	-	5.0
7	0.25	10	48	-	10.0
7	0.5	10	48	-	25.0
7	0.7	10	48	-	35.0
7	0.9	10	48	-	50.0
7	1.0	10	48	-	35.0
Control	-	-	-	-	0.0

Culture duration - 45 days; Number of explants per treatment - 30
Regeneration medium - ½ MS + 1 mg l^{-1} BA + 1 mg l^{-1} IAA + Kanamycin 25 mg l^{-1} + Cefotaxime 250 mg l^{-1}

4.4.4.2 Standardisation of infection time

Infection time 2, 5, 10, 15 and 30 minutes were given for strain EHA105 for infecting leaf and zygotic embryo explants. A period of two days of co-cultivation and a bacterial density of 0.9 at OD_{600} nm were fixed for the study. The data is given in Table 19 and 20. Transient gus assay was performed three days after co-cultivation.

There was no gus expression in leaf explants infected with shorter intervals of 2 to 5 min. But callus induction of 10 and 40 per cent was observed. The blue coloured areas were found on the transformed explants that were co-cultivated for 10 min., 15 min and 30 min. No blue staining was detected in control plant tissues. The survival rate of transformed explants after 60 days after infection was 80 per cent with callus induction in 10 min infection time. Increasing the infection time to 15 min. decreased percentage of callusing cultures to 60. Further increase in infection time to 30 min. resulted in cultures with no callus induction. However gus expression was visible in the infected leaves. Over growth of the *Agrobacterium* was visible with increase in infection time (Fig. 4). The control explants did not show any gus expression and callus induction in the media containing kanamycin 50 mg l^{-1} + cefotaxime 250 mg l^{-1}.

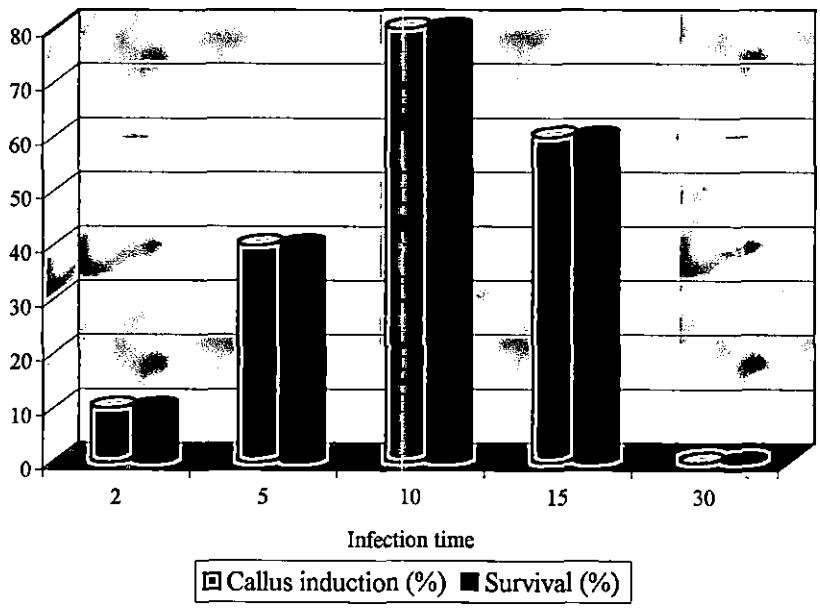


Fig. 4. Effect of infection time on callus induction/survival percentage

Table 19. Standardisation of infection time using *Agrobacterium* strain EHA105 for leaf explants

Pre-culture (days)	Bacterial density (OD_{600} nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Callus induction (%)
2	0.9	2	48	-	10
2	0.9	5	48	-	40
2	0.9	10	48	+	80
2	0.9	15	48	+	60
2	0.9	30	48	+	0
Control	-	-	-	-	0

Culture duration - 60 days; Number of cultures - 40
Regeneration medium - ½ MS + 1 mg l^{-1} BA + .1 mg l^{-1} IAA+ kanamycin 50 mg l^{-1} + cefotaxime 250 mg l^{-1}

In zygotic embryo explants no blue staining was observed in any of the treatments, however, survival percentage ranging from 10 to 80 was noticed. Maximum survival of zygotic embryo was observed with 10 min. infection. Increasing the infection time to 15 min reduced the survival percentage to 60. At 30 min infection time none of the cultures survived due to bacterial over growth.

Based on the above observations infection time of 10 min was found optimum for transformation using leaf and zygotic embryo explants.

Table 20. Standardisation of infection time using *Agrobacterium* strain EHA105 for zygotic embryo

Pre-culture (days)	Bacterial density (OD_{600} nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Survival (%)
7	0.9	2	48	-	10
7	0.9	5	48	-	40
7	0.9	10	48	-	80
7	0.9	15	48	-	60
7	0.9	30	48	-	0
Control	-	-	-	-	0

Culture duration - 60 days; Number of cultures - 40
Regeneration medium - ½ MS + 1 mg l^{-1} BA + 1 mg l^{-1} IAA + kanamycin 25 mg l^{-1} + cefotaxime 250 mg l^{-1}

4.4.4.3 Standardisation of co-cultivation period

The duration of co-cultivation with *Agrobacterium* is one of the factors affecting transformation efficiency. The effect of co-cultivation duration on black pepper was examined using leaf segments and zygotic embryo. A 10 min. infection time and a bacterial density of 0.9 OD_{600} nm were given based on the previous experiments. The explants were co-cultivated with *Agrobacterium* strain EHA105 for 0, 24, 48, 72 and 96 h. The data pertaining to gus expression and callus induction in kanamycin medium is given in Table 21 and 22.

Table 21. Standardisation of co-cultivation period using *Agrobacterium* strain EHA105 for leaf explants

Pre-culture (days)	Bacterial density (OD_{600} nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Callusing cultures (%)
2	0.9	10	0	-	40
2	0.9	10	24	+	55
2	0.9	10	48	+	80
2	0.9	10	72	+	55
2	0.9	10	96	-	40
Control	-	-	-	-	0

Culture duration - 60 days; Number of cultures - 40
Regeneration medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA+ kanamycin 50 mg l^{-1} + cefotaxime 250 mg l^{-1}

When infected leaf explants were transferred to selection medium without any co-cultivation no gus expression was noticed. However percentage of callusing cultures to the extent of 40 per cent was noticed. The callus induction rate of transformed explants was 80 per cent for 48 h co-cultivation period; this was followed by 24 and 72 h and then 96 h co-cultivation period. Bacterial overgrowth was seen when there was a prolonged co-cultivation period of more than 48 h.

For zygotic embryo gus expression was not observed in any of the treatments. Survival percentage ranging from 40 to 80 was observed with maximum after 48 h co-cultivation. Bacterial overgrowth was seen when co-cultivation period exceeded 48 h.

Based on the above observations, co-cultivation period of 48 h was found optimum with leaf and zygotic embryo explants for strain EHA105.

Table 22. Standardisation of co-cultivation period using *Agrobacterium* strain EHA105 for zygotic embryo

Pre-culture (days)	Bacterial density (OD_{600} nm)	Infection time (min)	Co-cultivation period (h)	GUS expression	Survival (%)
7	0.9	10	0	-	40
7	0.9	10	24	-	55
7	0.9	10	48	-	80
7	0.9	10	72	-	55
7	0.9	10	96	-	40
Control	-	-	-	-	0

Culture duration - 60 days; Number of cultures - 40
Regeneration medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA+ kanamycin 25 mg l^{-1} + cefotaxime 250 mg l^{-1}

4.4.4.4 Influence of acetosyringone as transformation enhancer

Influence of acetosyringone to enhance transformation was assessed by inclusion of acetosyringone in the bacterial inoculum for two different periods, 0.5 h and 1 h and at two concentrations 50 µM and 100 µM. The results are presented in Table 23 and 24.

Table 23. Effect of acetosyringone in enhancing transformation efficiency of EHA105 for leaf explant

Incubation period (h)	Concentration of acetosyringone (μM)	GUS expression	Callus induction rate (%)
0	0	+	50
0.5	50	+	80
1.0	100	+	80
Control	-	-	0

Culture duration - 60 days; Number of cultures - 40
Regeneration medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA+ kanamycin 50 mg l^{-1} + cefotaxime 250 mg l^{-1}

Results showed that 50-100 μM acetosyringone treated *Agrobacterium* enhanced the transformation efficiency by higher survival rate and callus induction of the infected tissues. Inclusion of acetosyringone in the co-cultivation medium increased the callus induction rate from 50 to 80 per cent.

For zygotic embryo in presence of acetosyringone no gus expression was observed but the cultures showed an increase in survival percentage from 50 to 80 at 50 to 100 μM acetosyringone.

Table 24. Effect of acetosyringone in enhancing transformation efficiency of EHA105 for zygotic embryo

Incubation period (h)	Concentration of acetosyringone (μm)	GUS expression	Survival (%)
0	0	-	50
0.5	50	-	80
1.0	100	-	80
Control	-	-	0

Culture duration - 60 days; Number of cultures - 40
Regeneration medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA+ kanamycin 25 mg l^{-1} + cefotaxime 250 mg l^{-1}

Based on the standardization experiments the parameters found best for *Agrobacterium* mediated genetic transformation of black pepper were infection time 10 min, co-cultivation period of 48 h, bacterial density 0.9 (OD_{600} nm) with acetosyringone in the induction medium at 100 μM.

4.4.5 Transformation using *Agrobacterium* strains, AGL.1.1303, GV2260 and LBA4404

Transformation was attempted with the *Agrobacterium* strains AGL.1.1303, GV2260 and LBA4404, keeping the conditions like infection time 10 min, co-cultivation period 48 h and bacterial density 0.9 (OD_{600} nm) constant and varying the explants.

4.5.5.1 Transformation with AGL.1.1303

The strain AGL.1.1303 contains the plasmid harbouring antibiotic resistant selectable marker genes (*npt II* and *hpt IV*) and gus and *gfp* as reporter genes. The data on transformation and regeneration with this strain after a culture period of three months in the screening medium is given in Table 25.

In the case of infected leaf disc explants, 50 per cent survived in the initial screening medium and the rest were necrotic. No callus induction/regeneration were observed in the surviving leaf bits, which become pale and dead after three months in the screening medium. The non-infected leaf explants maintained in antibiotic medium did not show any callus induction/ regeneration and were dead after three months. The non-infected leaf explants maintained in antibiotic free medium showed callus induction in 50 per cent of cultures and were green and proliferating after three months in the medium.

Seventy five percentage of the cotyledonary node explants survived in the initial screening medium and rest became brownish. Fifty percentage of the surviving cotyledonary node explants exhibited callusing at the base and bud initials at the nodal area. However, after three months in the screening medium the bud initials were remaining stunted. The non-infected cotyledonary nodal explants in antibiotic medium

did not show any regeneration and were brownish and dead after three months. The non-infected cotyledonary nodal explants in antibiotic free medium exhibited regeneration in 50 per cent of cultures and all were green and proliferating after three months.

Table 25. Results of transformation experiment with *Agrobacterium* strain AGL.1.1303 with selected explants

Explant	Results			
	Survival (%) in initial screening media	Callus induction (%)	Regenerat-ion (%)	Nature of explant
Leaf disc	50	Nil	Nil	All dead
Cotyledonary node	75	Nil	50	Green stunted
Zygotic embryo	100	Nil	Nil	Pale
Control-I (non-infected) with antibiotics (Leaf)	100	Nil	Nil	All dead
Control-I (non-infected) with antibiotics (Cotyledonary node)	100	Nil	Nil	All dead
Control-I (non-infected) with antibiotics (Zygotic embryo)	100	Nil	Nil	All dead
Control-II (non-infected) without antibiotics (Leaf)	100	50	Nil	All green & proliferating
Control-II (non-infected) without antibiotics (Cotyledonary node)	100	Nil	50	All green & proliferating
Control-II (non-infected) without antibiotics (Zygotic embryo)	100	Nil	100	All green & proliferating

Number of cultures - 50; Varieties - P_1, P_4 and P_6
Infection time - 10 min ; Co-cultivation - 48 h; Bacterial density - 0.9
Screening medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA + 250 mg l^{-1} cefotaxime + 25/ 50/ 100 mg l^{-1} kanamycin + 15/ 25 mg l^{-1} hygromycin

In the case of zygotic embryo 100 per cent survival was noted in the initial screening medium but all of them become pale without further growth in subsequent subculturing. In control zygotic embryo explants (non-infected) survived in the initial

screening medium but failed to survive in subsequent subculturing. Control explants maintained in the regeneration medium without antibiotic showed 100 percentage survival and proliferation. No varietal difference was observed.

Results indicate that among the explants cotyledonary node is better for transformation by the *Agrobacterium* strain AGL.1.1303.

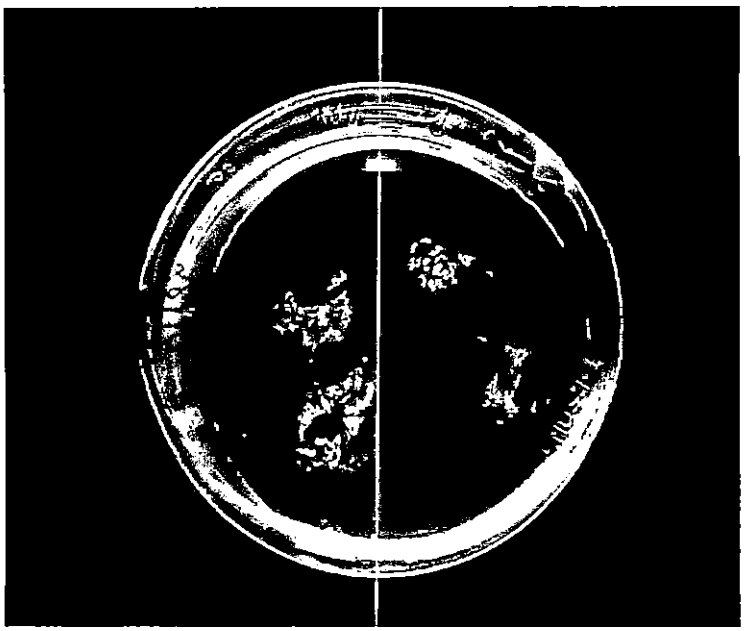
4.4.5.2 Transformation with GV2260

The strain GV2260 contains the plasmid pGV2260 with osmotin gene and *npt II* gene. Table 26 shows the results of the transformation experiments with GV2260. Leaf disc, zygotic embryo and cotyledonary nodal explants were transferred to the screening medium containing 50, 25 and 100 mg l^{-1} of kanamycin respectively. Cefotaxime at 250 mg l^{-1} was also included in the screening medium as bactericidal agent.

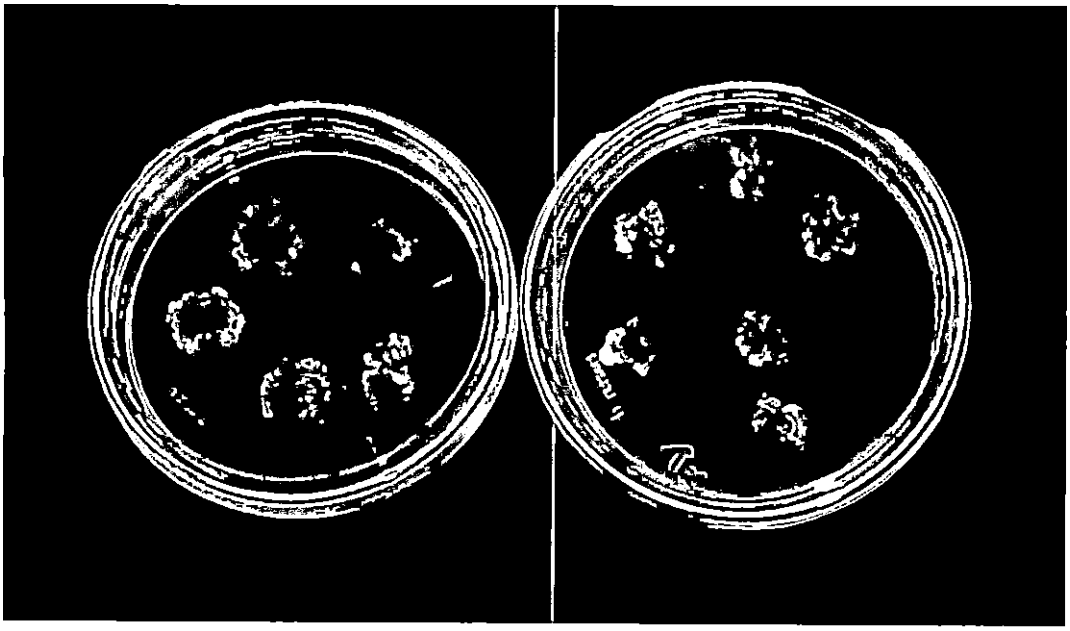
In the case of leaf disc explants, though 100 per cent survival in the initial screening medium was seen, only 50 per cent exhibited callus induction after a period of three months (Plate 9b). Noninfected leaf explants (control-I) did not show any callus induction in the screening medium and were pale coloured after three months. Non-infected leaf explants (control-II) in antibiotic free medium exhibited 100 per cent callusing and were green and proliferating after three months.

In the case of cotyledonary nodal explants 75 percentage survival was observed in the initial screening medium. After three months in the screening medium only 50 percentage of the surviving cotyledonary node explants were remaining green. But the growth of the surviving cotyledonary nodal explants was stunted. Non-infected cotyledonary nodal explants (control-II) in antibiotic free medium survived with good proliferation after three months. Non-infected cotyledonary nodal explants in selection medium (control-I) did not exhibit any regeneration.

Zygotic embryo explants infected with GV2260 exhibited 100 percentage survival in the initial screening medium. After three months of screening only 50 per cent of the surviving explants exhibited regeneration but the growth was stunted. All



pBZ 100
Variety Panniyur 4 **(*Agrobacterium* mediated)**



pGV 2260
Variety Panniyur 4 **(*Agrobacterium* mediated)**

Plate 9. Callus induction of infected leaves in selection media with kanamycin

the control (non-infected) explants survived in the initial screening medium but all of them became pale in later screening. In control-II (non-infected) explants maintained in antibiotic free medium remained green with proliferation of growth. No varietal difference was observed in the response of the explants.

Table 26. Transformation experiment with *Agrobacterium* strain GV2260 with selected explants

Explant	Results			
	Survival (%) in initial screening media	Callus induction (%)	Regeneration (%)	Nature of explant
Leaf disc	100	50	Nil	All green with callus induction
Cotyledonary node	75	Nil	50	Green, stunted
Zygotic embryo	100	Nil	50	Green, stunted
Control-I (non-infected) with antibiotics (Leaf)	100	Nil	Nil	All pale coloured
Control-I (non-infected) with antibiotics (Cotyledonary node)	100	Nil	Nil	All pale coloured
Control-I (non-infected) with antibiotics (Zygotic embryo)	100	Nil	Nil	All pale coloured
Control-II (non-infected) without antibiotics (Leaf)	100	100	Nil	All green and proliferating
Control-II (non-infected) without antibiotics (Cotyledonary node)	100	Nil	100	All green and proliferating
Control-II (non-infected) without antibiotics (Zygotic embryo)	100	Nil	100	All green and proliferating

Number of cultures - 50; Varieties - P_1, P_4 and P_6
Infection time - 10 min ; Co-cultivation - 48 h; Bacterial density - 0.9
Screening medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA + 250 mg l^{-1} cefotaxime + 25/ 50/ 100 mg l^{-1} kanamycin

Based on survival percentage in screening medium followed by callus induction/regeneration all explants were found equally good in transformation using GV2260.

4.4.5.3 Transformation with LBA4404

The strain LBA4404 contains the plasmid pBZ100 with alfalfa glucanase gene, rice chitinase gene and *npt II* gene. The data on transformation after a culture period of three months in the screening medium is given in Table 27.

Table 27. Results of transformation experiment with *Agrobacterium* strain LBA4404

Explant	Results			
	Survival (%) in initial screening media	Callus induction (%)	Regeneration (%)	Nature of explant
Leaf disc	100	50	Nil	All green with callus induction
Cotyledonary node	75	Nil	50	Green, stunted growth
Zygotic embryo	100	Nil	100	Green to pale stunted growth
Control-I (non-infected) with antibiotics (Leaf)	100	Nil	Nil	All pale coloured
Control-I (non-infected) with antibiotics (Cotyledonary node)	100	Nil	Nil	All pale coloured
Control-I (non-infected) with antibiotics (Zygotic embryo)	100	Nil	Nil	All pale coloured
Control-II (non-infected) without antibiotics (Leaf)	100	50	Nil	All green and proliferating
Control-II (non-infected) without antibiotics (Cotyledonary node)	100	Nil	50	All green and proliferating
Control-II (non-infected) without antibiotics (Zygotic embryo)	100	Nil	100	All green and proliferating

Number of cultures - 50; Varieties - P_1, P_4 and P_6
Infection time - 10 min ; Co-cultivation - 48 h; Bacterial density - 0.9
Screening medium - ½ MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA + 250 mg l^{-1} cefotaxime + 25/ 50/ 100 mg l^{-1} kanamycin

Leaf disc explants exhibited 100 percentage survival in the initial screening medium. In subsequent screening 50 per cent of the leaf disc survived with callus

induction. Non-infected (control-I) leaf explants did not exhibit callus induction/ regeneration and were pale coloured in the screening medium. Non-infected (control-II) leaf explants in antibiotic free medium exhibited callus induction in 50 per cent of cultures (Plate 9a).

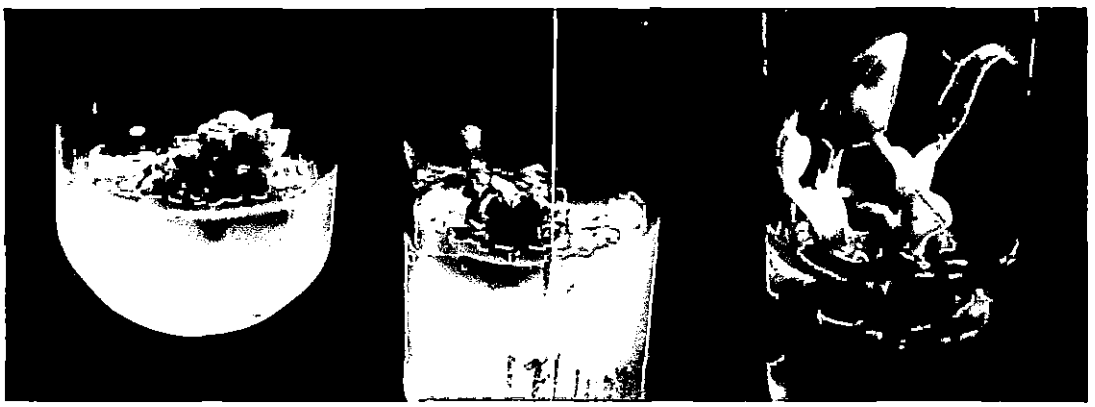
In the case of cotyledonary node explants, 75 per cent of the explants survived initially. Of this 50 per cent was surviving in the screening medium after three months of selection with greenish colour and stunted growth. Non-infected (control-I) explants did not show any regeneration in the screening medium and became pale. The control-II explants in antibiotic free medium exhibited regeneration in 50 per cent of cultures.

Survival rate of 100 per cent was observed in the case of zygotic embryos infected with LBA4404 which remained green with stunted initials after three months of screening. Control-I explants on screening medium become pale coloured. Whereas control-II explants on regeneration medium without antibiotics showed good proliferation of growth. No varietal difference was observed in the response of explants (Plate 10).

Results indicated that zygotic embryo are the best explants for transformation studies using LBA4404 followed by cotyledonary node and leaf explants.

4.5 PARTICLE BOMBARDMENT - MEDIATED TRANSFORMATION

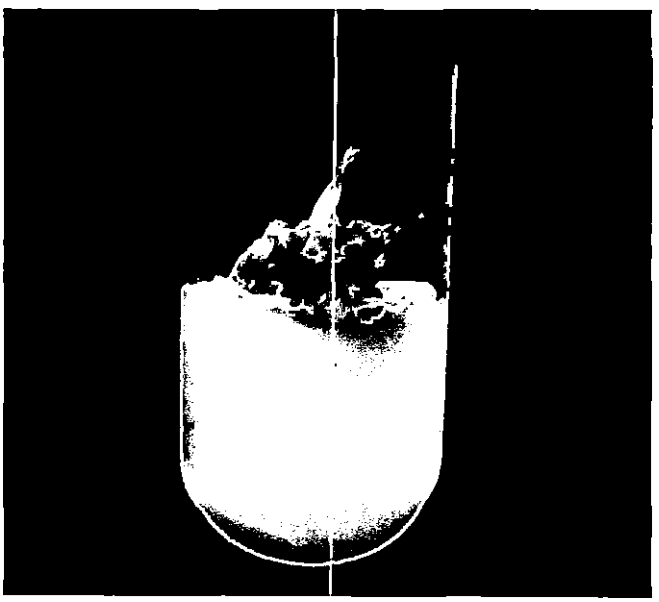
Nodal segments with adventitious buds and cotyledonary nodal segments of variety Panniyur 4 were shot with plasmid DNA pBZ100 using the particle delivery system. Ten explants each were shot and in selection medium all of them survived for three months. Bud initials were emerging but were stunted. So transferred to regeneration medium free of kanamycin. Only cotyledonary nodal segments survived with bud initials (Plate 11). Others were lost due to bacterial contamination.



Putative transformant (in antibiotic medium) | Control | Control in antibiotic free medium

Variety: Panniyur 4
Explant: Zygotic embryo

Plate 10. Transformation with pBZ 100



pBZ 100
Variety Panniyur 4

Plate 11. Putative transformant obtained through particle bombardment

4.6 MOLECULAR ANALYSIS OF TRANSFORMATION

a) Isolation of genomic DNA

Genomic DNA from five cultures transformed with LBA4404 having pBZ100 with glucanase and chitinase genes surviving in screening medium and one control were isolated following method by Doyle and Doyle (1987). Upon electrophoresis on one per cent agarose gel, intact DNA was observed in all the wells.

b) PCR analysis of *npt II* gene

The isolated DNA of the selected surviving cultures were screened by PCR. PCR was conducted with genomic DNA from putatively transformed and from nontransformed cultures as negative control. The plasmid DNA pGV2260 was used as positive control. Two specific primers derived from *npt II* gene sequences were used to detect a 600 bp fragment. The amplified DNA samples were electrophoresed on 0.7 per cent agarose gel. The results showed that only DNA from the positive control showed amplification of the 600 bp fragment. The control plant and the putative transformants did not amplify 600 bp fragment corresponding to *npt II* gene.

Discussion

5. DISCUSSION

Black pepper, a popular spice crop grown in India and traded throughout the world plays an important role in the country's agrarian economy. Among the major factors responsible for low productivity of black pepper, crop losses due to biotic and abiotic stresses are of foremost importance (Sarma and Kalloo, 2004). Lack of resistance to biotic and abiotic stresses in the available cultivars of black pepper and limitations in utilizing the resistance found in related species makes genetic engineering a viable option for developing disease resistant cultivars in black pepper.

PR proteins play significant role in the defense mechanism of plants against biotic and abiotic stresses. Genes encoding chitinases, glucanases, lysozymes, osmotin etc. were constitutively expressed in some model systems as well as crops under the control of different promoters. Glucanases belong to group PR-2 of PR proteins and break down β-1,3 glucans which are components of fungal cell wall. Yoshikawa *et al.* (1993) have produced transgenic tobacco plants with glucanase driven by 35S promoter showing increased resistance to *Rhizoctonia solani*. It was proposed that the degraded glucans act as elicitors to trigger the defense responses of the host systems thereby arresting the pathogen ingress. Chitinases were isolated and characterised from several sources like bacteria, fungi, insects and plants. They have shown to inhibit fungal growth by degrading newly synthesised chitin at the hyphal tips. The enzyme chitinase catalyses the hydrolysis of chitin. Osmotin is a small basic pathogenesis related protein produced by plants when challenged by biotic and abiotic stresses. Substantial accumulation of osmotin promoter occurs only in response to osmotic stress and ethylene. In tobacco cultivar W.38, Wolo Shuk *et al.* (1991) and La-Rosa *et al.* (1987) demonstrated *in vitro* that Osmotin has antifungal activity against a variety of fungi including *Phytophthora* infestans.

Among the available gene delivery systems, *Agrobacterium tumefaciens* mediated system is most widely used to introduce foreign genes into dicotyledonous plant species (Weising *et al.*, 1988) and some monocotyledonous species (Bytebier

et al., 1987). Biolistic approach has been used extensively for transformation of monocot species (Gopalakrishnan *et al.*, 2000).

Black pepper, *Piper nigrum* an intermediary of monocot and dicot with chromosome number 2n=54 belongs to the family Piperaceae. Micropropagation has been standardized in this crop (Philip *et al.*, 1992; Joseph *et al.*, 1996). Indirect organogenesis with leaf explants has also been reported (Nazeem *et al.*, 1990; Philip *et al.*, 1995; Shylaja, 1996). Reports about somatic embryogenesis are also available (Joseph *et al.*, 1996; Nair and Gupta, 2003). Cheriyan (2000) has attempted standardization of *Agrobacterium* mediated genetic transformation in black pepper using leaf and calli explants. Putative transgenics harboring osmotin gene were successfully regenerated from hypocotyl segments of *in vitro* derived seedlings of black pepper (IISR, 2004).

In the present study an attempt was made to standardize an *in vitro* regeneration protocol through somatic embryogenesis and to develop an *Agrobacterium tumefaciens* mediated genetic transformation system with suitable explants in three black pepper varieties viz., Panniyur - 1, Panniyur - 4 and Panniyur 6.

Agrobacterium tumefaciens strains EHA105, AGL-1 .1303 ,GV2260 , and LBA4404 were used for the study. The EHA105 contains the plasmid p35SGUSINT. This plasmid contains *gusA* gene fused to the CaMV35S promoter and the *npt II* gene controlled by the nos promoter. The *gusA* gene has an intron preventing its expression in *Agrobacterium*. The GV2260 contains the plasmid pGV2260. This plasmid contains osmotin gene and *npt II* gene tagged to the CaMV35S promoter. The AGL-1:1303 contains the plasmid harbouring antibiotic resistant selectable marker genes (*npt II* and *hpt IV*) and GUS and GFP reporter genes. These genes were under the control of CaMV35S promoter. The LBA4404 contains the plasmid pBZ100 containing alfalfa glucanase gene, rice chitinase gene and *npt II* gene tagged to the CaMV35S promoter.

5.1 SOURCE OF EXPLANTS FOR REGENERATION AND GENETIC TRANSFORMATION

Explants from mature vine and juvenile seedlings were used for the study. Tissue culture plantlets of the three selected varieties were raised from nodal segments to provide sufficient source of explants for transformation studies. The protocol standardised by Joseph *et al.* (1996) was found viable for all the three varieties. Among the varieties, P_6 gave maximum culture establishment and highest average number of multiple shoots/explant.

Axenic seedlings were raised by germinating seeds collected from ripe berries under *in vitro* condition to generate the explants, with reduced contamination for use in transformation work and related studies. Within a period of 15-18 days 100 per cent germination was noticed for all the three varieties on sterilized moist sand. However, recovery of good seedlings was restricted to 50 per cent due to fungal contamination. *In vitro* cultures were maintained by subculturing at biweekly intervals. Cheriyan (2000) also reported similar observations in the germination of seeds of black pepper variety Panniyur 1.

5.2 STANDARDIZATION OF SOMATIC EMBRYOGENESIS

Somatic embryogenesis is a process in which single somatic cells are induced to produce embryos, which in turn can germinate and produce a complete plant. Somatic embryogenesis protocol, if available in a crop, has two distinct advantages in transformation work. Firstly, because of its single cell origin, the development of chimeric plants is avoided. Secondly, through, secondary somatic embryogenesis thousands of transformed plants can be raised within a short period of time.

Nair and Gupta (2003) have reported somatic embryogenesis of black pepper in SH basal. So attempts were made to generate somatic embryos by culturing ripe seeds in SH basal and ½ MS basal medium with Inositol 1.0 g l^{-1}. Among the varieties P_4 and P_6 exhibited formation of proembryos at the micropylar end of the germinating seeds in 16.36 per cent of the seeds cultured in SH. Subculturing in the

same medium did not promote further development of the proembryos. The variety P_6 showed proembryo production in ½ MS medium with 1.0 g l^{-1} of Inositol also. Further development of the embryo to somatic embryo and plantlets was achieved in liquid shaking cultures in SH medium at 100 rpm. The variety P_1 showed only one per cent germination and no proembryos were produced.

Effect of genotypes on primary somatic embryogenesis in black pepper was assessed by Nair and Dutta (2005) and of the various genotypes tested, the cultivar Karimunda was found to be highly embryogenic. Variety P_6 has been evolved from Karimunda and in the present study also somatic embryogenesis was observed only in P_6. The percentage of cultures with somatic embryogenesis induction was low. Secondary embryogenesis though highly favourable for genetic transformation was not induced in the primary embryos formed. As per the report of Nair and Dutta (2005). subculturing the globular proembryo to SH with 1.5 per cent sucrose induced secondary somatic embryogenesis. In the present study embryo maturation and germination was achieved in liquid SH with three per cent sucrose. This result is in confirmation with that of Nair and Dutta (2005).

Attempt was also made for somatic embryogenesis by culturing zygotic embryo and zygotic embryo with endosperm in SH basal and MS basal with and without growth regulators. Potential growth regulators like 2,4-D, dicamba and thidiazuron at various concentration regimes were included for the study. Explants from the selected varieties were used. Culturing was done under dark and were observed for one year. None of the explants induced somatic embryogenesis in various media combinations tested. But culturing of embryo and embryo with endosperm resulted in embryo germination as well as callus induction in suitable media combinations and the study was helpful in understanding the regeneration path way of embryo.

Joseph *et al.* (1996) had reported obtaining embryogenic calli in black pepper cultivar Karimunda in SH basal without growth regulators. The failure in

obtaining embryogenic calli as reported by Joseph *et al.* (1996) in the present study could be due to the genotypic variation present in the different varieties and also some subtle differences in cultural condition which cannot be defined. Cheriyan (2000) attempted to generate embryogenic calli by culturing zygotic embryos of Panniyur I in SH medium but did not succeed.

Variation in response to somatic embryogenesis between different genotypes of the same species has been reported earlier. Raemakers *et al.* (1993) reported differences in somatic embryo production in six different lines of *Manihot esculenta* varying from 35 per cent in the line M. Col 22 to nil in other lines.

5.3 *IN VITRO* REGENERATION SYSTEM

Regeneration path way of different explants in various media combinations were also documented. Multiple shoot induction from nodal segments and cotyledonary node explants were observed in all the selected varieties in MS + 1.0 mg l^{-1} each of BA and IAA. In the same media organogenesis through multiple shoot production was observed in the zygotic embryo cultures. Direct organogenesis from *in vitro* established leaf segments from mature tissues of the variety P_4 was also obtained upto 17.5 per cent. Response of embryo germination and callusing was influenced by media and genotype. Variety P_6 was the most regenerative followed by P_4 and P_1. SH basal medium with dicamba 0.5 mg l^{-1} supported both germination and callusing of zygotic embryo in 66.66 per cent cultures. In this medium, embryo endosperm also callused in 72.2 per cent of cultures.

Based on the regeneration potential cotyledonary node, zygotic embryo and leaf segments and regeneration medium ½ MS with 1.0 mg l^{-1} BA and IAA were selected for the transformation study.

5.4 AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION

Agrobacterium mediated transformation was attempted using leaf segments and zygotic embryo as explants. The strains used include EHA 105, AGL.1.1303, GV 2260 and LBA 4404. For standardization of conditions for transformation the strain EHA 105 with P35 GUS INT was used.

5.4.1 Sensitivity of host tissues to antibiotics

Selection of the transformed cells is a key factor in developing successful methods for genetic transformation, otherwise the untransformed cells would overgrow leading to the loss of transformed cells. This is done by the use of certain selectable marker genes that are present in the vector along with gene of interest. Genes conferring resistance to antibiotics are generally used as selection markers. Once the tissue is transformed with the antibiotic resistance genes, it is grown in the medium containing antibiotic which is resistant to introduced genes. The cells which are transformed with the antibiotic resistance gene would multiply and grow normally, whereas, the untransformed cells are killed.

The usefulness of a particular antibiotic depends on several factors. The best selection agents are those that either inhibit growth or slowly kill the non transformed cells so that the dying cells do not overwhelm the transformed ones. Optimal selection pressure will use the low level of antibiotic needed to kill untransformed tissues. This necessitates the evaluation of sensitivity of plant tissue to antibiotics and the minimum concentrations of antibiotics required to inhibit growth (Cheriyan, 2000).

In the present study sensitivity of leaf segments, zygotic embryo and nodal segments of three selected varieties of black pepper to different antibiotics viz., kanamycin, hygromycin, cefotaxime and carbenicillin at different doses were evaluated. The gene constructs used for the transformation work carries kanamycin and hygromycin resistance as the plant selection markers. Cefotaxime and carbenicillin were also included for the study as the infected explants have to be made free of bacteria with suitable antibiotic at appropriate concentrations.

The application of kanamycin to the regeneration medium above 50 mg l^{-1} concentration inhibited the induction of callus in leaf segments and above 25 mg l^{-1} blackened the zygotic embryo explants. Nodal segments were sensitive to kanamycin from 100 mg l^{-1} onwards. Hence kanamycin 25 mg l^{-1}, 50 mg l^{-1} and 100 mg l^{-1} were

selected as cut off level for the selection of transofrments from zygotic embryo, leaf explants and nodal segments respectively. Kanamycin is the most commonly used selection agent for plant transformation (Owens, 1981). Sasikumar and Veluthambi (1994) and Cheriyan (2000) also reported that callus induction on black pepper leaf was completely inhibited at 50 mg l^{-1} kanamycin, whereas 100 mg l^{-1} was required to prevent fresh growth of the callus. This is in consensus with the findings in the present study. During transformation of black pepper using leaf, petiole and stem explants, Sin *et al.* (1998) have reported to have used 75 mg l^{-1} kanamycin for selection of transformed cells.

Hygromycin at concentration of 25 mg l^{-1} prevented callusing of leaf segments and for zygotic embryo, hygromycin at 10 mg l^{-1} resulted in blackening of embryos. Cheriyan (2000) reported complete arrest of callus induction on black pepper leaf at a minimum dose of 10 mg l^{-1} hygromycin.

Complete elimination of the bacteria from the explant after co-cultivation is very essential, otherwise it will interfere with growth and organogenesis of the explant. Overgrowth of the bacteria causes death of the explant and disrupts the experiment. Elimination of the bacteria from the explant is done by the use of antibiotics. The antibiotic chosen should be such that it efficiently kills the bacteria, at the same time it does not affect the growth and morphogenesis of the explants. The most commonly used antibiotics for this purpose are carbenicillin and cefotaxime. However, its effect on the explants has to be studied before choosing any one of them as they are also reported to have detrimental effect on some species (Cheriyan, 2000).

The antibiotics cefotaxime and carbenicillin were not toxic to explant tissues of black pepper within the range of concentrations (50-400 mg-l) tested. Carbencillin induced callusing in all the leaf explants and sprouting of the zygotic embryo at all the concentrations tested. Similarly callusing of the tissue by carbenicillin was reported in *Antirrhinum majus* and *Nicotiana* (Holford and Newbury, 1982; Pullock *et al.*, 1983). Holford and Newbury (1992) concluded that the break down product of carbenicillin with auxin activity, phenyl lactic acid was responsible for stimulatory effects in *Antirrhinum majus* callus growth. Such a mechanism could

partly explain the results observed with carbenicillin in the present study. The antibiotic cefotaxime also induced callusing in leaf explants and sprouting of zygotic embryo at all concentrations tested. Cheriyan (2000) studied the bacteriocidal activity of cefotaxime on *Agrobacterium tumefaciens* strain AGL-1:1303 and found that cefotaxime at 500 mg l^{-1} killed the Agrobacterium in pure bacterial suspension cultures with density 1.0 (OD_{560} nm). He also reported that cefotaxime at 500 mg l^{-1} could not eliminate the bacteria after co-cultivation from leaf derived calli.

In the present study the antibiotic concentrations found optimum for selection of the transformed tissues of nodal segments were kanamycin 100 mg l^{-1} and hygromycin 25 mg l^{-1}. Carbenicillin and cefotaxime did not inhibit the growth of various explants tested at all concentrations used.

5.4.2 Sensitivity of Agrobacterium strains to antibiotics

The pre requisite steps in *Agrobacterium* mediated transformation are an efficient system to produce transformed tissue and subsequent killing of *Agrobacterium* effectively. Cefotaxime and carbenicillin have been most widely used for killing *Agrobacterium* because of their broad spectrum bactericidal activity. The elimination of *Agrobacterium* is important because the continued presence of *Agrobacterium* can present a problem for identifying transformants or interferes with the growth and development of the transformed plant cells or cause the death of the cultures (Tang *et al.*, 2000).

All the four *Agrobacterium* strains tested were sensitive to cefotaxime at 50 mg l^{-1} onwards indicating that this antibiotic at this concentration could be used for killing the bacteria after co-cultivation. But a higher concentration of 250 mg l^{-1} was fixed for effective elimination of bacteria.

5.4.3 Standardisation of *Agrobacterium* mediated transformation

Agrobacterium mediated genetic transformation of *Piper nigrum* L. var. P_1, P_4 and P_6 was standardized with the *Agrobacterium* strain EHA 105 having gus reporter gene and *npt II* as plant selection marker gene. Humara *et al.* (1999) and Fatima *et al.* (2005) also used the same strain for standardization of transformation

experiments in *Pinus pinea* and *Capsicum* respectively. The selectable marker gene present in the T-DNA was *npt II* (neomycin phosphotransferase 2), which confers resistance to kanamycin. The T-DNA also harbours gene conferring resistance to rifampicin and this acts as bacteria selection agent to prevent contamination of bacterial strain. Once the T-DNA is transferred to the plant genome, the transformed plant cells are expected to develop resistance to kanamycin and will be capable of normal growth in the medium containing kanamycin, in contrast the non-transformed cells will be susceptible to kanamycin. The transformed cells thus developed will also show gus activity, which can be detected by histochemical gus assay.

The bacterial gene *uid A* encoding ß-glucuronidase (gus) is the most frequently used reporter gene for the analysis of plant gene expression. There is little or no detectable endogenous ß-glucuronidase activity in most of the higher plants allowing the easy detection of chimaeric gene expression. Glucuronidase activity can be detected easily and sensitively. Histochemical localization is possible in cells and tissues due to the blue colour developed with the substrate X-gluc (5-bromo-4-chloro-3-indolyl glucuronide). The substrate is cleaved by the enzyme within the plant cells (transformed/transgenic), and the product is converted into an insoluble blue precipitate at the site of enzyme activity. This colour so developed is visible to the naked eye and under microscope in sectioned tissues.

5.4.3.1 Standardisation of inoculum density

Concentration of bacterial cells in the inoculation medium is an important factor to be considered for efficient transformation. Among the different inoculum densities tried, $O.D_{600}nm$ = 0.9 was found optimum. Transient Gus expression after three days of co-cultivation showed blue areas with bacterial density 0.9 and 1.0 ($OD_{600}nm$). But none of the transformed explants survived after 60 days when bacterial density was 1.0. This is in conformity with the observations of Humara *et al.* (1999). Very high bacterial density leads to necrosis and death of the explant. This is due to the hypersensitive response of the tissue as a part of the plant defense against pathogen.

5.4.3.2 Standardisation of infection time

The time of infection of leaf explants of black pepper was standardized with P35 GUS INT construct. The infection times viz. 2, 5, 10, 15 and 30 minutes were tried and 10 minutes was found optimum. Lee *et al.* (1996) reported infection time of 1 h for successful transformation of *Casuarina glauca* whereas Humara *et al.* (1999) observed 5 minutes infection time giving better transformation efficiency in *Pinus pinea.*

5.4.3.3 Standardisation of co-cultivation period

Co-cultivation period plays an important role in the success of the transformation. It is during this period that the *vir* genes are activated and the T-DNA transferred into the plant cell. However, increasing the co-cultivation period might lead to necrosis and death of the explant due to the hypersensitive response of the tissue. Hence the length of co-cultivation period should always be the shortest interval necessary to obtain the maximum frequency of transformation in terms of the number of transgenic plants recovered following co-cultivation.

In the present study, a co-cultivation period of 48 h was the best for transformation when compared to 0, 24, 72 and 96 h. These differences could be explained by the fact that the concentration of *Agrobacterium* was optimum during 48 h co-cultivation than after 24 h and that could considerably increase the probability of gene transfer. Similar results were also reported by Pawlicki *et al.* (1992) in carrot and Suma (2006) in ginger.

Bacterial cells get multiplied in the co-culture medium and after 48 h, the optimum quantity of bacteria were available for transformation and hence a higher survival rate in the selection medium was achieved.

5.4.3.4 Influence of acetosyringone as transformation enhancer

Acetosyringone is a phenolic compound produced during wounding of plant cells that induces the transcription of the virulent genes of *A. tumefaciens.* Its beneficial role has been demonstrated in the genetic transformation of some woody

fruit species such as kiwifruit (Jansen and Gardner, 1993) and citrange (Cervera *et al.*, 1998).

In the present investigation also 50-100 µM acetosyringone treated *Agrobacterium* enhanced the transformation frequency by higher survival rate and callus induction of the transformed tissues.

5.4.3.5 Histochemical Gus assay

ß-glucuronidase enzyme activity was used to monitor the transformation. Blue staining was observed when explants were examined three days after co-cultivation with EHA 105. Only a diffuse blue staining was observed on the leaf explants. In the case of zygotic embryo no blue staining was observed due to heavy phenolic interference.

5.4.4 Transformation with *Agrobacterium* strains AGL 1.1303, GV 2260 and LBA 4404

Genetic transformation of selected explants leaf disc, cotyledonary node and zygotic embryo of black pepper was carried out with strains AGL.1.1303, GV2260 and LBA4404. Parameters standardized with strain EHA105 were followed for transformation. Bacterium inoculum density, 0.9 were made to infect the explant for 10 min and a cocultivation of 48 h given for multiplication of bacteria. Bacterial elimination from the infected cultures was done with cefotaxime 250 mg l^{-1}. Transformants was screened by kanamycin sensitivity and gus assay. Molecular analysis of selected putative transformants was also done by PCR.

Transformation experiments using leaf disc explants; cotyledonary node and zygotic embryo of black pepper with *Agrobacterium* strain AGL 1.1303 harbouring *nptII* and gus reporter gene revealed that cotyledonay node explants are better for transformation with this strain.The putative transformants have to be verified for gene integration using PCR Leaf disc explants and zygotic embryo were not surviving in the screening medium whereas cotyledonary node survived in subsequent

screening medium. Cheriyan (2000) reported failure of leaf explants of black pepper to survive in the screening media after co-cultivation which become dead or showed bacterial over growth on withdrawl of cefotaxime from screening media.

In the case of GV 2260 carrying osmotin gene, all the three explants viz. leaf disc, cotyledonary node and zygotic embryo were found to be transformed with this strain. This is based on survival of these explants on screening meida. Leaf disc explants were found to induce callus to the extent of 50 per cent. In the case of cotyledonary node also 50 per cent was surviving after three months in the screening medium. For zygotic embryo, 100 per cent survival was observed. However, regeneration was arrested or slow. PCR analysis for confirmation of transformation has to be done.

Transformation experiments with the Agrobacterium strain, LBA 4404 having glucanase and chitinase gene revealed that zygotic embryos are the best explants for this strain followed by cotyledonary node and leaf disc explants. 100 per cent survival of the zygotic embryo was observed in the selection media and all of them remained green with stunted bud initials. Here also confirmation for gene integration has to be made by PCR analysis

Strain specificity has been reported by different workers. In carrot, Pawlicki (1992) found that Agrobacterium strain C 58 C, was more active than the LBA 4404 strain in the explants, Likewise, Olikowska *et al.* (1995) reported that Agrobacterium strain EHA 105/p35SGUS INT was more infective than LBA 4404 with safflower cultivar 'Centennial'.

Strain specificity has also been reported in black pepper (Sasikumar and Veluthambi, 1996). Here binary vector (LBA 4404 an EHA 105 harbouring PGA 472) was found to successfully transform black pepper whereas co-integrate vector (p GV 2260-PGSFR 280) did not produce transformation. However, these results were based only on phenotypic observation.

5.4.5 Direct gene transfer using particle gun

Cotyledonary nodal segments and nodal segments with adventitious buds were used for direct gene transfer using particle gun delivery system. Only cotyledonary nodal segments were found surviving in the initial screening medium containing kanamycin. Adventitious buds were discarded due to interference of endogenous bacteria.

5.5 MOLECULAR ANALYSIS OF CONFIRMATION OF TRANSFORMED TISSUE

Zygotic embryo derived tissues infected by LBA4404 with glucanase and chitinase genes were subjected to PCR analysis along with a positive control. PCR analysis revealed that *npt II* gene integration has not happened in the infected tissues subjected to PCR analysis. Sample size for PCR analysis was four. Analysis of more number of regenerants may reveal the transformation efficiency.

Conclusion

Somatic embryogenesis was induced on ripe seeds of black pepper at mycropylar region in the variety P_6 and whole plantlets were developed and successfully planted out. From the regeneration trials explants, cotyledonary node and zygotic embryo were identified as suitable for transformation. Parameters for genetic transformation of black pepper was standardized with strain EHA105 harbouring *npt II* and gus reporter genes. *Agrobacterium* inoculum density 0.9 (OD_{600}), infection time 10 min and co-cultivation period of 48 h were identified. Genetic transformation using *Agrobacterium* strains AGL.1.1303, GV2260 and LBA4404 revealed that there exist explant specificity for the different strains. LBA4404 with glucanase and chitinase genes gave a better survival percentage of zygotic embryo explants. PCR analysis of selected putative transformants infected with LBA4404 did not show integration of *npt II* gene. Transformation efficiency can be assessed only after analysis of more number of cultures surviving in the screening medium. Transformation using gene gun with cotyledonary node explants led to survival in the screening medium for four months.

Future line of work

Somatic embryogenesis system is the ideal one for genetic transformation studies. The rate of somatic embryogenesis induction observed in this study was low. Somatic embryogenesis system has to be perfected with secondary embryogenesis. Among the *Agrobacterium* strains LBA4404 with glucanase and chitinase genes gave a better survival percentage of zygotic embryo explants in the selection medium. Future work should concentrate more on embryogenesis induction and transformation using glucanase and chitinase genes. Direct gene transfer method using gene gun should also be intensified.

Summary

6. SUMMARY

Investigations on Genetic transformation of black pepper for *Phytophthora* foot rot resistance/tolerance were carried out at the Department of Plantation Crops and Spices and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2001-2006. The objective of the study was to develop an *in vitro* regeneration protocol for somatic embryogenesis in black pepper and to develop an efficient vector mediated genetic transformation system in black pepper varieties P_1, P_4 and P_6 for *Phytophthora* foot rot resistanc/tolerance. The salient findings of the study are summarized below.

Axenic seedlings of the selected varieties were raised under *in vitro* conditions to generate explants with reduced contamination for transformation works. 100 percentage germination was obtained in sterilized moisture sand for all the varieties within a period of 15-18 days.

Axenic plantlets from nodal segments of the selected varieties P_1, P_4 and P_6 were raised for using as explant source for embryogenesis and transformation studies. Among the varieties, average number of multiple shoot/explant was high for variety P_6 in ½ MS medium with BA and IAA 1.0 mg l^{-1}.

Somatic embryogenesis was attempted using different explants like seeds from ripe berries, zygotic embryo, zygotic embryo with endosperm, cotyledonary node, hypocotyl and cotyledonary leaf segments of black pepper. MS and SH basal media with different growth regulators like, 2.4-D, BA, dicamba and thidiazuron were used for somatic embryogenesis in black pepper. SH basal medium was found better than MS basal for germination of seeds of black pepper. P_4 and P_6 varieties gave a germination percentage of 16 per cent whereas variety P_1 gave only 1 per cent germination. Development of proembryos at the micropylar region of germinating seeds was observed in the varieties P_4 and P_6 in SH basal medium which however failed to develop further in subsequent subculturing. In variety P_6 2.5 per cent of cultures developed proembryos in ½ MS medium with Inositol 1.0 g l^{-1} and further

development of the proembryo into different stages of somatic embryos was observed in liquid shaking cultures of SH medium with three per cent sucrose. Regenerated plantlets were hardened and successfully established under *ex vitro* conditions.

Growth regulators like BA, 2,4-D, dicamba and thidiazuron did not give rise to embryogenesis in the different explants of black pepper.

Regeneration path way of different explants in various media combinations were also documented. Multiple shoot induction from nodal segments and cotyledonary node explants were observed in all the selected varieties in MS + 1.0 mg l^{-1} each of BA and IAA. In the same media organogenesis through multiple shoot production was observed in the zygotic embryo cultures. Direct regeneration from *in vitro* established leaf segments from mature tissues of the variety P_4 was also obtained upto 17.5 per cent. Response of embryo germination and callusing was influenced by media and genotype. Variety P_6 was the most regenerative followed by P_4 and P_1. SH basal medium with dicamba 0.5 mg l^{-1} supported both germination and callusing of zygotic embryo in 66.66 per cent cultures. In this medium embryo endosperm also callused in 72.2 per cent of cultures .Based on the regeneration potential cotyledonary node, zygotic embryo and leaf segments and regeneration medium ½ MS with 1.0 mg l^{-1} BA and IAA were selected for the transformation study.

Agrobacterium tumefaciens strains EHA 105, AGL 1.1303, GV 2260 and LBA 4404 were used for the transformation work. Strain EHA 105 contains the plasmid p35SGUSINT with *gus A* gene and *npt II* gene. The AGL 1.1303 contains the plasmid harbouring antibiotic resistant selectable marker genes (*npt II* and *hpf IV*) and GUS and GFP reporter genes. The GV 2260 contain the plasmid pGV2260 with osmotin gene and *npt II* gene. The LBA 4404 contains the plasmid pBZ100 containing alfalfa glucanase gene, rice chitinase gene and *npt II* gene.

Sensitivity of leaf segments, nodal segments and zygotic embryos of pepper to kanamycin and hygromycin was tested as they were the plant selection

markers in the gene constructs used. Kanamycin 25 mg l^{-1}, 50 mg l^{-1} and 100 mg l^{-1} were selected as the cut of level for the selection of transformants from zygotic embryo, leaf segments and nodal segments respectively. Leaf segments exhibited callus induction and zygotic embryo sprouted in kanamycin free regeneration medium. Hygromycin at concentrations of 25 mg l^{-1} prevented callusing of leaf segments. For zygotic embryo at 10 mg l^{-1} and above blackening of the freshly scooped embryo was noticed. Cefotaxime and carbenicillin did not inhibit growth of leaf segments and zygotic embryo at all concentrations tested and a concentration of 250 mg l^{-1} was selected for transformation experiments.

Sensitivity of the *Agrobacterium* strains to different antibiotics was evaluated and all the four strains were found sensitive to cefotaxime. Cefotaxime at 250 mg l^{-1} was used for the elimination of bacteria from the transfomed explants.

Genetic transformation was standardized with *Agrobacterium* strain EHA 105 using leaf disc explants and zygotic embryo. Tentative protocol for the transformation of black pepper include *Agrobacterium* inoculum density 0.9 (OD_{600}), infection time 10 min, and co-cultivation period of two days. Addition of acetosyringone at a concentration of 50-100 µM favoured transformation. Transient GUS assay revealed faint blue staining on the transformed leaf explants. Transformed leaf explants induced callus on screening medium ½ MS with 1.0 mg l^{-1} BA and IAA, cefotaxime 250 mg l^{-1} and kanamycin 100 mg l^{-1}.

Explants leaf segments, cotyledonary node and zygotic embryo were used for transformation with *Agrobacterium* strains, AGL 1.1303, GV 2260 and LBA 4404. In the case of AGL 1.1303, only cotyledonary node explants survived in the screening medium with cefotaxime 250 mg l^{-1}, kanamycin 100 mg l^{-1} and Hygromycin 30 mg l^{-1}, whereas leaf explants and zygotic embryo did not survive. With GV 2260, leaf explants, cotyledonary node and zygotic embryo survived in the screening medium. With LBA 4404 zygotic embryo explants gave 100 per cent survival in the screening medium followed by cotyledonary node and leaf explants. There is explant specificity

for different *Agrobacterium* strains used. In the screening medium the transformed explants survived even after three months but failed to regenerate due to inhibitory effect of kanamycin.

Direct gene transfer method using gene gun (PDS/1000 He) was also attempted with pBZ100 (Glucanase and chitinase) and cotyledonary node/adventitious bud explants. Bombarded cotyledonary node explants were found surviving in the screening medium with kanamycin.

Molecular analysis of confirmation of tissue transformed with strain LBA4404 using zygotic embryo explants was done using PCR. PCR analysis revealed that *npt II* gene integration has not happened in the tissues subjected to PCR analysis. Analysis of more number of regenerants will reveal the transformation efficiency.

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* Originals not seen

Appendices

APPENDIX - I

Composition of MS basal medium (Murashige and Skoog, 1962)

Components	Quantity ($mg\ l^{-1}$)
a) Major nutrients (Stock solution I)	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSo_4.7H_2O$	370
KH_2PO_4	170
b) Minor nutrients (Stock solution II)	
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
c) Stock solution III	
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
d) Organic constituents (Stock solution IV)	
Myoinositol	100
Pyridoxine HCl	0.5
Glycine	2.0
Thiamine	0.1
Nicotinic acid	0.5
e) Sucrose	30 g/L
f) Agar	8 g/l

APPENDIX - II

Media composition (YEP)

Peptone	- 10.0 g.
Yeast	- 10.0 g
NaCl	- 5.0 g
$MgCl_2$	- 1.0 g
Distilled water	- 1 litre
pH at 25°C	- 7.

Media composition (YEM)

K_2HPO_4	- 5.0 g
$MgSO_4.7H_20$	- 2.0 g
NaCl	- 1.0 g
Manitol	- 10.0 g
Yeast	- 1.0 g
Distrilled water	- 1 litre
pH	- 7.

Luria Bertani (LB) medium

Tryptone	- 10.0 g
Yeast extract	- 5.0 g
NaCl	- 5 .0 g
Glucose	- 1.0 g
Distilled water	- 1 litre
pH	- 7.

APPENDIX - III

Reagents for GUS assay

X-gluc	:	10 mg
Dimethyl sulfoxide (DMSO)	:	100 µl
1 M sodium phosphate (pH 7)	:	1 ml
Triton	:	1 ml
Sterile distilled water	:	18 ml

Ten mg X-gluc was dissolved in 100 µl DMSO. Sodium phosphate and triton were added and finally the volume was made up to 20 ml with sterile distilled water. Aliquots of 1 ml were taken in storage vial, wrapped with aluminium foil and stored at -20°C.

GENETIC TRANSFORMATION OF BLACK PEPPER (*Piper nigrum* L.) FOR *PHYTOPHTHORA* FOOT ROT RESISTANCE / TOLERANCE

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ABSTRACT OF THE THESIS

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ABSTRACT

Investigations on "Genetic transformation of black pepper (*Piper nigrum* L.) for *Phytophthora* foot rot resistance/tolerance" were carried out at the Department of Plantation Crops and Spices, and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during 2001-2006. Three selected black pepper varieties Panniyur 1, 4 and 6 were utilized for the study.

Axenic cultures of the selected varieties were raised from nodal segments as well as ripe seeds for embryogenesis and transformation studies. Among the varieties average number of multiple shoot/explant was high for variety P_6 in ½ MS medium with BA and IAA 1.0 mg l^{-1}.

Somatic embryogenesis was induced on ripe seeds of the variety P_6 at the micropylar region in ½ MS medium with inositol 1.0 g l^{-1}. Further development of the somatic embryo observed in liquid shaking cultures of SH basal. Growth regulators like BA,2,4-D ,dicamba and thidiazuron did not give rise to embryogenesis in the different explants of black pepper.

Multiple shoot induction from cotyledonary nodal explants and zygotic embryo explants were observed in all the varieties in ½ MS with 1.0 mg l-1 BA and IAA. Direct regeneration from leaf segments was also observed in the same media combination.

Agrobacterium tumefaciens strains EHA105, AGL.1.1303, GV2260 and LBA4404 were used for the transformation work. Strain EHA105 contains the plasmid p35SGUSINT with *gus A* gene and *npt II* gene. The AGL.1.1303 contains the plasmid harbouring antibiotic resistant selectable marker genes (*npt II* and *hpt IV*) and GUS and GFP reporter genes. The GV2260 contain the plasmid pGV2260 with osmotin gene and *npt II* gene. The LBA4404 contains the plasmid pBZ100 containing alfalfa glucanase gene, rice chitinase gene and *npt II* gene.

Sensitivity studies of black pepper tissues to various antibiotics resulted in selecting the optimum threshold level of antibiotic to be used in the screening medium. Kanamycin 25 mg l^{-1}, 50 mg l^{-1}, and 100 mg l^{-1} were selected as the cut off level for the selection of transformants from zygotic embryo, leaf segments and nodal segments respectively. Cefotaxime at 250 mg l^{-1} was selected for the effective elimination of *Agrobacterium* after infection.

Genetic transformation was standardized with *Agrobacterium* strain EHA105 using leaf disc and zygotic embryo explants. Tentative protocol for transformation in black pepper include *Agrobacterium* inoculum density 0.9, infection time 10 min and co-cultivation period of 48 h. Acetosyringone at 50-100 µM favoured transformation. Transient gus assay revealed faint blue staining on the infected leaf explants.

Explants, leaf segment, cotyledonary node and zygotic embryo were used for transformation with *Agrobacterium* strains AGL.1.1303, GV2260 and LBA4404. There was explant specificity for the different *Agrobacterium* strains used. With LBA4404 zygotic embryo explants gave maximum survival in the screening medium containing 50 mg $^{l-1}$ kanamycin and 250 mg^{-1} cefotaxime.

Direct gene transfer using gene gun attempted with pBZ100 and cotyledonary node explants. Bombarded explants were found surviving in the screening medium with kanamycin for four months. However molecular analysis of selected transformants through PCR revealed that *npt II* gene integration has not happened in the tissues subjected to PCR analysis.