AGROBACTERIUM - MEDIATED GENETIC TRANSFORMATION IN BLACK PEPPER

(Piper nigrum L.)

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy in Agriculture

Faculty of Agriculture
Kerala Agricultural University

DEPARTMENT OF PLANT BREEDING AND GENETICS
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VELLANIKKARA, THRISSUR - 680 656
KERALA, INDIA

2000

DECLARATION

I hereby declare that this thesis entitled "***Agrobacterium* mediated genetic transformation in black pepper (*Piper nigrum* L.)**" 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, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled "***Agrobacterium* mediated genetic transformation in black pepper (*Piper nigrum* L.)**" is a record of research work done independently by **Shri.Homey Cheriyan,** 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 him.

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ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude and indebtness to ***Dr.N.K.Vijayakumar****, Professor, College of Forestry, Kerala Agricultural University and Chairman of the Advisory Committee for his valuable guidance, constant encouragement, thought provoking discussion and forbearance throughout the course of this investigation and preparation of this thesis. I consider myself fortunate to have been guided by him.*

I am grateful to ***Dr.K.Pushkaran****, Professor and Head, Department of Plant Breeding and Genetics, College of Horticulture for his guidance and timely help in completing this work successfully.*

I am deeply indebted to ***Dr.P.A.Nazeem****, Associate Professor, CPBMB, College of Horticulture, who played a key role right from the formulation of this project to the completion of this manuscript. I thankfully acknowledge her sincere and much needed help and guidance at every stage of this work.*

I take pleasure in expressing my thanks to ***Dr.R.Vikraman Nair****, A.D.R., Vellayani, for his constant encouragement and useful advice during the course of this investigation.*

I acknowledge, the encouragement and guidance rendered by ***Dr.C.R.Elsy****, Associate Professor, Department of Plant Breeding and Genetics, College of Horticulture during this study.*

I am obliged to Dr.D.Girija, Assistant Professor, CPBMB, College of Horticulture for her timely help and guidance in the conduct of experiments and correction of the manuscript.

I am respectfully thankful to Dr.P.V.Prabhakaran, Associate Dean, College of Horticulture, for the keen interest he had shown for the successful completion of this work.

I express my heartfelt gratitude to all the members and staff of the Department of Plant Breeding and Genetics, College of Horticulture, for their goodwill and co-operation in completing this programme successfully.

I am thankful to Dr. Luckin C. Babu, Associate Dean, College of Forestry, for permitting me to do my research work in the Tissue Culture Laboratory, College of Forestry. My thanks are also due to Dr. P.K. Asokan for the help rendered during the investigations.

Let me place on record my profound gratitude and indebtness to Shri. V.Jayashankar, I.A.S., Chairman, Spices Board for being magnanimous in granting me study leave for undertaking this programme. His inspiration and good wishes have helped me in successfully completing this programme.

I am also obliged to M/s SPIC Research Foundation, Chennai for having supplied the Agrobacterium tumefaciens strain AGL-1;1303 which was used for this study.

The co-operation and help rendered by Shri.S.R.Natesh, Smt.Sathi Devi, smt.P.G.Khadija and Shri. Davis in the day to day work during the course of this investigation is thankfully acknowledged.

I also thank Sri. Joy for neat and speedy typing of this manuscript.

I am thankful to my wife, my little daughter, mother, sisters and brother-in-laws for their perseverance throughout this programme.

The support rendered by my father-in-law and mother-in-law is thankfully acknowledged.

I am also obliged to Shri. Arjun Singh for the support he rendered to help me complete this programme.

My father would have been the happiest person to see this manuscript. I am sure his soul is brim with joy in heaven at this moment. He was instrumental in my doing the Ph.D. I pay my obeisance to him.

I bow my head before The Almighty, whose blesssing were with me throughout this venture.

HOMEY CHERIYAN

in loving memory of my father

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ABBREVIATIONS

IAA	-	indole – 3 – acetic acid
BA	-	benzyl amino purine
Kn	-	kinetin
2,4-D	-	2,4-dichlorophenoxy acetic acid
NAA	-	naphthalene acetic acid
min.	-	minutes
MS	-	Murashige and Skoog's (1962) medium
SH	-	Schenk and Hildebrandt (1972) medium
Kana	-	kanamycin
Cefo	-	cefotaxime
Hygro	-	hygromycin
Carb	-	carbenicillin
h	-	hours
O.D.	-	optical density
µm	-	micrometer
µl	-	microlitre
$mg\ l^{-1}$	-	milligram per litre

Introduction

INTRODUCTION

Black pepper (*Piper nigrum* L.), the king of spices, is an important cash crop of India. Today it's cultivation is spread over an area of 1,81,550 ha mainly in the states of Kerala, Tamil Nadu and Karnataka producing around 57,270 tonnes of black pepper annually (Ghosh *et al.*, 1999). Often referred to as black gold, it fetches Rs.600 to 700 crores of foreign exchange annually through export.

The productivity of black pepper in India, however, is recorded to be as low as 315 kg/ha as against around 3954 kg/ha in Thailand or about 1888 kg/ha in Malaysia (Madan *et al.*, 2000). Of the various reasons cited for low productivity of black pepper in India, crop loss due to pests and diseases is considered to be a major factor and foot rot disease caused by *Phytophthora capsici* is one of the most important among them. An annual loss of 905 and 119 tonnes of black pepper, respectively, was reported from Kannur and Kozhikode districts of Kerala by Balakrishnan *et al.* (1986) and Anandaraj *et al.* (1988). Prabhakaran (1997) estimated an unavoidable loss of 10 per cent of annual pepper yield due to *Phytophthora capsici* by way of stand loss, which has serious implication in future yields also.

To overcome this problem, development of varieties resistant to foot rot disease is considered as an important objective in breeding of black pepper. The most important breeding strategy in this context is to incorporate genes for resistance/tolerance to the promising cultivars. Several workers have screened the germplasm for resistance to *Phytophthora* foot rot (Holliday and Mowat, 1963; Leather, 1967; Kueh and Khew, 1980; Vilasini, 1982; Sarma *et al.*, 1982) but none of the cultivated types was found to be resistant. Sarma *et al.* (1991) suggested that since high degree of resistance is lacking in the available germplasm, available tolerance should be utilized in the hybridisation programme. They also emphasised that the immunity exhibited by the distant related wild species *Piper colubrinum* to *Phytophthora capsici* should be exploited.

The development of reproductive isolation barriers between species during the course of evolution, put limitations in utilising resistance found in related wild species by conventional method of hybridisation and selection. The barriers can be overcome with the application of techniques availble in molecular biology and genetic engineering for genetic transformation.

Development of disease resistance by transfer of foreign genes through genetic transformation has been reported in a number of crops. Several crops like tomato (Heath, 1985; Jongedijk *et al.*, 1995), tobacco (Howie *et al.*, 1994; Jach *et al.*, 1995), rice (Lin *et al.*, 1995), brassica (Grison *et al.*, 1996), carrot (Punja and Raharjo, 1996) and cucumber (Tabei *et al.*, 1998), were transformed with genes for chitinases from different sources like bacteria, rice, barley, tomato, petunia, tobacco and bean. The transgenics so developed were found to show enhanced disease resistance.

This suggests that there is a possibility of exploiting the potential of transformation in black pepper for development of resistance to *Phytophthora capsici* by introducing resistant genes believed to be present in *Piper colubrinum* or even genes conferring disease resistance reported in other crops.

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 present study was undertaken to standardise the process of genetic transformation in black pepper, variety Panniyur-1, through *Agrobacterium tumefaciens* strain AGL-1; 1303 using antibiotic resistant genes as selectable markers and *GUS* and *GFP* genes as reporter genes.

Review of Literature

2. REVIEW OF LITERATURE

Foot rot disease of black pepper, caused by *Phytophthora capsici*, is the biggest constraint in the cultivation of the crop. The conventional breeding programmes aimed at producing genetically resistant lines of pepper mainly through screening the available germplasm as well as open pollinated and irradiated seedling progenies for their relative resistance/tolerance to the disease, have been proved unsuccessful.

The available cultivars of black pepper and related species of *Piper* were screened for resistance to *Phytophthora* foot rot by several workers (Holliday and Mowat, 1963; Ruppel and Almeyda, 1965; Leather, 1967; Turner, 1973; Kueh and Khew, 1980; Sarma *et al.*, 1982; and Vilasini, 1982) and none of the cultivars were resistant to the disease. However, related wild species like *Piper colubrinum* and *Piper obliqum* showed resistance (Purseglove *et al.*, 1981).

Sarma *et al.* (1982) screened around 73 wild types of *Piper* spp. and found resistance in none. However, cultivars like Narayakodi, Kalluvally, Uthirankota and Balankota were found to show some tolerance in this study. Sarma *et al.* (1991) suggested that since high degree of resistance is lacking in available germplasm, available tolerance should be utilized in hybridisation programmes. The immunity of related species of *Piper colubrinum* to *Phytophthora capsici* should be exploited.

The process of speciation leads to the development of reproductive isolation barriers that maintain the integrity of the species by restricting the flow of genes from one to another (Hadley and Openshaw, 1980). This has put limitations to the conventional breeding methods in utilising the resistance found in related wild species by the process of hybridisation and selection.

In vitro culture technique have been applied to develop foot rot tolerant lines. Somaclonal variants and variants induced by gamma irradiation have been screened against toxic metabolites of the pathogen for identifying cell lines showing tolerance to the pathogen (Shylaja, 1996 and Nazeem *et al.*, 1997). However, this approach has not yet yielded positive results.

To overcome the difficulties associated with conventional hybridization the possibility of genetic transformation of black pepper with a gene which imparts resistance against *Phytophthora capsici* is seriously looked into.

Genetic transformation can be defined as the transfer of foreign genes isolated from plants, viruses, bacteria or animals into a new genetic background, so that a normal fertile organism which expresses the newly inserted genes is obtained (Webb and Moris, 1992). For successful plant genetic transformation, there are three essential requirements, like, (a) a foreign gene imparting desired characters, (b) a well developed and efficient regeneration protocol for the crop under consideration and (c) an efficient gene delivery system.

2.1 Application of *in vitro* culture techniques in transformation

Development of efficient *in vitro* regeneration techniques is an important milestone in streamlining a procedure for the production of genetically transformed plants. Direct organogenesis, indirect organogenesis and somatic embryogenesis have been effectively utilized in production of genetically transformed plants.

2.1.1 Callus culture

The response of cultured tissue depends on both the ratio and absolute concentration of auxins and cytokinins. The concentration of auxins and cytokinins are equally as important as their ratio. Callus initiation generally occurred when ratio of auxins to cytokinins was high (Torres, 1989).

Bennici *et al.* (1997) reported that the same hormonal combination under same cultural conditions produced different responses to callus induction and proliferation in different selection lines of the same species of *Amaranthus*, ranging from 100 per cent to nil.

Protocol for callus mediated organogenesis developed in *Moricandia arvensis* was utilised by Rashid *et al.* (1996) in producing genetically transformed plants using *Agrobacterium tumefaciens*. Similarly, Igasaki *et al.* (1996) after transforming the leaf disc and stem segments of *Robinia pseudoacacia*, induced the transformed cell to produce callus which later regenerated to produce transformed plants.

2.1.2 Organogenesis

The ratio of auxins to cytokinins influence the balance between root and shoot organogenesis from cultured tissues. Generally, a higher auxin relative to cytokinin favoured root formation and the converse situation favoured shoot formation (Warren, 1991).

Gana *et al.* (1995) reported highly significant variation in plant regeneration from callus and suspension culture exhibited by different genotypes within a species of *Avena* spp.

Phenol exudation from explant has a detrimental effect on regeneration. Raghuvanshi and Srivastava (1995) reported that phenol exudation from cut ends of mango explants greatly hindered their regenerative ability in any *in vitro* growth medium. Chatelet *et al.* (1992) reported phenolic exudation prevented development of proembryos into somatic embryos and plantlets. Phenolic exudation had been a serious problem in woody species. Oxidation of phenolic substances leaching out from cut surface of the explant which turns the medium dark brown often led to the death of the explant (Razdan, 1993). Polyphenol interference in culture establishment of different woody plant species had been reported by several

workers (Anderson, 1975; Lloyd and McCown, 1980, Amin and Jaiswal, 1988 and Gill and Gill, 1994).

Orlikowska *et al.* (1995) utilised the technique of direct organogenesis in safflower to produce genetically transformed plants. The leaf segments were inoculated with *Agrobacterium tumefaciens* and the transformed cells screened by selecting agent in shoot regeneration medium. Transformed shoots were produced directly from the explants.

In Regal pelargonium, transformed plant could be produced by Boase *et al.* (1996) because of the well developed direct organogenesis protocol standardised in the crop. Similarly direct shoot organogenesis protocol was made use of by Voisey *et al.* (1994) to develop transgenic plants in white clover.

2.1.3 Somatic embryogenesis

Somatic embryogenesis had been well standardised in a number of crops. Tomar and Gupta (1988) described somatic embryogenesis in callus culture of a tree legume *Albizia richardiana* king. Hypocotyl explants 1-10 mm were excised from 12 day old *in vitro* grown seedlings of the tree legume and cultured on B_5 medium supplemented with BA. Some of the green calli formed started producing somatic embryos with the second subculture.

Vieitez and Barciela (1990) reported somatic embryogenesis and plant regeneration from embryogenic tissues of *Camellia japonica* from immature and mature zygotic embryos cultured on MS medium without growth regulators or with combination of IBA and BA. The dependence of embryogenesis rates on growth regulator levels was not clear, though high levels were inhibitory.

Somatic embyros of oak (*Quercus robur* L.) were produced from immature zygotic embryos cultured on MS or WPM containing 1 mg l^{-1} BA + 1 mg l^{-1} GA or BA and IBA. Germination and conversion into plantlets was achieved

on WPM containing reduced concentration of cytokinins. In Linden (*Tilia cordata* Mill.) somatic embryos were obtained from embryogenic tissue initiated from immature zygotic embryos cultured on MS + 0.3-2 mg l^{-1} 2,4-D. Germination and plantlet formation occurred on MS containing low concentration of IBA (Chalupa, 1990).

Lambe *et al.* (1999) reported that embryogenic tissues were induced from shoot apices excised from 2 to 10 days after *in vitro* germination of seeds of *Pennisetum glaucum* when cultured in MS medium supplemented with 22.6 µM 2,4-D and 5 per cent coconut milk. These embryonic tissue when transferred to MS or N_6 medium containing 9 µM 2,4-D alone or in combination with 1.6 µM BA and 2.7 µM NAA gave rise to somatic embryos. Significant differences were observed in somatic embryogenesis among three different genotypes tried.

Significant differences in 2,4-D induced somatic embryogenesis between different cultivars in cassava has also been reported by Raemakers *et al.* (1993). Variation in response to somatic embryogenesis within breeding lines of different botanical varieties of peanut was observed by Mckently (1995).

Somatic embryogenesis had been very well standardised in coffee. Embryogenic calli was transformed and then regenerated through the production of somatic embryos by Hatanaka *et al.* (1999) to obtain transgenic plants. In carrot, leaf segments and petiole were transformed and the transformed explants cultured in medium standardised for the induction of embryogenic calli. The transformed embryogenic calli were then transferred to another medium standardised for development of somatic embryos and regeneration of plants to obtain transgenic plants (Pawlicki *et al.*, 1992).

2.2 Status of *in vitro* techniques in black pepper

Mathew and Rao (1984) reported good callus growth from explants of *in vitro* raised seedlings. Good callus growth was noticed from explants taken from

mature vines of black pepper also (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), though with a 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-1 showing the least response.

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

Of all the regeneration techniques reported, the one through somatic embryos is viewed with great interest. Joseph *et al.* (1996) reported production of embryogenic callus on culturing zygotic embryo in a liquid basal medium without hormones. As per the report 1500 numbers of plants were produced from 5 mg of the 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.

2.3 *Agrobacterium* mediated gene delivery system

Once an efficient *in vitro* regeneration protocol has been developed in a crop, the next requirement for a successful genetic transformation, is the development of an efficient gene delivery system. There are different methods for introducing a foreign gene into plants. Of the various methods available, the one utilising *Agrobacterium tumefaciens* is the most widely used for introducing foreign genes into dicotyledonous plant species (Weising *et al.*, 1988) and some monocotyledolonous species (Bytebier *et al.*, 1987; Eady *et al.*, 2000). Both the efficiency of transformation and the broad host range of *Agrobacterium tumefaciens* among the dicot species contribute to its widespread use. Also the frequency of obtaining gene insertions which get expressed in plants is higher than other gene transfer systems in use today.

2.3.1 *Agrobacterium tumefaciens*: a tool for genetic manipulation

The genes bracketed by the border sequence in a T-DNA, eventhough they are of prokaryotic origin, contain eukaryotic promoters. As a result these genes do not express inside the bacterial cell. It is precisely because of this, 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 T_i plasmid and replacement with the desired gene permits the use of this bacterium for genetic transformation of plant tissue. The genetically transformed plant tissue can be regenerated, by *in vitro* techniques into whole plants, thus producing transgenic plants.

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).

In Cis or co-integrate vectors (plasmid), the novel, foreign genes replace the oncognes between the T-DNA border fragments on the virulent T_i plasmid. The

foreign DNA which resides between the T-DNA border is then transferred to the plant cell genome by the normal process of bacterial infection. The limited space available for the new genes in these vectors has restricted the size and number of genes that can be transferred.

In trans vectors an extra non-virulent plasmid is transferred into the *A. tumefaciens* strain, where a separate disarmed T_i plasmid already exists with a virulence region. Thus, transfer of T-DNA is mediated by activity of the Vir region from one plasmid acting on another plasmid (Webb and Morris, 1992).

2.3.1.1 Constitution of foreign gene

The foreign gene(s) to be placed in the T_i plasmid should be capable of expressing itself in the plant cells, i.e., it should have a constitution common to plant cells. The nuclear plant genes consist of distinct regions, each with different functions, involved in transcription and translation of mRNA. Starting with the 5'end, these are: a promoter region which is involved in the initiation of transcription, together with enhancer/silencer regions which confer environmental or developmental regulation of expression, a transcriptional start or cap site and the so called *CAAT* and *TATA* boxes which are located upstream from the cap site and which probably function in RNA polymerase II binding. Following the cap site is an untranslated region preceding the translational initiation codon (ATG). Within the transcribed region one or more untranslated or intron regions may be present. The end of the translated region is determined by a stop (TAA, TAG or TGA) codon, followed by an untranslated region and finally terminate at the 3'end by a polyadenylation signal.

2.3.1.2 Promoters and other regulatory DNA sequences

For expression in plant cells, foreign genes need to have appropriate promoters. The regulatory sequences found in prokaryotes may not work in eukaryotes. Exception to this are the regulatory elements of certain genes of

Agrobacterium tumefaciens and *A. rhizogenes* which are active on transfer to plant cells. Thus the *nos* (nopaline synthase), *ocs* (octopine synthase) and *mas* (mannopine synthase) gene promoters have been used successfully to direct expression of genes in plant cells.

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 gene of the cauliflower mosaic virus (*CaMV*). Evidences have cropped up showing less efficient functioning of 35S promoters in monocot cells. However, doubling of certain enhancer regions of the 35S promoters has increased the level of expression in both dicot and monocot cells.

The promoter derived from the maize alcohol dehydrogenase 1 (*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 1 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 were obtained without the intron region. Similar results were obtained with the first intron of the maize shrunken-1 locus (Vasil *et al.*, 1989).

2.3.1.3 Reporter genes

The expression of a foreign gene by transformed plant cells can best be assessed by determining the abundance or activity of its products. The coding sequences of genes for bacterial enzymes, which are easily assayed and whose activity is not normally found in plants, form the basis of the reporter genes. The reporter genes commonly used are bacterial nopaline synthase (*nos*), chloramphenicol acetyltransferase (*CAT*), neomycin phosphotransferase (*npt II*) and ß-glucuronidase (*GUS*). These reporter genes are used to analyse the function of promoters and other gene regulatory sequences.

2.3.1.4 Selectable marker gene

Selection of transformed cells is a key factor in developing successful methods for genetic transformation. Single dominant genes encoding suitable resistance to a selective agent are generally used. These genes do not disrupt plant regeneration, but allow selection of transformed cells.

The antibiotics kanamycin, G418 and hygromycin are currently amongst the most widely used selection agents. All the three are aminoglycoside antibiotics which affect translation activities of the cells.

Neomycin phosphotransferase II (*npt II*) gene from transposon Tn5, detoxify neomycin, kanamycin and G418 by phosphorylation. It is widely used in dictoyledon systems, including tobacco, potato and tomato (An *et al.*, 1986), legumes, such as white clover (White and Greenwood, 1987) and pea (Puonti-Kaerlas *et al.*, 1989) and woody species, such as *Pseudostuga menziesii* (Ellis *et al.*, 1989). But this marker genes has proved unsuitable for some dicotyledonous species, such as *Arabidopsis*, in which large number of non-transformed cells survive and for many monocotyledons species whose growth is not significantly inhibited by the antibiotic (Potrykus *et al.*, 1985; Hauptmann *et al.*, 1988; Dekeyser *et al.*, 1989).

Hygromycin phosphotransferase (*hpt IV*) governs resistance to hygromycin. This gene isolated from *E. coli* has been placed under various promoters and has been successfully used in strawberry (Nehra *et al.*, 1990) and rice (Dekeyser *et al.*, 1989; Shimamoto *et al.*, 1989).

Genes conferring resistance to herbicide are also used as selectable marker. Modified version of *EPSPS* isolated from *E. coli* confer resistance to glyphosate in transformed plants. Such transformants have been produced in tobacco (Comai *et al.*, 1985), tomato (Filatti *et al.*, 1987), flax (Jordan and McHughen, 1988) and soybean (Hinchee *et al.*, 1988).

Another, more recently developed system uses the bar gene isolated from *Streptomyces hygroscopicus*, which confers resistance to the herbicide, phosphinothricin (PTT), the active ingredient of Bilaphos and Basta. The bar gene codes for phosphinothricin acetyltransferase (PAT), which inactivates PTT, an irreversible inhibitor of glutamine synthase. This gene has been inserted and expressed in several crop plants, including tomato, tobacco, potato (DeBlock *et al.*, 1987); rape (DeBlock *et al.*, 1989), alfalfa (Krieg *et al.*, 1990), maize (Spencer *et al.*, 1990) and rice (Dekeyser *et al.*, 1989).

Comparison of these different selection agents under the control of different promoters has allowed optimal system to be developed for many species. Working with rice, Dekeyser *et al.* (1989) evaluated the efficiency of various selectable markers for transformation. They found that, while phosphinothricin and bleomycin are effective at low concentrations, G418 and hygromycin are required at higher concentrations and kanamycin by contrast only partially inhibit growth of the rice cells.

2.3.2 *Agrobacterium* mediated plant genetic transformation

The development of plant gene delivery system was one of the key breakthroughs enabling rapid progress in the field of plant biotechnology.

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 import produced (Lindsey, 1992).

2.3.2.1 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.

The technique of tissue culture makes it possible to use diverse plant tissues for transformation. The wounded edge of cotyledon (Fillatti *et al.*, 1987; Michelmore *et al.*, 1987), hypocotyl (Radke *et al.*, 1988), leaf (Horsch *et al.*, 1985) or stem (An *et al.*, 1986) have been used for genetic transformation. Genes have also been introduced into plants following co-cultivation of seeds (Feldman and Marks, 1987), protoplast (Herrera-Estrella *et al.*, 1983); suspension cells (Scott and Draper, 1987); callus (Pollock *et al.*, 1985; Arokiaraj *et al.*, 1996; Dillen *et al.*, 1997; Belarmino and Mii, 2000), somatic embryos (McGranahan *et al.*, 1988), embryogenic calli (Hatanaka *et al.*, 1999) and shoot apices (Ulian *et al.*, 1988). *In vitro* regeneration systems based on somatic embryogenesis has an added advantage in transformation because of the single cell origin of the somatic embryos which reduces the possibility of recovery of chimeric plants.

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.

The rate of transformation with *Agrobacterium tumefaciens* can also vary with the age and physiological status of the explant. Braun (1975) reported that actively dividing cells are more susceptible to *Agrobacterium* infection. Preculturing of the explant in callusing or regeneration media prior to *Agrobacterium* infection may result in increased transformation rate. Alternatively, providing a hormone pulse to stimulate cell division in the explant probably enhances the transformation rate. However, Pawlicki *et al.* (1992) found no effect of preculture of explants on the rate of transformation in carrot.

Chikrizova and Polyakov (1996) found that in transformation of flax, the most effective inoculation method involved removing about 50 per cent of the epidermis from 78 day old hypocotyl segments.

Gould *et al.* (1997) successfully transformed the shoot apices of loblolly pine, cotton and maize using a supervirulent strain of *Agrobacterium*. This helped in reducing the culture induced mutations. Explants for transformation work are generally generated by raising *in vitro* seedling. Normal germination of seeds in MS media has been reported by Sunilkumar *et al.* (2000) in *Hopea parviflora*.

2.3.2.2 *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).

2.3.2.2.1 Strain specificity

Agrobacterium tumefaciens mediated transformation had been successful with a broad range of dicotyledonous plants but had shown a poor response with the monocotyledons. Even among dicots, there are differences in susceptibility between species and even between cultivars and genotypes of the species. Differences in infectivity are also seen between bacterial strains on the same genotypes.

One way of arriving at the best bacterial strain and plant genotype compatible system, is to test the genotype with different strains of the bacteria, based on tumor production. Then utilize the system for the transformation work. This approach was used successfully in soyabean where compatible systems were found (Owen and Cress, 1985; Byrne *et al.*, 1987) and subsequently used to obtain transgenic plants (Hinchee *et al.*, 1988). However, this method may not be useful for all species. The tumor formation 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. It is therefore possible to have T-DNA transfer but an incompatbile hormonal regime

and therefore little or no gall formation. Hence, the alternative left is to try transformation with different strains harbouring a good selectable marker, till we get the genotype/strain combination.

In a study with *Robinia pseudoacacia*, when the same binary vector pSMAH704 was put in three disarmed strains of *Agrobacterium tumefaciens*, namely, LBA4404 (pAL4404); EHA101 (pEHA101) and GV3101 (pMP90), tremendous differences were seen in the rate of transformation. Strain GV3101 (pMP90) gave the highest frequency of transformation of *R. pseudoacacia* (Igasaki *et al.*, 2000). Similarly, in another experiment, *Pinus pinea* L. was transformed with three different strains of *Agrobacterium tumefaciens*, namely, EHA105, LBA4404 and C58.GU3850 harbouring the binary vector p35SGUSint. EHA105, an agropine type bacteria alone showed significantly different rate of transformation compared to the other strains (Humara *et al.*, 1999).

Strain specificity has also been reported in black pepper (Sasikumar and Veluthambi, 1996). Here binary vector (LBA4404 and EHA105 harbouring pGA472) was found to successfully transform black pepper whereas cointegrate vector (PGV2260-PGSFR280) did not produce transformation. However, these results were based only on phenotypic observation.

Desgagnes *et al.* (1995) conducted an extensive experimentation to select the best combination of genotype, bacterial strain and plasmid vector to develop a highly efficient transformation system for alfalfa (*Medicago sativa*). Out of a thousand genotypes available in 11 breeding lines, they selected three highly regenerative genotypes belonging to three breeding lines. Three *Agrobacterium tumefaciens* strains namely, C58, A281 and LBA4404 which belong to three major classes of the bacteria, namely, nopaline, agropine and octopine type, respectively were used for the experiment. The vectors used were pGA643, pBinKan and pGA482. All combinations of genotypes (3) x expression vectors (3) x bacterial

strains (3) were tested for their ability to produce stable transformation. Each of the 27 possible combination was tested with 48 leaf discs.

Within each selected genotype, combination of strain/vector had a drastic effect. There was no single most efficient combination that could be derived from transformation trial, since a specific vector/strain combination seemed better suited for each genotype.

The *Agrobacterium* strain used in this study are said to have wide host range, as they are efficient at transferring genes to genotypes of many plant species. However, it has been shown in the experiment that their virulence can be restricted to a few genotypes within a single plant species. In the current study, the interaction between bacterial strains and plant genotypes were highly significant.

Another aspect revealed in the study is the difference in ability of the genotypes to retain the regenerative potential after bacterial infection. Here one of the genotype which produced transformed calli failed to show embryogenesis as it should have done in its normal course without bacterial infection.

Strain specificity was also reported in alfalfa (*Medicago sativa*). Samac (1995) have reported that *Agrobacterium tumefaciens* strain AGL-1 had a detrimental effect on leaf explant tissue. Majority of the leaf explant belonging to nine germplasm that were co-cultivated with this strain had reduced amounts of callus and a greater degree of tissue browning than the explants co-cultivated with strain LBA 4404. However Chabaud *et al.* (1988) had reported that strain A281, from which AGL-1 is derived gave better transformation in *Medicago varia* than LBA 4404.

Reduced recovery of transgenic plants due to reduced regeneration of transformed cells is also reported by Donaldson and Simmonds (2000) during their transformation experiments in short season soybean. Of the 12 genotypes used all of them produced GUS sectors after 20 days in selective media, ranging from 27

per cent to 92 per cent. However, only one regenerant was recovered in variety AC Colibri. The authors attributed this to inefficient transformation of regenerable cells and/or poor selection or survival of such cells and not to poor susceptibility to *Agrobacterium.*

The key elements involved in the transfer of T-DNA from the bacteria to the plant cells are the T-DNA borders, vir gene complements and the bacterial chromosomal backgrounds. Manipulations of these three elements could help in the design of a strain that could transform an otherwise recalcitrant genotype.

The host range of *A. tumefaciens* has been extended by using super virulent strains of *A. tumefaciens*, that have highly expressed vir genes and induces unusually large tumors (Hood *et al.*, 1986). Jin *et al.* (1987) cloned a portion of the vir region of pT_iB_0542, responsible for supervirulent phenotype and then introduced this fragment back into the supervirulent strain A281, creating a new extrasupervirulent (XSV) strain. This new XSV strain when inoculated on a previously recalcitrant tobacco species, now produced galls.

Hood *et al.* (1987) during their experiments with soybean could find that the combination of T_i plasmid with certain chromosamal backgrounds, can markedly influence virulence. For example, the *A. tumefaciens* strains B_0542 (containing pT_iB_0542 in the B_0542 chromosomal background) is at best weakly tumorigenic on soybean, yet the strain A281 (containing pT_iB_0542 in the C58 chromosomal background) is highly virulent upon this plant species.

In their studies on the effect of T-DNA borders in transformation, Li *et al.* (1992) found that T-DNA borders from octopine type T_i plasmid pT_iB6 and nopaline type T_i plasmid pT_iT37 showed differences in transformation efficiency in rice.

Increasing the copy number of Vir G could improve the transformation efficiency. However, the effect was unpredictable. For some plant species increasing the copy number of an octopine type Vir G gene enhanced transformation, whereas for other plant species this enhancement could be detected using multiple copies of an agropine type Vir G gene.

Increasing the copy number of Vir G in *Agrobacterium tumefaciens* had an additional unexpected effect. Under normal circumstances Vir G gene induction by acetosyringone can only be effected in minimal medium at a pH less than 6.0. Increasing Vir G copy permitted Vir gene induction in enriched medium and at an alkaline pH (Liu *et al.*, 1992). For many plant species, however, efficient regeneration requires medium supplemented with amino acids. This enriched medium may inhibit efficient Vir gene induction using *A. tumefaciens* strain harbouring a single copy of Vir G, but may not affect to such a great extent the Vir gene induction in strains harbouring multiple copies of Vir G.

Agrobacterium strain containing nopaline type T_i plasmid are able to transfer DNA to maize whereas strains containing octopine type T_i plasmid cannot. Boulton *et al.* (1993) using agro-infection of maize streak virus (MSV) DNA as a reporter system, tried to find out the reason for strain specificity. The striking difference between the two T_i plasmids is that the presence of Vir H and Vir F regions are specific to octopine type T_i plasmid and the presence of tzs specific to nopaline type T_i plasmid. They found that mutation at Vir F locus of the octopine type T_i plasmid improved transformation efficiency slightly, showing that the locus played a small part in strain specificity. However, when Vir A region of the nopaline type T_i plasmid was transferred to octopine type T_i plasmid, the transformation efficiency of octopine type T_i plasmid improved to the level of nopaline types. Thus strain specificity was due to proper Vir gene induction which depended on Vir A locus.

2.3.2.2.2 Inoculation

This is a part of the transformation process, where the explant is exposed to bacterial culture raised in liquid medium called induction medium. Here attention is given to create a favourable environment to induce the Vir genes of the bacteria as well as to maintain the explant in the most receptive condition to facilitate high rate of T-DNA transfer.

Agrobacterium tumefaciens is attracted to wounded plants presumably due to the signal molecules in wound exudates (Zambryski, 1992). These signal molecules activate the Vir genes leading to the processing and transfer of T-DNA from bacterium to the plant cell. The signal molecules identified from tobacco wound exudates are acetosyringone and hydroxy-acetosyringone (Stachel *et al.*, 1985). These exudates stimulate transcription of Vir genes on the bacterial plasmid for over 8-16 hours (Hooykaas and Schilperoort, 1984). Monosaccharides like D-galacturonic acid and glucose enhance the activation of Vir A by the phenolic compounds.

Lewi *et al.* (1995) categorised sunflower (*Helianthus annuus*) genotypes into two-high phenol producers (HPP) and low phenol producers (LPP), and exposed them to *Agrobacterium* infection. The HPPs showed transformation efficiencies of 92 per cent and 73 per cent. The LPP genotypes, however, showed transformation efficiency of 52 per cent only. The results confirm the importance of the presence of phenolic compounds in the interaction between *Agrobacterium* and sunflower cells.

2.3.2.2.2.1 Wounding of explant

Wounding of explants with the help of a needle/scalpel is routinely done in transformation experiments prior to inoculation, for exudation of wound sap that could trigger Vir genes of the *Agrobacterium* thereby increase the efficiency of

transformation. Choi *et al.* (1994) produced successful transformation protocol in watermelon by wounding the cotyledonary explant with the tip of the scalpel.

However, Confalonieri *et al.* (1995) did not find any marked difference in transformation when punctured and unpunctured cotyledon were used in black poplar clones. Instead a reduction in regeneration of transformed explant was noticed.

Humara *et al.* (1999) evaluated different wounding procedure to enhance the efficiency of *Agrobacterium* mediated transformation in *Pinus pinea* L. They used ultra sound wounded (SAAT - Sonication assisted *Agrobacterium* mediated transformation), particle gun wounded, scraped and unwounded cotyledon in their experiments. During transient GUS expression analysis, they found that maximum GUS expression was seen in ultra sound wounded cotyledon (27%), followed by unwounded cotyledon (16%), scraped cotyledon (8.4%) and particle bombarded (5.3%). The SAAT procedure was found to be efficient in promoting transformation events. However, none of the SAAT treated explants produced buds. Only scraped explants produced 1-2 per cent buds. The unwounded explant, with only the excision wound, gave 11 per cent of buds. This was attributed to hypersensitive response of the tissues as part of the plant defence against pathogens.

In *Casuarina glauca*, transformation by *Agrobacterium tumefaciens* was reduced when explants were wounded by microprojectile bambardment (Le *et al.*, 1996).

Potrykus (1991) suggested that only plants with an appropriate wound response develop large populations of wound adjacent competent cells for regeneration and transformation. Therefore, although excessive wounding is probably detrimental to stable transformation, the frequency of gene transfer

mediated by *Agrobacterium tumefaciens* can in some species be significantly enhanced by inducing wounds in the target tissue.

2.3.2.2.2.2 Use of attractants to activate Vir genes

To improve the efficiency of transformation, addition of phenolic compounds to the induction medium and co-cultivation medium, had been widely used, with a view to activate or induce Vir gene of the T_i plasmid. The compounds include potato wound exudate for yam (Schafer *et al.*, 1987) and rice (Chan *et al.*, 1993) and acetosyringone and nopaline in maize (Gould *et al.*, 1991).

Joao and Brown (1993) reported that in tomato transformation, addition of acetosyringone in induction medium as well as in co-cultivation medium helped not only in increasing transformation frequencies but also in increased expression of the foreign gene inserted (*npt II*). This they said, could be due to multiple T-DNA insertions into the host genome.

In Agrobcterium transformation of white clover, Voisey *et al.* (1994) found that acetosyringane (100 µM) when added to co-cultivation medium helped in stabilizing rate of transformation, which varied considerably in the absence of it.

Transformation experiments in phalaenopsis orchid showed that co-cultivation of suspension culture derived cells with 200 µM acetosyringone pre-activated *A. tumefaciens* and inclusion of 500 µM acetosyringone in agar solidified co-cultivation medium enhanced the rate of infection (Belarmino and Mii, 2000).

Activation of Vir genes by acetosyringone depended on the bacterial strain in use. In his studies in black poplar clones, Confalonieri *et al.* (1995) reported that when 200 µM acetosyringone was added to the bacterial suspension of A281 pKIWI1105, the frequency of leaf disc transformation was significantly enhanced compared to the control. However, acetosyringone produced no effect when added to the bacterial suspension of GV2260/35SGUS strain.

Acetosyringone showed no effect in some cases and in some cases inhibitory effect on regeneration as in carrot (Pawlicki *et al.*, 1992). Torres *et al.* (1993) found no effect of acetosyringone or syringaldehyde during their transformation works in lettuce.

In many cases where wounded tissues produce sufficient phenolic exudates to activate a vir response, acetosyringone may not have a role in the process of transformation. Further more, all the strains of *Agrobacterium* may not respond to acetosyringone. Workers in the field of *Agrobacterium* mediated genetic transformation also use tobacco feeder layer during co-cultivation in order to activate the vir genes. Sarmento *et al.* (1992) reported significant improvement in transformation efficiency in pickling cucumber, when explants were co-cultivated with *Agrobacterium* on a tobacco feeder layer than in its absence. Attempting *Agrobacterium* mediated transformation in *Moricandia arvensis*, Rashid *et al.* (1996) found that addition of acetosyringone in co-cultivation media did not help in attaining the objective and no GUS expression was obtained. On the other hand supplementing co-cultivation medium with tobacco feeder cells alone could give transient GUS expression. It may be presumed that tobacco suspension culture contains some substances, other than acetosyringane, which may facilitate T-DNA transfer.

2.3.2.2.2.3 Bacterial density

Concentration of bacterial cells in the induction medium is another important factor to be considered for efficient transformation. Very low density of bacterial population could lead to ineffective transformation, whereas very high density may lead to necrosis and death of the explant. Some species are very sensitive to bacterial infection and hence very low density of bacterial population is used.

Sarmento *et al.* (1992) evaluated bacterial concentration ranging from 10^5 cells ml^{-1} to 10^8 cells ml^{-1} in their experiments with pickling cucumber. They

found a transformation frequency of 14 per cent when the lowest concentration was tried and 26 per cent with the highest concentration tried.

Humara *et al.* (1999) tried different bacterial density ranging from $O.D_{600nm} = 0.01$ to $O.D_{600nm} = 1.0$ in an effort to find the optimum bacterial density giving maximum transformation in *Pinus pinea*. The transient GUS expression after 7 days of inoculation showed very high transformation events when bacterial density was 1.0 ($O.D_{600nm}$). However, none of transformed cotyledon tissue survived after 30 days. The cause for this reduction in the number of cotyledon forming buds was related to the hypersensitive response as part of the plant defense against pathogen. However, when bacterial density was 0.01 ($O.D_{600nm}$) transformed buds were recovered at a frequency of 4 per cent. Hence lower bacterial density was recommended.

Transformation studies in safflower cv. centennial, showed that bacterial infection had hypersensitive reaction on the explant which reduced its organogenetic potential (Orlikowska *et al.*, 1995).

Archilletti *et al.* (1995) working on *Agrobacterium* mediated transformation of almond leaf pieces found that bacterial density 0.6 ($O.D_{600nm}$) was best, eventhough a higher density did not cause necrosis on explant. Similarly for white mustard (*Sinopis alba* L.), Hadfi and Batschauer (1994) found that the bacterial density of 0.15 ($O.D_{600nm}$) was best suited for *Agrobacterium* transformation of hypocotyl segments. In coffee, Hatanaka *et al.* (1999) used an $O.D_{600nm} = 0.6$ for the leaf disc transformation experiments, whereas Torres *et al.* (1993) used $O.D_{600nm} = 0.7$ for their experiments in lettuce.

2.3.2.2.2.4 Induction time and media

The explant chosen, in their most receptive stage, is exposed to the *Agrobacterium* culture in the induction media at an optimum bacterial density.

Both the composition of the induction media and time of induction may have a role in the efficiency of transformation.

For induction, regeneration medium of the explant which can support bacterial growth like Murashiege and Skoog medium is used. It can also be the bacterial culture medium. The pH of the medium usually maintained range from 5.5 to 5.7. For inoculation the explants are mostly immersed in the induction medium for a time varying 30 s to 1 hour.

In an attempt to standardise the process of *Agrobacterium* mediated transformation, Sarmento *et al.* (1992) used MG/L nutrient medium (Garfinkel and Nester, 1980) for induction and evaluated the efficiency of induction time ranging from 5 minutes to 60 minutes. He found callusing frequencies at 5, 10, 30, 45 and 60 minutes as 35, 30, 24, 19 and 9 per cent, respectively, the best being 5 minutes of exposure. With prolonged exposure (>45 min), explants became necrotic and dried or were colonized by *Agrobacterium* even in the presence of 500 mg l^{-1} carbenicillin.

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

Transformation works in *Pinus pinea* L. revealed that the induction time of 5 minutes gave better transformation frequency compared to 30 minutes of induction time (Humara *et al.*, 1999).

Murashiege and Skoog salts and vitamins were used for preparation of induction medium by Nagaraju *et al.* (1998) during their experiments with *Gerbera hybrida*. They immersed the explants in induction medium for 5 minutes and got successful transformation rate.

Induction time of 1 hour was given by Le *et al.* (1996) for their reported successful transformation of *Casuarina glauca.*

Desgagnes *et al.* (1995) while describing their transformation experiment in alfalfa mentioned that they grew the bacterial population in L.B. medium to a $O.D_{600nm}$ = 0.17-0.2 and then the cells were pelleted at 4°C by centrifugation at 5000xg and resuspended in an equal volume of 0.85 per cent NaCl. The leaf explants were immersed in this for 20 s for inoculation. Similarly, Voisey *et al.* (1994) had resuspended the bacterial pellet in 5 ml of 10 mM $MgSO_4$ prior to inoculation during their experiment with white clover.

A different inoculation method was employed by Eady *et al.* (2000) in his experiments with onion. Here the embryos used as explants (40 Nos) were transferred into 0.8 ml of *Agrobacterium* suspension and vortexed for 30 s. In the case of cassava, Sarria *et al.* (2000) had just applied few drops of the bacterial suspension on the explants.

2.3.2.2.3 Co-cultivation

After inoculation, the explants are blot dried on sterile filter paper to remove excess of bacterial suspension and then co-cultivated in the regeneration medium without selecting agent. Co-cultivation period plays a very important role in the success of the transformation. It is during this period that the vir genes are activated and T-DNA transferred into plant cell. However, increasing the co-cultivation period might lead to necrosis and death of the explant due to hypersensitive reaction of the tissue. Sometimes it might also lead to uncontrollable bacterial overgowth. Hence the length of the co-cultivation period should always be shortest interval necessary to obtain the maximum frequency of transformation in terms of the number of transgenic plants recovered following co-cultivation. The optimal co-cultivation time for tomato, tobacco, rapseed and poplar was no more than 48 hours and for cotton 40 hours was optimal (Fillatti, 1990).

Pawlicki *et al.* (1992) studying the factors affecting transformation in carrot, tried different co-cultivation period of 1, 2, 3 and 7 days to get the best transformation efficiency. The best results were obtained with two or three days of co-cultivation. Co-cultivation for one day produced no callus whereas by co-cultivating for seven days the frequency of transformation was very less.

Hassan *et al.* (1993) reported four days to be optimum for transformation of *Rubus* genotypes. In lettuce, the percentage of explant producing transformed calli were similar at 48, 72, 96 and 120 h of co-cultivation. The co-cultivation period of 48 h produced the maximum transgenic shoots followed by 24 and 72 h of co-cultivation. Co-cultivation of 96 and 120 h had an inhibitory effect on shoot formation giving no shoots at all (Torres *et al.*, 1993). Hadfi and Batschauer (1994) reported four to five days co-cultivation to be optimum for white mustard and any decrease in the co-cultivation period reduced the frequency of transformation.

Le *et al.* (1996) during their transformation experiments in *Casuarina glauca* found that the transformation frequency were at their maximum value when three days co-cultivation period was given. After seven days, the explants were damaged by co-cultivation and turned brown, thus reducing transformation efficiency. In *Gerbera hybrida*, Nagaraju *et al.* (1998) found 48 h to be the optimum co-cultivation period and a period beyond that resulted in bacterial over growth and inhibition of callus formation and shoot regeneration.

A slightly different method of co-cultivation was adopted by Belarmino and Mii (2000) in *Phalaenopsis* orchid. Here a two step co-cultivation method was adopted. In the first step, 1 g suspension cells of *Phalaenopsis* orchid used as explant where added to 20 ml of the *A. tumefaciens* suspension and kept in shaker at 30 rpm for 10 h. The suspension cells were collected on a nylon mesh washed and blotted dry with sterile filter paper. In the second step, the cells were spread on a piece of sterile filter paper placed on solidified regeneration media without drugs

in a petriplate for a period of three days. The authors found that co-cultivation for one day did not produce any transient expression in cells. But a three day co-cultivation produced transient GUS activity. Extending the period to five to seven days did not increase GUS activity but caused necrosis and death of cells on the selective medium.

Fillatti (1990) mentions that co-cultivating the inoculated explant on a sterile filter paper placed on top of the medium enhances the rate of transformation.

2.3.2.3 Elimination of bacteria after co-cultivation

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. Overgrowth of 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 organogenesis of the explants. The most commonly used antibiotics for this purpose are carbenicillin and cefotaxime. However, its effect on the explant has to be studied before choosing any one of them as they are also reported to have detrimental effect on some species.

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), 250 mg l^{-1} in *Casuarina glauca* (Le *et al.*, 1996), 300 mg l^{-1} in *Phalaenopsis* orchid (Bilarmino and Mii, 2000) and 500 mg l^{-1} in commercial melon (Valles and Lasa, 1994).

The effect of carbenicillin and cefotaxime on leaf morphogenesis in apple was studied by Yepes and Aldwinckle (1994). They found carbenicillin has a detrimental effect at 500 mg l^{-1} concentration, whether alone or in combination

with cefotaxime. Cefotaxime on the other hand significantly enhances regeneration at a concentration of 250 mg l^{-1} but at concentration of 500 mg l^{-1} it causes 40-70 per cent reduction in regeneration.

Carbenicillin was also found to be inhibiting callus formation in almond leaf pieces. Hence cefotaxime, which did not affect callus formation at a concentration of 0.5 mM was used in transformation work (Archilletti *et al.*, 1995). However, carbenicillin was found to stimulate growth of callus and shoot regeneration in safflower cv. Centennial (Orlikowska *et al.*, 1995) and *Antirrhinum majus* L. (Holford and Newbury, 1992).

Cefotaxime enhanced shoot proliferation in *Rubus* and hence used for transformation work. Here, the explant after co-cultivation was rinsed in 0.5 mM cefotaxime for 30 s and then placed in regeneration medium containing 0.4 mM cefotaxime (Hassan *et al.*, 1993).

Combination of carbenicillin and cefotaxime have also been used in eliminating the bacteria from explant. In lettuce, Torres *et al.* (1993) first immersed the co-cultivated explant in 200 ml basal medium supplemented with 100 mg l^{-1} cefotaxime and 500 ml^{-1} of carbenecillin for 30 minutes and then transferred into agar solidified regeneration medium containing 100 mg l^{-1} of cefotaxime and 500 ml^{-1} carbenicillin. Similarly, Igasaki *et al.* (2000) in *Robinia psuedoacacia* eliminated bacteria from the co-cultivated explant by first washing the explant with regeneration medium containing 500 mg l^{-1} of cefotaxime and 500 mg l^{-1} of carbenicillin and then transferring it into the screening medium containing 500 mg l^{-1} cefotaxime and 500 mg l^{-1} carbenicillin.

Vergauwe *et al.* (1996) working on transformation of *Artemisia annua* L. found that cefotaxime at -500 mg l^{-1} was effective as a decontaminating antibiotic but it caused retardation in callus formation. They then tried

Vancomycin at 750 mg l^{-1} which was not toxic to the tissue. But vancomycin could not control the bacteria effectively.

Ling *et al.* (1998) reported ticarcillin potassium clavulanate to be very effective combination of antibiotics to eliminate *Agrobacterium tumefaciens*. They were studying transformation in tomato and found that cefotaxime though by itself did not inhibit callus growth in culture medium, it decreased shoot differentiation together with kanamycin. Cefotaxime showed a strong negative effect on callus growth, shoot regeneration and transformation efficiency. Ticarcillin potassium clavulanate had no toxic effect on tomato tissues at a concentration of 150 mg l^{-1}, instead, significantly promoted callus growth and shoot proliferation. It was more economical than cefotaxime or carbenicillin.

Hammerschlag *et al.* (1997) found difficulty in eliminating the *Agrobacterium* during transformation work in apple. They tried carbenicillin, cefotaxime and cefoxitinmefoxin at various concentrations. They found that cefotaxime at 2000 mg l^{-1} when applied by vacuum infiltration for 30 minutes did not inhibit the percentage of regeneration and was more effective than the other two drugs.

In the transformation experiments with short season soybean, Donaldson and Simmonds (2000) used a slightly different method in eliminating *Agrobacterium*. The cocultured explants were washed for four days (with medium changed each day) in medium supplemented with 500 mg l^{-1} of cefotxaime and 100 mg l^{-1} of vancomycin and placed the explants with adaxial side down on selective medium with cefotaxime and vancomycin as in wash medium, plus 30 mg l^{-1} of Timentin.

Samac (1995) used two different antibiotics for two different strains of *Agrobacterium tumefaciens* during his transformation experiments in alfalfa. He

used 500 mg l^{-1} of ticarcillin for LBA4404 and ABI bacterial strains whereas 500 mg l^{-1} of claforan for AGL1 strain.

Aliev *et al.* (1997) studied different antibiotics in their role in eliminating bacteria during their transformation works in tobacco. Of the six antibiotics capable of completely suppressing *A. tumefaciens* they found ampiox to be the only drug which did not affect morphogenesis.

Zhou *et al.* (1997) used 250 mg l^{-1} of amino penicillanic acid to control bacterial growth in their transformation work with *Orychophraginus violacens.* Hadfi and Batschauer (1994) used betabactyl at 500 mg l^{-1} for elimination of the bacteria during the transformation work with white mustard. In onion, Eady *et al.* (2000) used 200 mg l^{-1} timentin to eliminate the bacteria during transformation.

2.3.2.4 Screening

Screening of untransformed cells or selection of transformed cells is an important aspect of transformation works. Several factors affect the choice of chemicals used for selection. The selection agent must be toxic to plant cells, though not so toxic that the products from the dying, non-transformed cells kill adjacent transformed cells. Thus the most effective toxins are those which either inhibit growth of untransformed cells or slowly kill the untransformed cells. Optimal selection pressure will use the lowest level of toxin needed to kill untransformed tissues.

2.3.2.4.1 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.

Orlikowska *et al.* (1995) during their experiments with safflower cv. Centennial, found that when the explants were transferred to screening media

immediately after co-cultivation, transformation frequency was not affected as seen by transient expression of GUS, but it failed to produce transgenic shoot. However, when explants were transferred to screening media two days after co-cultivation direct shoot regeneration was obtained. Pre-selection above 2 days did not improve recovery of transgenic shoots.

Transformation studies in *Liquidambar styraciflua* by Sullivan and Lagrimini (1993) revealed that when co-cultivated explants were directly placed on screening media no shoot proliferation was obtained. However, when explants were put in non-selective media, after co-cultivation, till callusing appeared in explants and then transferred to selection medium, shoots resistant to kanamycin were obtained.

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 preselection of ten days had deleterious effect on transformation efficiency.

Based on the experiments with *Moricandia arvensis*, Rashid *et al.* (1996) observed that a pre-selection period after co-cultivation was essential for successful transformation. When selection using 200 mg l^{-1} kanamycin was started 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. Pre-selection for a prolonged period of ten days reduced transformation efficiency.

2.3.2.4.2 Selection of transformed cells

Careful timing of the application of selection pressure is critical in order to limit the number of non-transformed cells which survive through cross-

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

Transformation work with different selectable marker genes are to be done to identify the selectable marker giving highest transformation frequency. Commonly used selectable markers are genes conferring resistance to antibiotics like kanamycin, hygromycin, paramomycin and genticin and herbicides like glyphosate and phosphinothricin.

Neomycin phosphotransferase II (*npt II*) confering 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.

Different strengths of selection of agents are used in different crops. Kanamycin was used at a strength of 20 mg l^{-1} for selection during transformation in *Gerbera hybrida* (Nagaraju *e al.*, 1998) and at a strength of 200 mg l^{-1} in *Moricandia arvensis* (Rashid *et al.*, 1996). Similarly hygromycin was used at a strength of 20 mg l^{-1} in *Robinia pseudoacacia* (Igasaki *et al.*, 2000) and at a strength of 256 mg l^{-1} in cowpea (Muthukumar *et al.*, 1996).

Joao and Brown (1993) reported that acetosyringone treated explants of tomato grew more vigorously in presence of kanamycin and synthesised a greater amount of *npt II* enzyme than control transformation.

There are instances in alfalfa where *npt II* gene even after integration into the 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 Chinese cabbage had to transfer the explants soon after induction of shoots from screening media to rooting medium without kanamycin, as kanamycin inhibited root formation.

During transformation of passion fruit, Manders *et al.* (1994) found that using 86 µM of kanamycin in screening media allowed good percentage of untransformed shoots and hence had to raise the concentration to 172 µM. However, prolonged culture of regenerated shoots in a medium with 172 µM caused vitrification of shoots. Hence after initial screening the regenerated shoot were grown at 86 µM of kanamycin.

In pickling cucumber, Sarmento *et al.* (1992) had noted that regeneration of transformed calli growing in 75 mg l^{-1} kanamycin was obtained only with difficulty. Quite frequently, calli were not transformed (no *npt II* activity detected) despite growth on 75 mg l^{-1} kanamycin. However, when shoots were formed, 91 per cent of them were transformed indicating there were few escapes.

Rashid *et al.* (1996) tried both kanamycin and hygromycin for their efficiency as selectable marker during transformation of *Moricandia arvensis*. They found that at 10-20 mg l^{-1} of hygromycin, transformed calli grew slightly, but the growth was halted and cells become necrotic. Finally no transgenic plants were obtained. In contrast growth and callus proliferation was observed with 100-200 mg l^{-1} of kanamycin and yielded the greatest transformation efficiency of 10.3 per cent. However, in *Robinia pseudoacacia*, Igasaki *et al.* (2000) found hygromycin to be more effective than kanamycin at similar concentration.

Barry (1976) has described the procedure for handling the antibiotic stock solution during experimentation. According to him antibiotic stocks can be stored at -20°C for a period of six months without loosing its activity. However, he cautions that antibiotic stock should be melted only before use and that once melted it should never be re-frozen.

Barbic *et al.* (1997) performed genetic transformation of *Brassica carinata* using *Agrobacterium tumefaciens*, harbouring a construct containing the marker genes neomycin phosphotransferase II, phosphinothricin acetyltransferase (*bar*) and the reporter gene beta-glucaranidase (*Uid A*). When selection was done with kanamycin 30 to 50 per cent of the explants produced GUS positive shoots, whereas when it was selected with L-phosphinothricin only one to two per cent resistant shoots were produced.

Transformation of white clover revealed that the *bar* gene and phosphinothricin selection system was more effective than *npt II* (kanamycin selection) or *aad A* (spectinomycin selection) in this crop (Larkin *et al.*, 1996).

Bretague-Sagnard and Chupean (1996) found that in transformation of flax, selection with different neomycin yielded transformed calli but failed to produce transformed shoots. On the other hand, selection with spectinomycin resistance allowed the growth of transformed shoots. Hence spectinomycin based

selection was more effective than kanamycin or other neomycin. In *Castanea sativa,* Costa-Seabra-R-dapais and da-Costa-Seabra-Rsalesses (1999) reported paramomycin to be better selecting agent than kanamycin and genticin during *Agrobacterium* mediated transformation.

Joersbo and Okkels (1996) reported a novel method of selection of transformed cells avoiding the use of antibiotics. Here an *E. coli* beta glucuronidase (*GUS*) reporter gene was used together with benzyladenine glucoronide derivative. Benzyladenine glucoronide is inactive as cytokinins, however, upon hydrolysis by GUS, active cytokinins was released which stimulated transformed cells to propagate whereas the untransformed cells do not multiply. This system was successfully used in tobacco and resulted in a 1.7 to 2.9 fold higher transformation rate compared to kanamycin selection. In this selection system, the *GUS* gene is used as a selectable marker, this eliminates the need for herbicide and antibiotic resistance gene.

Joersbo *et al.* (1998) utilized phosphomannose isomerase (*PMS*) gene as selectable maker 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.

2.3.2.5 Escapes in the screening medium

Even after growing the inoculated explant in selection media containing suitable selectable markers, some untransformed cells may also show growth. This could be because of the cross-protection provided by transformed cells. Hence all tissues showing growth in the selective medium cannot be considered as trangenic and further confirmatory tests are to be done to confirm the transfer and stable integration of the foreign genes.

In commercial melon, Valles and Lasa (1994) found that inoculated explants when placed in screening media containing 100 mg l^{-1} of kanamycin showed bud formation in 10 per cent of the explants. However, shoots from buds developed only in 50 per cent of the buds. Further, when shoots were excised and put on rooting media containing kanamycin, only 50 per cent of the shoots produced roots. They found all the shoots which did not root in kanamycin were untransformed and the ones which rooted in kanamycin were all transformed, showing *npt II* activity.

Pena *et al*. (1995) found that in sweet orange, 92.1 per cent of the shoots regenerated in screening media containing kanamycin were escapes. Of the 168 shoots that regenerated in screening media, only 7.9 per cent showed GUS activity. When inoculated explants were grown in a medium without kanamycin none of the shoots produced were GUS positive. Hence kanamycin screening was necessary.

Material and Methods

3. MATERIALS AND METHODS

The present investigation was carried out at the Plant Tissue Culture Laboratory, College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur, during November 1997 to August 2000. The study was aimed at developing a procedure for *Agrobacterium tumefaciens* mediated genetic transformation in black pepper. 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

In vitro raised seedlings of cultivar Panniyur-1 were used as the source of explant for the study.

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 Glasswares

Borosilicate glasswares of Cornings/Borosil brand were used for the experiment.

3.2.3 Composition of media

Basal MS medium (Murashige and Skoog, 1962), SH medium (Schenk and Hildebrandt, 1972) and modifications of the two 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) was used for

bacterial culture during the study. 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 using 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 250 ml conical flask and 250 ml in 500 ml conical flask. The test tube 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 minutes (Dodds and Roberts, 1982) to sterilize the medium. The medium was allowed to cool to room temperature and stored in culture room until used.

For bacterial culture, the pH of the YEP 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 for extra protection.

3.4 Culture conditions

The cultures were incubated at 26±2°C in an 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 according to the climatic conditions prevailed.

3.5 Production of axenic seedlings

Ripe berries were collected from mature vines of the cultivar Panniyur-1 maintained by the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara. Seeds were extracted after removing the pulp, thoroughly washed in water and dried in shade for a period of two days. The dried seeds were washed in water containing few drops of teepol and rinsed with four or five changes of water to remove the teepol. The seeds were taken to the laminar flow and surface sterilized with $HgCl_2$ (0.1 per cent) for ten minutes. It was then washed free of $HgCl_2$ by rinsing with five changes of sterile distilled water. The seeds were drained on sterile blotting paper. The surface sterilised seeds were inoculated in four different media; viz., double sterilised moist sand, half MS semisolid medium with 15 g l^{-1} sucrose, semisolid SH medium and liquid SH medium. Filter paper bridge was provided in liquid medium to support the explant. The cultures were incubated in the culture room. Observations on time taken for germination, number of seeds germinated and number of seedlings recovered were recorded.

3.6 Establishment of callus cultures

Cotyledonary leaves of the *in vitro* raised seedlings were used as explant for establishing callus cultures. The *in vitro* raised seedlings were taken to the laminar flow and leaves separated from the seedlings using sterilised scissors. The excised leaves were surface sterilised with $HgCl_2$ (0.1 per cent) for 3 minutes and then washed thoroughly with five changes of distilled water to remove all traces of $HgCl_2$. The leaves were then cut along the breadth into small pieces of size 1.5 x 0.5 cm. The leaf pieces were drained on sterile filter paper. The surface sterilised cotyledonary leaf pieces were cultured in test tubes in the medium reported by Nazeem *et al.* (1990) which consisted of half MS supplemented with 1.0 mg l^{-1} each of IAA and BA. The explants were also cultured in a medium consisting of half MS supplemented with 1.5 mg l^{-1} of IAA and 1.0 mg l^{-1} of BA. The cultures were maintained in the culture room. The explants were subcultured as and when

the phenolic exudates appeared in the medium. The observations on time taken for callusing, number of explants callusing and callus growth in the two media combinations were recorded. Based on percentage of explant callusing and callus growth score, callus index (CI) was calculated as given below.

$$CI = P \times G$$

Where 'P' is the percentage of explant callusing and G is the average growth score of the callusing explants. The maximum growth score of '4' was given when the surface of the media in the tube was fully covered by callus.

3.7 Multiplication of callus

The primary callus obtained from culturing the leaf explants were cut into smaller pieces using sterile scalpel blade and cultured in their respective media, prepared freshly in test tubes, for multiplication of the callus (secondary callus). The cultures were maintained in the culture room. The cultures were changed to fresh media as and when phenolic exudations were seen in the medium. Observations on number of calli showing fresh growth and amount of growth achieved were recorded in each of the two growth media.

3.8 Regeneration from callus

Nazeem *et al.* (1990) had reported the same medium for callus induction and regeneration in black pepper which consisted of half MS supplemented with 1.0 mg l^{-1} each of IAA and BA.

Callus was multiplied in half MS supplemented with two different combinations of IAA and BA, viz., 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA and 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA. The calli produced in the two media combinations were used for regeneration experiments in nine different combinations of IAA, BA and kinetin in half MS medium.

The nine growth regulator combinations tried are given below.

Table 1. Growth regulator combination in half MS used for indirect organogenesis in *Piper nigrum* L. variety Panniyur-1

Treatments	Growth regulator combinations (mg l^{-1})		
	IAA	BA	kinetin
1	1.0	1.0	0.0
2	1.0	1.0	0.1
3	1.0	1.0	0.5
4	1.0	1.5	0.0
5	1.0	1.5	0.1
6	1.0	1.5	0.5
7	1.0	2.0	0.0
8	1.0	2.0	0.1
9	1.0	2.0	0.5

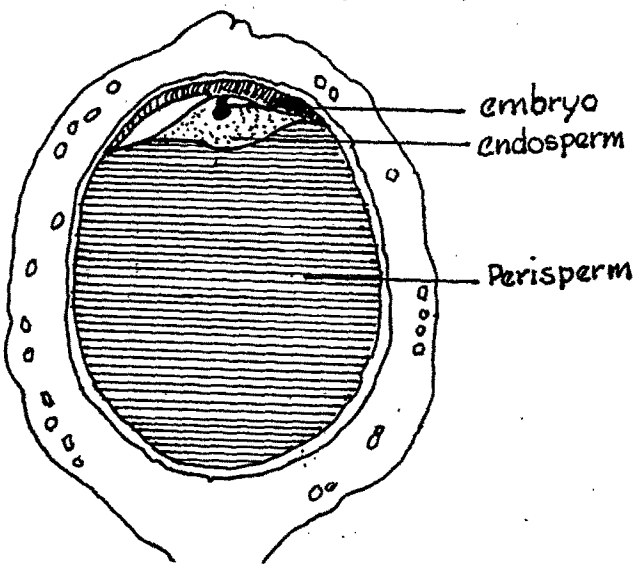
The cultures were subcultured in the respective medium as and when phenolic exudation appeared in the medium. Observations on regeneration were recorded for the eighteen treatment combinations.

3.9 Somatic embryogenesis

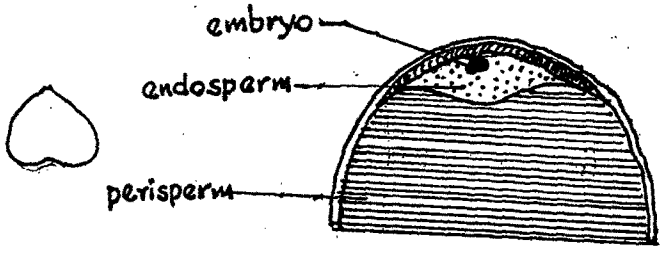
The protocol reported by Joseph *et al.* (1996) was followed to initiate embryogenic calli. In addition, certain variations in culture condition were also attempted.

The seeds collected from dry berries were surface sterilised as given in section 3.5. The embryos with endosperm were dissected out aseptically using sterile scalpel and used as explant to initiate the culture. Cultures were also initiated using embryo alone (embryos taken out from the endosperm) or by cutting one third of the seed containing the embryo and using them as explants.

Diagramatic representation of the seed as well as the three explants are given in Fig.1 below (adapted from Bhojwani and Bhatnagar, 1990).



(a) LS of seed



(b) Embryo with endosperm

(c) Embryo alone

(d) One third seed

Fig.1. Diagramatic representation of black pepper seed showing the three explants used for culturing.

The different explants were cultured in SH medium. The procedures adopted are tabled below:

Table 2. Details of experimental procedure adopted for somatic embryogenesis in *Piper nigrum* L. var. Panniyur-1

Sl. No.	Explant	Cultural conditions			
		Incubation light/dark	Growth regulator 2,4-D in mg l^{-1}	Consistency liquid/semi solid	Static/ shake culture
1	Embryo with endosperm	Dark	Nil	Liquid	Static
2	Embryo with endosperm	Light	Nil	Liquid	Static
3	Embryo with endosperm	Dark	0.5	Liquid	Static
4	Embryo with endosperm	Dark	1.0	Liquid	Static
5	Embryo with endosperm	Dark	2.0	Liquid	Static
6	Embryo with endosperm	Dark	3.0	Liquid	Static
7	Embryo with endosperm	Dark	4.0	Liquid	Static
8	Embryo with endosperm	Dark	5.0	Liquid	Static
9	Embryo with endosperm	Dark	Nil	Semi solid	Static
10	Embryo with endosperm	Light	Nil	Semi solid	Static
11	Embryo with endosperm	Dark	Nil	Liquid	Shake culture
12	Embryo alone	Dark	Nil	Liquid	Static
13	$1/3^{rd}$ seed with embryo	Dark	Nil	Liquid	Static
14	$1/3^{rd}$ seed with embryo	Light	Nil	Liquid	Static

The liquid static cultures were raised in 60 ml test tubes containing 15 ml of the liquid medium and the explants were supported by filter paper bridge. The explants were taken in conical flasks when cultured on rotary shaker. For culturing explants in semi solid medium, 60 ml test tubes were used.

All the cultures were maintained at 26±2°C and observations on growth of zygotic embryos and production of embryogenic calli/somatic embryos were recorded.

3.10 *Agrobacterium* mediated genetic transformation

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

Both callus and leaf segments were tested for their sensitivity to various antibiotics. For callus studies, it was cut into size of 1 cm^3 (approximately) and precultured for a period of 10 to 12 days until they resumed growth. In case of leaf segments (size – 2.0 x 0.5 cm), preculturing of 4 to 5 days was given.

The callus induction/proliferation medium, viz., half MS supplemented with 1.0 mg l^{-1} each of IAA and BA, was prepared in conical flask of suitable size and stored in culture room until use. On the day of the experiment, the medium was melted and then cooled to a temperature of 40° to 50°C. It was then taken to the laminar flow cabinet where required filter sterilised (pore size – 0.2µm) antibiotic was added and mixed thoroughly by vigorous shaking. The medium containing the antibiotic was dispensed to sterilised empty culture tubes and allowed to cool and solidify. When the medium in the test tube cooled down to room temperature, the explant was inoculated.

Six experiments were carried out to evaluate the antibiotic sensitivity of black pepper tissues. Details of the experiments are given in Table 3.

Observation on fresh growth of callus in callus explant and induction of callus in case of leaf explant were recorded.

3.10.2 Culture and maintenance of *Agrobacterium*

Agrobacterium tumefaciens strain AGL-1; 1303 received from the Centre for Biotechnology, SPIC Science Foundation, Chennai, was used for the study. The T-DNA of the T_i plasmid of this strain harboured antibiotic resistance genes for kanamycin, hygromycin and carbenicillin along with reporter genes *GUS* and *GFP* (Green fluorescent protein). These genes were under the control of *CaMV 35S* promoter and *Nos* terminator.

Table 3. Details of experiments conducted for evaluating antibiotic sensitivity of leaf/callus tissues of *Piper nigrum* L. variety Panniyur-1

Expt. No.	Explants	Antibiotics (mg l^{-1})					Nature of antibiotic stocks	Nature of application	Subculture interval (days)
		Kana	Hygro	Cefo	Rif	Amp			
1	Callus	100	10	250	50	50	Stored in frozen condition	Single	-
		200	20	500	100	100			
		400	40	750	200	200			
		500	50	-	400	500			
		-	-	-	500	-			
2	Leaf	50	5	-	-	-	Stored in frozen condition	Multiple	14
		-	10	-	-	-			
	Callus	50	5	-	-	-			
		-	10	-	-	-			
3	Callus	50	-	-	-	-	Freshly prepared	Multiple	14
4	Leaf	-	10	-	-	-	Freshly prepared	Multiple	14
		-	20	-	-	-			
		-	30	-	-	-			
		-	40	-	-	-			
	Callus	-	10	-	-	-			
		-	20	-	-	-			
		-	30	-	-	-			
		-	40	-	-	-			
5	Leaf	25	-	-	-	-	Freshly prepared	Multiple	14
		50	-	-	-	-			
		75	-	-	-	-			
		100	-	-	-	-			
	Callus	25	-	-	-	-			
		50	-	-	-	-			
		75	-	-	-	-			
		100	-	-	-	-			
6	Callus	100	-	-	-	-	Freshly prepared	Multiple	8
		125	-	-	-	-			
		150	-	-	-	-			
		200	-	-	-	-			

Kana – kanamycin; Hygro – hygromycin; Cefo – cefotaxime
Rif – rifampicin; Amp – ampicillin

The bacterial culture was maintained in semi solid YEP (An *et al.*, 1988) medium containing 100 mg l^{-1} of carbenicillin and 50 mg l^{-1} of kanamycin.

The YEP medium 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, filter sterilised kanamycin and carbenicillin were added to the 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 culture developed in five to six days (Plate 1). The bacteria was subcultured once in a month.

3.10.3 Preparation of bacterial suspension

Liquid YEP medium was prepared in required volume in conical flask and kept in culture room until use. On the day of use, Kanamycin and carbenicillin were added to the medium and distributed to sterilised test tube in a laminar flow. One ml of sterile distilled water was taken in a 1.5 ml eppendorf tube. Using a sterile bacterial loop, a single colony of the bacteria from the culture was taken and added to the distilled water in the eppendorf tube and shaken well to form a uniform bacterial suspension. 100-200 µl of this bacterial suspension was added to the test tube containing YEP medium. The test tubes were then plugged and kept in shaker (110 rpm) at 28°C for 12 to 24 hrs according to the required density of the bacteria. The bacterial density was measured in Spectronic 20 spectrophotometer at a wavelength of 560 nm.

3.10.4 Test for bacteriocidal activity of cefotaxime

Twenty five ml of the regeneration media of black pepper (half MS supplemented with 1.0 mg l^{-1} each of IAA and BA) was taken in three 100 ml conical flasks. To these 2.5 ml of the liquid bacterial suspension raised in YEP medium and reading an $O.D_{560nm}$ = 0.9 was added and incubated in shaker (rpm 110) at 28°C for 48 hrs. All the three cultures developed an OD_{560nm} = 1.09. Cefotaxime at a concentration of 500 and 1000 mg l^{-1} were added to the two of these cultures. The third was kept as control. The three cultures were again incubated in shaker (rpm 110) at 28°C for 48 hrs.

Two hundred µl of the bacterial suspension from each of these cultures were added to YEP medium in different culture vials. After incubation in shaker for 48 hours, observation on bacterial growth were recorded. The experiment was repeated thrice.

3.10.5 Effect of proline on bacterial multiplication

Equal volumes of regeneration medium for black pepper (½ MS supplemented with 1.0 mg l^{-1} each of IAA and BA) with and without proline were prepared. In proline containing medium it was added prior to autoclaving. A definite volume (1.0-2.5 ml) of bacterial suspension culture raised in YEP medium was added to both media. These were then incubated in shaker for a definite period of time (24-54 h). The $O.D_{560nm}$ reading developed in regeneration media of the two media with and without proline were recorded. The experiment was repeated with different concentrations of proline as well as incubation time.

3.10.6 Genetic transformation of black pepper

Genetic transformation experiments with leaf and callus were carried out using *Agrobacterium tumefaciens* strain AGL-1; 1303. There were 16 experiments done, of which 11 were with leaf and five with callus. They are designated as leaf transformation-I, II, ... XI and callus transformation

I, II, ... V. Each experiment consisted of one to four treatment combinations, along with two controls, which were common for the experiment. In one of the controls, the explants without bacterial treatments were placed in regeneration medium containing antibiotics used for screening in that experiment and the other control consisted of explants without bacterial treatment placed in the regeneration medium without antibiotics. The regeneration medium used for transformation work was the one reported by Nazeem *et al.* (1990), except in leaf transformation VII, where, callusing medium reported by Sasikumar and Veluthambi (1996) was used which consisted of MS medium supplemented with 3.0 mg l^{-1} BA, 2.0 mg l^{-1} NAA and 1.0 mg l^{-1} 2,4-D. The general methodology adopted for these experiments is detailed below.

3.10.6.1 Preparation of the explants

The axenic seedlings raised in test tubes were brought to the laminar flow cabinet. Cotyledonary leaves were cut and separated from the seedlings. The leaves were surface sterilized and explants prepared as in section 3.6. The leaf segments were then transferred to regeneration media (½ MS supplemented with 1.0 mg l^{-1} each of IAA and BA) in sterile petriplates. The petriplates were sealed with cling film and incubated in culture room. The period of preculture tried varied from 1 to 4 days in different experiments.

In callus transformation experiments, good growing calli were selected and cut into appropriate size and cultured in regeneration media for one to two weeks until it resumed growth. The callus was wounded with sterile needle just prior to inoculation with the induction media. Transformation was also tried without wounding the callus.

3.10.6.2 Preparation of induction media

Regeneration media (liquid) taken in conical flask was used for the preparation of induction media. Bacterial stimulants like proline and

acetosyringone were added to the regeneration medium at varying concentration in different experiments. Bacterial suspension raised in YEP medium was added (1-3 ml) to the regeneration medium in the conical flask along with 50 mg l^{-1} kanamycin and 100 mg l^{-1} carbenicillin. The conical flask was kept in shaker (rpm 110) at 28°C for a period varying from 4 h to 48 h as per the bacterial density required for the experiments. Transformation was tried at different bacterial density ranging from $O.D_{560nm}$ = 0.2 to 1.5 in both leaf and callus experiments.

3.10.6.3 Induction

The explants were immersed in the induction media. The induction time tried in various experiments ranged from 5 to 30 minutes. The explants were then blot dried with sterile filter paper.

3.10.6.4 Co-cultivation

The blot dried explants were transferred to regeneration medium (without antibiotics) in petriplates. Few experiments were conducted adding attractants like proline and acetosyringone to the regeneration medium at varying concentrations. The period of co-cultivation tried ranged from one to four days.

3.10.6.5 Screening

After co-cultivation of the explant with the *Agrobacterium*, the explants were transferred to screening media in culture tubes or petriplates. The screening media consisted of regeneration media containing antibiotics used as selecting agent and the bacteriocidal agent (used for killing the bacteria). The concentration of the selecting agent and the bacteriocidal agent varied in the experiments to study their relative efficiency.

The regeneration media was prepared in required volume in conical flask and kept in culture room. On the day of use, the media was melted and cooled to 40-50°C. Filter sterilized antibiotics were added to the medium under aseptic

conditions and dispensed to the empty sterilized test tubes or sterile petriplates where it was allowed to cool to room temperature and solidify. The co-cultivated explants were then transferred to the solidified screening media.

The cultures in the screening media were subcultured every 10 to 14 days. All the cultures were maintained in the culture room.

The details of treatments given in the individual experiment are given below. The data on callus growth, induction of callus on leaf segments and bacterial over growth were recorded in all the callus and leaf transformation experiments.

3.10.6.6 Leaf transformation experiments

3.10.6.6.1 Leaf transformation-I

In leaf transformation-I a single treatment combination was tried. Here, the leaf explants were precultured for four days in the regeneration medium. They were treated with induction medium containing 20 mg l^{-1} acetosyringone and having a bacterial density of $O.D_{560nm}$ = 0.25 for 30 minutes. The explants were then co-cultivated with bacteria in the regeneration medium for three days and transferred to screening medium containing 500 mg l^{-1} cefotaxime, 50 mg l^{-1} kanamycin, 100 mg l^{-1} carbenicillin and 20 mg l^{-1} hygromycin. The explants were subcultured at two weeks interval. Cefotaxime was withdrawn from the screening medium after third subculture. Bacterial over growth was seen and hence cefotaxime treatment was continued from next subculture onwards. Here only the control with antibiotics was laid.

3.10.6.6.2 Leaf transformation-II

Leaf transformation-II consisted of four treatment combinations and two controls. The four treatment combinations varied among themselves with respect to the preculture of explants (1-4 days), proline (0-200 mg l^{-1}) and acetosyringone (0-20 mg l^{-1}) added to the induction media.

The precultured explants were treated with their respective induction media having a bacterial density of $O.D._{560nm}$ = 0.3 for 15 minutes. The explants were blot dried and co-cultivated with bacteria in regeneration medium containing 100 mg l^{-1} of proline for two days. The explants were then transferred to screening medium containing 500 mg l^{-1} cefotaxime, 50 mg l^{-1} kanamycin, 20 mg l^{-1} hygromycin and 100 mg l^{-1} carbenicillin. Subculturing was done on to fresh screening medium every two weeks. Cefotaxime in this group of experiments was withdrawn during second subculture (28^{th} day), however, due to bacterial overgrowth, cefotaxime was continued from third subculture onwards.

3.10.6.6.3 Leaf transformation-III

This consisted of four treatment combinations and two controls. In this experiment, proline (100 to 300 mg l^{-1}) and acetosyringone (20-30 mg l^{-1}) added to the induction media and the bacterial density ($O.D._{560nm}$ = 0.19 to 0.26) of the induction media were varied in the four treatment combinations tried.

After preculture for three days the explants were treated with their respective induction media for 15 minutes. The explants were then blot dried and co-cultivated with bacteria in regeneration medium containing 100 mg l^{-1} proline and 10 mg l^{-1} acetosyringone for two days. The co-cultivated explants were transferred to screening medium containing 500 mg l^{-1} cefotaxime, 50 mg l^{-1} kanamycin, 20 mg l^{-1} hygromycin and 100 mg l^{-1} carbenicillin. The explants were subcultured after every two weeks into fresh screening medium for a period of three months. Cefotaxime was withdrawn from the screening medium during fourth subculture.

3.10.6.6.4 Leaf transformation-IV

In this experiment, four treatment combinations were tried, which varied among themselves with respect to proline (100-200 mg l^{-1}) and acetosyringone

(20-30 mg l^{-1}) added to the induction media and the bacterial density ($O.D._{560nm}$ = 0.31 to 0.45) of the induction media. Two controls were also laid.

The explants were precultured for two days and treated for 20 minutes in their respective induction media. The explants were then blot dried and co-cultivated with bacteria for four days in regeneration medium containing 100 mg l^{-1} proline and 10 mg l^{-1} acetosyringone. The co-cultivated explants were transferred to screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. The explants in the screening medium were subcultured at an interval of two weeks. Cefotaxime was withdrawn from screening medium during fourth subculturing (56^{th} day).

3.10.6.6.5 Leaf transformation-V

Leaf transformation-V consisted of four treatments and two controls. Acetosyringone (0-30 mg l^{-1}) added to the induction media, the bacterial density ($O.D._{560\ nm}$ = 0.32 to 0.45) of the induction media and induction time (5-30 min.) had been varied in the four treatment combinations tried in this experiment.

The leaf explants were precultured for five days and treated with their respective induction media for a period ranging from 5 to 30 minutes. The explants were blot dried and then co-cultivated with bacteria in regeneration medium for three days. After co-cultivation, the explants were transferred to screening medium containing 500 mg l^{-1} cefotaxime, 75 mg l^{-1} kanamycin and 20 mg l^{-1} hygromycin. The explants were subcultured at an interval of two weeks. Cefotaxime was withdrawn on 42^{nd} day. The cultures were maintained for three months.

3.10.6.6.6 Leaf transformation-VI

This consisted of two treatments, each replicated twice, along with two controls. The two treatment combinations differed among themselves with respect to the presence (200 mg l^{-1}) and absence of proline in the induction media.

The explants were precultured for 40 days and were treated for five minutes with induction media with and without proline and having a bacterial density of $O.D._{560nm}$ = 1.2. The treated explants were blot dried and co-cultivated with the bacteria for four days. After co-cultivation the explants were transferred to screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. The explants were subcultured at an interval of five to eight days for the first 25 days and then at an interval of 10 to 13 days. Cefotaxime was withdrawn from the screening medium on 60th day.

3.10.6.6.7 Leaf transformation-VII

Leaf transformation-VII consisted of two treatments, each with two replications. The two treatments differed with respect to the presence (300 mg l^{-1}) or absence of proline in the induction media and the bacterial density ($O.D._{560nm}$ = 0.5/1.4) of the induction medium.

The leaf explants were precultured for three days in callusing medium consisting of MS medium supplemented with 3.0 mg l^{-1} BA, 2.0 mg l^{-1} NAA, 1.0 mg l^{-1} 2,4-D and 30 mg l^{-1} sucrose. The precultured explants were treated for three minutes with the two induction media tried in this experiment. In treatment-1 the induction medium consisted of liquid callusing medium containing no attractants and having a bacterial density of $O.D._{560nm}$ = 0.5 and in treatment-2 the induction medium consisted of liquid callusing medium containing 300 mg l^{-1} proline and a bacterial density of $O.D._{560nm}$ = 1.4. The leaf explants treated with the induction media were blot dried and co-cultivated with the bacteria in callusing medium for a period of two days. These were then transferred to the screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. The explants were subcultured at an interval of five to seven days during the first 18 days and then at an interval of 8 to 14 days. Cefotaxime was withdrawn from the screening medium on the 37th day.

3.10.6.6.8 Leaf transformation-VIII

Leaf transformation-VIII consisted of two treatments, each replicated twice and two controls. The two treatments varied with respect to the presence (200 mg l^{-1}) or absence of proline in the induction medium.

Leaf explants were precultured for 32 days and then immersed for 5 minutes in the induction medium with and without proline and having a bacterial density of $O.D._{560nm}$ = 1.0. The blot dried explants were then co-cultivated with the bacteria in the regeneration medium for two and a half days. These were then transferred to the screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. The explants were subcultured on to fresh screening medium after two days. Thereafter subculturing was done at an interval of 8 to 13 days. Cefotaxime was withdrawn from the screening medium on 52nd day.

3.10.6.6.9 Leaf transformation-IX

Two treatments replicated twice were conducted under leaf transformation-IX. Presence (400 mg l-1) or absence of proline in the induction media and the bacterial density of the induction media were the two factors that were varied in the two treatments. Two controls were also maintained.

Leaf explants precultured for four days were used for this experiment. In treatment-1, the precultured leaf segments were immersed for three minutes in the induction medium containing no attractants and having a bacterial density of $O.D._{560nm}$ = 0.98, whereas, in treatment-2, the precultured leaf segments were immersed for the same period of time in the induction medium containing 400 mg l^{-1} proline and having a bacterial density of $O.D._{560nm}$ = 1.25. The treated explants in both the treatments were blot dried and co-cultivated with the bacteria in regeneration medium for three days. These were then transferred to screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. The explants were subcultured on to fresh screening medium at an interval ranging from 5 to 12

days. The concentration of cefotaxime in the screening medium was increased to 1000 mg l^{-1} on 12th day and then to 1500 mg l^{-1} on 34th day. Cefotaxime was withdrawn from the screening medium on 42nd day.

3.10.6.6.10 Leaf transformation-X

Leaf transformation-X consisted of two treatments replicated twice and two controls. The two treatments differed with respect to the presence (400 mg l^{-1}) or absence of proline and the bacterial density ($O.D._{560nm}$ = 1.02/1.3) of the induction medium.

The leaf explants were precultured for five days. In treatment-1, the precultured explants were treated for three minutes with induction medium containing no attractants and having a bacterial density of $O.D._{560nm}$ = 1.02, whereas, in treatment-2, the precultured explants were treated for the same period of time with induction medium containing 400 mg l^{-1} and having a bacterial density of $O.D._{560nm}$ = 1.30. In both the treatments, the leaf explants after induction were blot dried and then co-cultivated for two days in regeneration medium. These were then transferred to screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. The explants were subcultured on to fresh screening medium after six days and thereafter at an interval of 10 to 13 days. Cefotaxime was withdrawn from the screening medium on 41st day.

3.10.6.6.11 Leaf transformation-XI

In leaf transformation-XI, two treatments were tried varying with respect to the presence (500 mg l^{-1}) or absence of proline in induction media and the bacterial density ($O.D._{560nm}$ = 1.0/1.3) of the induction media. The two experiments were replicated twice. Two control were also maintained.

The leaf explants were precultured for 32 days. In treatment-1, the precultured explants were immersed for three minutes in the induction medium containing no attractants and having a bacterial density of $O.D._{560nm}$ = 1.0,

whereas, in treatment-2, the precultured explants were immersed in the induction medium containing 500 mg l^{-1} proline and a bacterial density of $O.D._{560nm}$ = 1.30 for the same period of time. The explants after treatment with induction medium were blot dried and then co-cultivated for two days in regeneration medium in both the treatments. After co-cultivation, the explants were transferred to screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. After three days, the explants were subcultured to fresh screening medium with an increased cefotaxime concentration of 1000 mg l^{-1}. Further subculturing was done at an interval of 11 to 14 days with the screening medium containing 1000 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin. Cefotaxime was withdrawn from the screening medium on 40th day.

3.10.6.7 Callus transformation experiments

3.10.6.7.1 Callus transformation-I

Vigorously growing calli were selected, cut into small pieces of size 0.75 cm, precultured for three days in regeneration medium and used for transformation. Callus transformation-I consisted of four treatments and two controls. The four treatments varied among themselves with respect to the absence or presence (500 mg l^{-1}) of proline in the induction medium, bacterial density ($O.D._{560nm}$ = 0.75/0.8) of the induction medium and innoculation time (5 and 15 min.).

The precultured calli were wounded with sterile needle and immersed for 5 to 15 minutes in induction medium with or without proline and having a bacterial density of $O.D._{560nm}$ = 0.75/0.8 in different treatments. The calli were air dried over a sterile filter paper. They were then co-cultivated with bacteria in regeneration medium for three days. The explants were then transferred to screening medium containing 500 mg l^{-1} cefotaxime, 50 mg l^{-1} kanamycin and 20 mg l^{-1} hygromycin. The explants were subcultured into fresh screening medium

every two days for first eight days and then at an interval of two weeks. Cefotaxime was withdrawn from screening medium on 42nd day.

3.10.6.7.2 Callus transformation-II

Vigorously growing calli were collected, cut into small pieces of size 1.0 cm^3 and precultured in regeneration medium for two to three weeks till fresh growth appeared. These were used for transformation work.

Callus transformation-II consisted of four treatments and two controls. The four treatments varied among themselves with respect to the concentration of proline (200/300 mg l^{-1}) added to the induction media, the bacterial density ($O.D._{560nm}$ = 1.43/1.45) of the induction media and induction time (5-30 min.).

The precultured calli were wounded with sterile needle and immersed for 5 to 30 minutes in induction media containing 200-300 mg l^{-1} proline and a bacterial density ranging from $O.D_{560nm}$ = 1.43-1.45 in different treatments. The calli were then air dried for 20 minutes and put in regeneration medium containing 200 mg l^{-1} proline for co-cultivation with the bacteria for three days. The co-cultivated calli were transferred to screening medium containing 500 mg l^{-1} cefotaxime, 50 mg l^{-1} kanamycin and 20 mg l^{-1} hygromycin. The calli were subcultured every day for four days because of high phenol exudation in the four experiments compared to the two controls. Thereafter they were subcultured once in two days for the next six days and later once in two weeks. Cefotaxine was withdrawn from screening medium on 38th day.

3.10.6.7.3 Callus transformation-III

Vigorously growing calli were selected and cut into small pieces of 0.75 cm^3 and cultured in regeneration medium for two weeks until it showed fresh growth. These calli were used for transformation in this experiment.

The callus transformation-III consisted of four treatments and two controls. The four treatments differed in two respects, either with the use of wounded or unwounded calli and the concentration of proline (100/400 mg l^{-1}) added in the induction medium.

Wound/unwounded calli were immersed for five minutes in induction media with 100 or 400 mg l^{-1} proline and having a bacterial density of $O.D_{560nm}$ = 0.5. The calli were then air dried for 20 to 30 minutes and put in regeneration medium for co-cultivation with the bacteria for four days. The calli were then transferred to screening medium containing 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. To prevent damage by phenols, the calli were subcultured twice daily for seven days and then daily for another seven days. One of the calli in control with antibiotics showed fresh growth and hence on 14^{th} day the concentration of kanamycin was increased to 100 mg l^{-1} in the screening medium. During the next 25 days subculturing was done once in two days and then once in three days for the next 18 days. Cefotaxime was withdrawn from the screening medium on 58^{th} day.

3.10.6.7.4 Callus transformation-IV

Vigorously growing calli were cut into pieces of 0.75 cm^3 and cultured in regeneration medium for two to three weeks till the calli showed fresh growth. These calli were used for transformation work in this experiment.

The callus transformation-IV consisted of four treatments and two controls. The four treatments varied among themselves in the type of calli used (wounded or unwounded), presence (500 mg l^{-1}) or absence of proline in the induction medium and the bacterial density ($O.D._{560nm}$ = 1.0/1.5) of the induction medium. In treatment-4, screening of untransformed cells by use of kanamycin was avoided so as to study growth of untransformed cells after infection in the absence of kanamycin.

Precultured calli either wounded or unwounded were immersed for five minutes in induction medium with or without proline and having a bacterial density of $O.D_{560nm}$ = 1.0 or 1.5. The infected calli were air dried for 30 minutes and transferred to regeneration medium for co-cultivation with the bacteria for two days. After co-cultivation the calli were either transferred to screening medium containing 500 mg l^{-1} cefotaxime and 75 mg l^{-1} kanamycin (treatment 1-3) or to regeneration medium containing only 500 mg l^{-1} cefotaxime (treatment-4). The calli were subcultured in their respective media every day for a period of nine days. For the next 17 days it was subcultured once in two to three days. On 26th day the concentration of cefotaxime was raised from 500 mg l^{-1} to 1000 mg l^{-1}.

3.10.6.7.5 Callus transformation-V

Vigorously growing calli were cut into small pieces of size 0.75 cm^3 and cultured in regeneration medium for two to three weeks till they showed fresh growth. These calli were used for the transformation work.

Callus transformation-V consisted of six treatments and two controls. This experiment was done primarily to find a way to control bacterial overgrowth after co-cultivation. The six treatments varied with respect to the presence (500 mg l^{-1}) or absence of proline in the induction media, bacterial density ($O.D._{560nm}$ = 1.0/1.2) of the induction media, co-cultivation period (1-2 days), pre-selection period (1 day or nil), air drying after co-cultivation of the calli (30 min. or nil), dose of cefotaxime (1000 or 1500 mg l^{-1}) used for killing bacteria and culturing of infected calli in the absence or presence of kanamycin.

Precultured calli were immersed for three minutes in the induction media with or without proline and having a bacterial density of $O.D_{560nm}$ = 1.0 or 1.2. The infected calli were co-cultivated with the bacteria for one or two days in the regeneration medium. After co-cultivation the calli were transferred, with or without preselection and air drying, to either screening media containing 1000/1500 mg l^{-1} cefotaxime and 100 mg l^{-1} kanamycin or to regeneration medium

containing only 1000/1500 mg l^{-1} cefotaxime. The calli were subcultured in their respective medium once in two days for 12 days and then once in three days. Cefotaxime was withdrawn from the medium from 13^{th} to 19^{th} day.

Results

4. RESULTS

The results of the investigations on *Agrobacterium* mediated genetic transformation in *Piper nigrum* L., carried out at the Plant Tissue Culture Laboratory, College of Forestry from November 1997 to August 2000 are presented in this chapter.

4.1 Production of axenic seedlings for transformation

The data on percentage of seeds germinated and recovery of seedlings in the media tried are given in Table 4. The percentage of germination in sterile moist sand was the highest with 73.89 per cent. The recovery of healthy seedlings in this medium was reduced to 57.26 per cent due to contamination and still was the highest among the four media tested (Plate 2). Though the germination percentage in semisolid half MS medium and liquid SH medium is seen to be 27.8 per cent and 38.88 per cent, the actual recovery of seedling were 1.6 per cent and 9.75 per cent, respectively. This is because the majority of the germinated seeds did not grow into normal seedlings in both the media. The seeds did not germinate at all in semisolid SH media.

Time taken for germination was only 18 days in sand whereas it was 50 and 45 days in semisolid modified MS medium and liquid SH medium, respectively.

4.2 Callus induction and growth rate

Cotyledonary explants were cultured in half MS medium under two different growth regulator combinations of IAA and BA. The data on percentage of callusing and callus index are given in Table 5.

The percentage of explant callusing was much higher (34.3) under the growth regulator combination of 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA compared to the combination of 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA (24.3).

Table 4. Germination of seeds and recovery of seedlings of *Piper nigrum* L. in different media under *in vitro* conditions

Medium	Time taken for germination (days)	Percentage of germination	Percentage of recovery of seedlings
Sterilised moist sand	18	73.89	57.26
Modified MS - semi solid	50	27.80	1.60
SH liquid	45	38.88	9.25
SH – semi solid	-	Nil	Nil

Table 5. Callus induction and callus growth rate in half MS with two different growth regulator combinations of IAA and BA (culture duration:80days)

Growth regulator combination	Percentage of explant callusing	Callus index
1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA	34.3	97.7
1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA	24.3	71.9

Table 6. Establishment and growth rate of secondary callus in half MS under two different combinations of IAA and BA (culture duration : 20 days)

Growth regulator combination	Percentage of calli showing fresh growth	Callus index
1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA	100	276.0
1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA	100	208.3

Callus growth was also better under the combination of 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA compared to 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA as indicated by the callus index of 97.7 for the former and 71.9 for the latter combination.

4.3 Multiplication of callus

The primary callus obtained were cut into smaller pieces and cultured in their respective medium (half MS + 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA and half MS + 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA). Effect of the two growth regulator combinations of IAA and BA on multiplication of secondary callus indicated by the percentage of callus showing fresh growth and callus index is given in Table 6.

Establishment of callus cultures as shown by the percentage of callus showing fresh growth was found to be 100 per cent under both the growth regulator combinations. However, growth rate indicated by callus index was much higher under the combination of 1.0 mg l^{-1} each of IAA and BA compared to the combination of 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA. Phenols were seen in callus cultures in both the media (Plate 3).

4.4 Regeneration

The data on regeneration of callus in half MS under 18 different treatment combinations involving IAA (1.0 mg l^{-1}), BA (1.0, 1.5, 2.0 mg l^{-1}) and kinetin (0.1, 0.5 mg l^{-1}) is given in Table 7. Establishment of callus as indicated by fresh growth of callus was 100 per cent in treatment combination 3, 7, 8, 9, 10, 13, 14 and 16. This was followed by 80 percent in treatment combination 2 and 4, 75 per cent in 1, 11 and 15, 60 per cent in 12 and 18, 50 percent in 5 and 40 per cent in 6. Treatment combination 17 did not show any fresh growth.

Regeneration to the extent of 3.0 per cent was noticed in the treatment combination 1, where calli cultured in half MS medium supplemented with 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA were transferred to half MS medium supplemented

Table 7. Regeneration trial with callus of *Piper nigrum* L., variety Panniyur – 1 in half MS supplemented with eighteen different treatment combinations of IAA, BA and kinetin

Treat-ments	Growth regulator combinations (mg l^{-1})		Percentage of calli with fresh growth	Percentage of calli regenerated
	Induction	Regeneration		
1	1.5 IAA + 1.0 BA	1.0 IAA + 1.0 BA	75	3.0
2	1.0 IAA + 1.0 BA	1.0 IAA + 1.0 BA	80	0.0
3	1.5 IAA + 1.0 BA	1.0 IAA + 1.0 BA + 0.1 Kn	100	0.0
4	1.0 IAA + 1.0 BA	1.0 IAA + 1.0 BA + 0.1 Kn	80	0.0
5	1.5 IAA + 1.0 BA	1.0 IAA + 1.0 BA + 0.5 Kn	50	0.0
6	1.0 IAA + 1.0 BA	1.0 IAA + 1.0 BA + 0.5 Kn	40	0.0
7	1.5 IAA + 1.0 BA	1.0 IAA + 1.5 BA	100	0.0
8	1.0 IAA + 1.0 BA	1.0 IAA + 1.5 BA	100	0.0
9	1.5 IAA + 1.0 BA	1.0 IAA + 1.5 BA + 0.1 Kn	100	0.0
10	1.0 IAA + 1.0 BA	1.0 IAA + 1.5 BA + 0.1 Kn	100	0.0
11	1.5 IAA + 1.0 BA	1.0 IAA + 1.5 BA + 0.5 Kn	75	0.0
12	1.0 IAA + 1.0 BA	1.0 IAA + 1.5 BA + 0.5 Kn	60	0.0
13	1.5 IAA + 1.0 BA	1.0 IAA + 2.0 BA	100	0.0
14	1.0 IAA + 1.0 BA	1.0 IAA + 2.0 BA	100	0.0
15	1.5 IAA + 1.0 BA	1.0 IAA + 2.0 BA + 0.1 Kn	75	0.0
16	1.0 IAA + 1.0 BA	1.0 IAA + 2.0 BA + 0.1 Kn	100	0.0
17	1.5 IAA + 1.0 BA	1.0 IAA + 2.0 BA + 0.5 Kn	0	0.0
18	1.0 IAA + 1.0 BA	1.0 IAA + 2.0 BA + 0.5 Kn	60	0.0

with 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA (Plate 4). However, none of the other treatment combinations produced regeneration even after 4 months of culture.

4.5 Somatic embryogenesis

Data on culture of zygotic embryos in SH medium under varying conditions is given in Table 8. 'Embryos with endosperm' when cultured in liquid SH medium supported by filter paper bridge gave the highest percentage of germination irrespective of whether it was incubated in dark or in light (69% and 70%, respectively) (Plate 5, 6 and 7). The time taken for germination was the shortest with 14 days in both the cases. However, leaf production and development into normal seedlings were seen only under light incubation conditions (Plate 8a and 8b). Germination was absent when 2,4-D was added to SH medium except at the lowest concentration of 0.5 mg l^{-1}. The percentage of germination at 0.5 mg l^{-1} was also very low (11%) and time taken for germination was higher (25 days). When 1/3rd of the seed containing the embryo, endosperm and a part of perisperm, was cut and cultured in liquid SH medium supported by filter paper bridge, there was 55.5 per cent germination in dark incubation and time taken was 25 days but the germination was absent in light incubation. Germination percentage was same (72.2%) when 'embryos with endosperm' were cultured in semi solid SH medium, irrespective of whether it was incubated in light or in dark and time taken was the lowest of 14 days in both the cases. Embryo alone when cultured in liquid SH medium supported by filter paper bridge gave a low germination percentage of 11.11 per cent and took higher time of 25 days for germination. When embryos with endosperm were cultured in SH liquid medium on rotary shaker, all of them germinated. But one of the germinated embryos produced six embryo like structures, one by one, from the junction of root and shoot, which eventually developed into normal seedlings (Plate 9). However, in none of the above cases could embryogenic calli be obtained even after a prolonged culture period of one year.

Table 8. Culture of zygotic embryos in SH medium under varying conditions for somatic embryogenesis

Explant	Culture conditions			Percentage of explants germinated	Time taken for germination (days)	Percentage of explants producing leaves	Percentage of explants producing embryogenic calli
	Medium/Solid /Liquid	Incubation Dark/Light	Growth regulator 2, 4-D (mg l^{-1})				
Embryo with endosperm	liquid	dark	Nil	69	14	Nil	Nil
Embryo with endosperm	liquid	dark	0.5	11	25	Nil	Nil
Embryo with endosperm	liquid	dark	1.0	Nil	Nil	Nil	Nil
Embryo with endosperm	liquid	dark	2.0	Nil	Nil	Nil	Nil
Embryo with endosperm	liquid	dark	3.0	Nil	Nil	Nil	Nil
Embryo with endosperm	liquid	dark	4.0	Nil	Nil	Nil	Nil
Embryo with endosperm	liquid	dark	5.0	Nil	Nil	Nil	Nil
Embryo with endosperm	solid	dark	Nil	72.20	14	Nil	Nil
1/3 seed with embryo	liquid	dark	Nil	55.50	25	Nil	Nil
Embryo alone	liquid	dark	Nil	11.11	25	Nil	Nil
Embryo with endosperm	liquid	light	Nil	70.45	14	13.63	Nil
Embryo with endosperm	solid	light	Nil	72.20	14	Nil	Nil
1/3 seed with embryos	liquid	light	Nil	Nil	-	Nil	Nil
Embryo with endosperm	liquid shake culture	diffused light	Nil	100 one of the explant gave rise to seven normal seedlings	14	100	Nil

Culture duration : one year

Explants placed on filter paper bridge wherever liquid medium used and subcultured bimonthly

4.6 *Agrobacterium* mediated genetic transformation

4.6.1 Sensitivity of host tissues to antibiotics

Six experiments were carried out to evaluate the sensitivity of both callus and leaf segments of black pepper to different antibiotics at varying concentrations.

4.6.1.1 Antibiotic sensitivity-1

Sensitivity of callus of *Piper nigrum* L. to five different antibiotics at varying concentrations was tested. Complete suppression of callus growth was not noticed in any of the concentrations tested for the five antibiotics (Table 9).

Kanamycin at the highest concentration of 500 mg l^{-1} showed fresh callus growth in 100 per cent of the calli tested. Hygromycin, though produced fresh growth in 100 per cent of the calli tested at 20 mg l^{-1}, at higher concentration of 40 and 50 mg l^{-1} the fresh callus growth was restricted to 66 per cent and 25 per cent of the calli tested, respectively.

Cefotaxime at the highest dose of 750 mg l^{-1} showed fresh callus growth in 83.3 per cent of the calli tested whereas at 500 mg l^{-1} it was 100 per cent. Rifampicin showed a variation from 83.3 to 100 per cent in the number of calli with fresh growth when its concentration was varied from 50 to 500 mg l^{-1}, whereas, in ampicillin the percentage of calli with fresh growth decreased from 100 per cent to 66 per cent when its concentration was raised from 50 to 500 mg l^{-1}. In the control 100 per cent of the calli produced fresh growth.

4.6.1.2 Antibiotic sensitivity-2

Sensitivity of leaf and callus to 5 and 10 mg l^{-1} of hygromycin and 50 mg l^{-1} of kanamycin were tested. The data is presented in Table 10.

Table 9. Sensitivity of black pepper callus to varying concentrations of five antibiotics

Antibiotics	Dosage (mg l^{-1})	Percentage of calli showing fresh growth
Kanamycin	100	100.0
	200	83.3
	400	100.0
	500	100.0
Hygromycin	20	100.0
	40	66.6
	50	25.0
Cefotaxime	500	100.0
	750	83.3
Rifampicin	50	100.0
	100	83.3
	200	100.0
	400	100.0
	500	83.3
Ampicillin	50	100.0
	100	100.0
	200	50.0
	500	66.6
Control	-	100

Antibiotic stock stored frozen were used
No periodic subculturing done
Culture duration : 4 weeks

Callus induction on leaf and callus growth at 5.0 mg l^{-1} of hygromycin was equal or even better than the control. Reduced callus induction and callus growth was observed when 10 mg l^{-1} of hygromycin was used. With kanamycin at 50 mg l^{-1} there was no fresh growth in callus and induction of callus on leaf was completely suppressed.

4.6.1.3 Antibiotic sensitivity-3

This was a short period trial to assess the sensitivity of callus to kanamycin at a concentration of 50 mg l^{-1}. The data for three weeks period is given in Table 11. After first week the calli were dark at the sides but had light coloured live tissues in the centre. After three weeks, 30 per cent of the calli were completely dark, with no phenols and looked almost dead. In the remaining 70 per cent of the calli darkening was extending to the centre and fresh growth could not be distinguished.

4.6.1.4 Antibiotic sensitivity-4

In this experiment sensitivity of leaf and callus to four different concentrations of hygromycin were tested. The data is given in Table 12.

In control, 100 per cent of the calli used showed fresh growth and 33 per cent of the leaves showed induction of callus. There was a progressive decrease in the percentage of calli showing fresh growth when concentration of hygromycin was increased. With 10 mg l^{-1} hygromycin, percentage of calli producing fresh growth was 77 and it decreased to 44 per cent at 20 mg l^{-1} hygromycin. The fresh growth noticed was brown in colour compared to white growth in the control. At higher concentrations of 30 and 40 mg l^{-1} of hygromycin, none of the calli showed fresh growth, indicating that the growth of callus is completely suppressed at 30 and 40 mg l^{-1} of hygromycin. With respect to leaf, there was no induction of callus in any of the leaves for all the concentrations tried from 10 mg l^{-1} to 40 mg l^{-1} of hygromycin.

Table 10. Sensitivity of leaf and callus to hygromycin and kanamycin at varying concentrations

Antibiotics and their concentrations	Explant used	Percentage of explant showing growth
Hygromycin 5 mg l^{-1}	Leaf	55.5
	Callus	75.0
Hygromycin 10 mg l^{-1}	Leaf	22.2
	Callus	11.1
Kanamycin 50 mg l^{-1}	Leaf	0.0
	Callus	0.0
Control	Leaf	40.0

Antibiotics stock frozen were used
Subculturing done at 2 weeks interval
Culture duration – 8 weeks

Table 11. Pilot test for sensitivity of callus to 50 mg l^{-1} of freshly prepared kanamycin when subcultured at two week interval.

Observation			
After one week		After three weeks	
Percentage of calli with sides dark and live tissue at the center	Completely dark	Percentage of calli with sides dark and line tissue at the center	Completely dark
100	Nil	70	30

Table 12. Sensitivity of leaf and callus to different concentrations of freshly prepared hygromycin

Explant	Dosage (mg l^{-1})	Percentage of explant showing growth	Appearance of the fresh growth
Leaf	10	0	-
Leaf	20	0	-
Leaf	30	0	-
Leaf	40	0	-
Callus	10	77	Brown
Callus	20	44	Brown
Callus	30	0	-
Callus	40	0	-
Leaf	Control	33	White
Callus	Control	100	White

Culture duration : 12 weeks
Subcultured at 2 weeks interval

4.6.1.5 Antibiotic sensitivity-5

Leaf and callus were tested for their sensitivity to various concentrations of kanamycin. The data is given in Table 13.

In the control 77 per cent of the calli showed fresh growth and 44 per cent of the leaf showed induction of callus. The fresh growth seen were all white. The number of calli showing fresh growth were 55, 66 and 44 per cent when kanamycin at concentrations of 25, 50 and 75 mg l^{-1}, respectively, were given. The colour of the fresh growth was brown. At a concentration of 100 mg l^{-1}, none of the calli showed fresh growth (Plate 10).

At 25 mg l^{-1} of kanamycin 22.2 per cent of the leaves showed callus induction. There was no induction at 50 mg l^{-1} and above concentrations.

4.6.1.6 Antibiotic sensitivity-6

Callus sensitivity to freshly prepared kanamycin at concentrations of 100 mg l^{-1} and above when subcultured at an interval of 8 days was tested. The data after nine weeks is given in Table 14. Fresh growth of calli was not seen in any of the concentration of kanamycin tested. Thus, kanamycin at a concentration of 100 mg l^{-1} and above completely suppressed callus growth.

4.6.2 Test for bacteriocidal activity of cefotaxime

The experiment was done to determine the sensitivity of *A. tumefaciens* strain AGL-1; 1303 to cefotaxime in pure bacterial suspension culture at an $O.D_{560nm}$ = 1.09 The data is given in Table 15. Both the doses of 500 and 1000 mg l^{-1} of cefotaxime tested were effective in killing the bacteria.

4.6.3 Effect of proline as a stimulant to *A. tumefaciens* strain AGL-1; 1303

Data on the effect of proline on multiplication of the bacteria is presented in Table 16. A slight increase in bacterial density was observed when

Table 13. Sensitivity of leaf and callus to varying concentrations of freshly prepared kanamycin

Explant	Dosage (mg l^{-1})	Percentage of explant showing growth	Appearance of the fresh growth
Leaf	25	22.2	Brown
Leaf	50	0	-
Leaf	75	0	-
Leaf	100	0	-
Callus	25	55	Brown
Callus	50	66	Brown
Callus	75	44	Brown
Callus	100	0	-
Leaf	Control	44	White
Callus	Control	77	White

Culture duration : 12 weeks
Subculturing at 2 weeks interval

Table 14. Sensitivity of callus to varying concentrations of freshly prepared kanamycin when subcultured at 8 days interval

Dosage (mg l^{-1})	Percentage of calli showing fresh growth
100	0
125	0
150	0
200	0

Culture duration : 9 weeks

Table 15. Bacteriocidal effect of cefotaxime on *Agrobacterium tumefaciens* strain AG L-1; 1303 at two different concentration

Concentration of cefotaxime (mg l^{-1})	Bacterial growth noticed	Inference
500	No	Bacteria completely dead
1000	No	Bacteria completely dead
Nil	Yes	Live bacteria present

Table 16. Effect of proline on multiplication of *Agrobacterium tumefaciens* strain AGL-1;1303

Sl. No.	Volume of regeneration medium	Concentration of proline (mg l^{-1})	Volume of bacterial suspension added (ml)	Incubation (h)	Bacterial density developed ($O.D_{560nm}$)
1	a) 25 ml	200	2.5	48	1.00
	b) 25 ml	0	2.5		1.00
2	a) 25 ml	200	2.5	58	1.20
	b) 25 ml	0	2.5		1.20
3	a) 20 ml	300	2.0	48	1.44
	b) 20 ml	200	2.0		1.43
4*	a) 25 ml	300	2.5	48	1.40
	b) 25 ml	0	2.5		0.50
5	a) 25 ml	400	2.5	48	1.26
	b) 25 ml	0	2.5		0.98
6	a) 25 ml	400	2.5	54	1.30
	b) 25 ml	0	2.5		1.02
7	a) 20 ml	500	1.0	24	0.80
	b) 20 ml	0	1.0		0.74
8	a) 25 ml	500	2.5	48	1.30
	b) 25 ml	0	2.5		1.00
9	a) 30 ml	500	3.0	48	1.20
	b) 30 ml	0	3.0		1.00
10	a) 25 ml	500	2.5	54	1.50
	b) 25 ml	0	2.5		1.10

*Regeneration medium used here was MS + BA 3 mg l^{-1} + NAA 2 mg l^{-1} + 2,4-D 1 mgl^{-1}

proline at 300 mg l^{-1} or above concentrations were added to the regeneration medium than when it was not.

4.6.4 Experiments on transformation

4.6.4.1 Leaf transformation experiment

4.6.4.1.1 Leaf transformation-I

The data on leaf transformation-I after a culture period of four and a half months in the screening medium is given in Table 17.

It was observed that 31.7 per cent of the explants developed bacterial overgrowth on withdrawal of cefotaxime from the screening media. The remaining explants were all dead with no callus induction. The control explants put in regeneration medium containing antibiotics did not show callus formation as was expected.

4.6.4.1.2 Leaf transformation-II

The data in Table 18 show that except for treatment-2, bacterial overgrowth was seen in all the other three treatments ranging from 33 to 55 per cent on withdrawal of cefotaxime. None of the explant in the four treatments showed callus induction on leaf in the screening medium. The leaf explants were dead within three months period. The control with antibiotics did not produce callus in any of its explants while the one without antibiotics produced callus in 55 per cent of the cultures.

4.6.4.1.3 Leaf transformation-III

The data on leaf transformation-III is given in Table 19. In none of the four treatments the leaf explants showed bacterial overgrowth. Callus induction in screening medium was not seen in any of the leaf explants in all the four experiments. The controls were normal, the control with antibiotics arresting

Table 17. Experimental details and results obtained in leaf transformation I

Treatment	Details of treatments given										Results		
	Preculture (days)	Induction medium		Induction time (min)	Co-culture time (days)	Concentration of antibiotics in the screening medium				Day of withdrawal of cefotaxime from screening media	Percentage of explants with bacterial overgrowth on withdrawal of cefotaxime	Percentage of explants producing callus	Nature of the explants
		Attractants: Acetosyringone ($mg\ l^{-1}$)	Bacterial density ($O.D_{560nm}$)			Cefotaxime ($mg\ l^{-1}$)	Kanamycin ($mg\ l^{-1}$)	Hygromycin ($mg\ l^{-1}$)	Carbenicillin ($mg\ l^{-1}$)				
1	4	20	0.25	30	3	500	50	20	100	42^{nd}	31.7	Nil	All dead
Control with antibiotics	4	20	-	30	-	500	50	20	100	42^{nd}	-	Nil	All dead

Table 18. Experimental details and results obtained in leaf transformation II

Treatments	Details of treatments given												Results		
	Preculture (days)	Induction medium			Induction time (min)	Attractants in co-cultivation medium (Proline mg l^{-1})	Co-cultivation time (days)	Concentration of antibiotics in the screening medium				Day of withdrawal of cefo	Percentage of explants with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of the explants
		Attractants		Bacterial density ($O.D_{560nm}$)				Cefo (mg l^{-1})	Kana (mg l^{-1})	Hygro (mg l^{-1})	Carb (mg l^{-1})				
		Proline (mg l^{-1})	Aceto (mg l^{-1})												
1	1	100	20	0.3	15	100	2	500	50	20	100	28^{th}	33	Nil	All dead
2	4	0	0	0.3	15	100	2	500	50	20	100	28^{th}	Nil	Nil	All dead
3	4	100	0	0.3	15	100	2	500	50	20	100	28^{th}	55	Nil	All dead
4	4	200	10	0.3	15	100	2	500	50	20	100	28^{th}	44	Nil	All dead
Control with antibiotics	4	0	0	-	15	100	-	500	50	20	100	28^{th}	Nil	Nil	All dead
Control without antibiotics	4	0	0	-	15	100	-	Nil	Nil	Nil	Nil	-	Nil	55	55% callusing

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Table 19. Experimental details and results obtained in leaf transformation III

Treatments	Details of treatments given													Results		
		Induction medium				Attractants in co-cultivation medium			Concentration of antibiotics in the screening medium							
		Attractants														
	Preculture (days)	Proline (mg l^{-1})	Aceto (mg l^{-1})	Bacterial density ($O.D_{560nm}$)	Induction time (min)	Proline (mg l^{-1})	Aceto (mg l^{-1})	Co-cultivation time (days)	Cefo (mg l^{-1})	Kana (mg l^{-1})	Hygro (mg l^{-1})	Carb (mg l^{-1})	Day of withdrawal of cefo	Percentage of explants with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of explants
1	3	200	30	0.26	15	100	10	2	500	50	20	100	56^{th}	Nil	Nil	All dead
2	3	300	20	0.26	15	100	10	2	500	50	20	100	56^{th}	Nil	Nil	All dead
3	3	200	20	0.19	15	100	10	2	500	50	20	100	56^{th}	Nil	Nil	All dead
4	3	100	30	0.19	15	100	10	2	500	50	20	100	56^{th}	Nil	Nil	All dead
Control with antibiotics	3	300	20	-	15	-	-	-	500	50	20	100	56^{th}	Nil	Nil	All dead
Control without antibiotics	3	300	20	-	15	-	-	-	-	-	-	-	56^{th}	Nil	60	60% callusing

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

induction of callus by leaf explants and control without antibiotics producing callus on 60 per cent of leaf explants.

4.6.4.1.4 Leaf transformation-IV

After a culture duration of three months in the screening media the results of leaf transformation-IV were recorded and is presented in Table 20. As in leaf transformation-III, none of the leaf explants in any of the four experiments produced callus in the screening medium. Bacterial overgrowth was also not seen on withdrawal of cefotaxime in any of the explants in this experiment. The leaf explants were all dead by three months. The control with antibiotics did not produce callus in any of the explants, whereas, the control without antibiotics produced callus in 42 per cent of the explants.

4.6.4.1.5 Leaf transformation-V

As with leaf transformation III and IV, here also none of the explants in any of the four experiments produced callus in the screening medium (Table 21). Bacterial overgrowth was not seen in any of the four experiments. Control with antibiotics was normal with none of the leaf explants producing callus. However, in control without antibiotics, against the expectation, none of the leaf explants callused. All the leaf explants in the four treatments were dead at the end of three months.

4.6.4.1.6 Leaf transformation-VI

The data of leaf transformation-VI is presented in Table 22. All the explants in the two treatments produced bacterial overgrowth 10 to 12 days after withdrawal of cefotaxime from the screening medium. The control with antibiotics did not produce callus, whereas, control without antibiotics showed 50 per cent of the explants callusing profusely.

Table 20. Experimental details and results obtained in leaf transformation IV

Treatments	Details of treatments given											Results		
	Preculture (days)	Induction medium			Induction time (min)	Attractants in co-cultivation medium		Co-cultivation time (days)	Concentration of antibiotics in the screening medium		Day of withdrawal of cefo	Percentage of explants with bacterial overgrowth on withdrwal of cefo	Percentage of explants producing callus	Nature of explants
		Attractants		Bacterial density (OD_{560nm})										
		Proline (mg l^{-1})	Aceto (mg l^{-1})			Proline (mg l^{-1})	Aceto (mg l^{-1})		Cefo (mg l^{-1})	Kana (mg l^{-1})				
1	2	100	10	0.45	20	100	10	4	500	50	56^{th}	Nil	Nil	All dead
2	2	100	30	0.45	20	100	10	4	500	50	56^{th}	Nil	Nil	All dead
3	2	200	10	0.31	20	100	10	4	500	50	56^{th}	Nil	Nil	All dead
4	2	200	30	0.31	20	100	10	4	500	50	56^{th}	Nil	Nil	All dead
Control with antibiotics	2	200	30	-	20	100	10	4	500	50	56^{th}	Nil	Nil	All dead
Control without antibiotics	2	200	30	-	20	100	10	4	-	-	-	Nil	42.8	42.8% callusing

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Table 21. Experimental details and results obtained in leaf transformation V

Treatments	Details of treatments given										Results		
	Preculture (days)	Induction medium: Attractants: Proline (mg l^{-1})	Induction medium: Attractants: Aceto (mg l^{-1})	Induction medium: Bacterial density ($O.D_{560nm}$)	Induction time (min)	Co-cultivation time (days)	Concentration of antibiotics in the screening medium: Cefo (mg l^{-1})	Concentration of antibiotics in the screening medium: Kana (mg l^{-1})	Concentration of antibiotics in the screening medium: Hygro (mg l^{-1})	Day of withdrawal of cefo	Percentage of explant with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of explants
1	5	500	0	0.32	5	3	500	75	20	42^{nd}	Nil	Nil	All dead
2	5	500	10	0.54	30	3	500	75	20	42^{nd}	Nil	Nil	All dead
3	5	500	20	0.54	5	3	500	75	20	42^{nd}	Nil	Nil	All dead
4	5	500	30	0.32	30	3	500	75	20	42^{nd}	Nil	Nil	All dead
Control with antibiotics	5	500	30	-	30	3	500	75	20	42^{nd}	Nil	Nil	All dead
Control without antibiotics	5	500	30	-	30	3	-	-	-	-	-	Nil	All dead

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

4.6.4.1.7 Leaf transformation-VII

The results of this experiment recorded after 85 days are presented in Table 23. None of the explants in any of the two treatments showed callus induction in the screening medium. Bacterial overgrowth was also not seen in any of the two treatments on withdrawal of cefotaxime from the screening media. The two controls were normal as expected. The control with antibiotics did not show any form of growth, whereas, in control without antibiotics 60 per cent of the explants produced callus.

4.6.4.1.8 Leaf transformation-VIII

The observations on leaf transformation-VIII were recorded after two months of culture in screening media (Table 24). The controls were normal. The control with antibiotics showed callus induction in none of the explants, whereas, in control without antibiotics, 60 per cent of the explants showed callus induction. On withdrawal of cefotaxime from the screening medium, all the explants in treatment-1 and 50 per cent of the explants in treatment-2 showed bacterial overgrowth. The remaining 50 per cent of the explants in treatment-2 which did not show bacterial overgrowth were all dead by the end of two months.

4.6.4.1.9 Leaf transformation-IX

The two treatments in leaf transformation-IX showed bacterial overgrowth in all explants after the withdrawal of cefotaxime from the screening medium (Table 25). None of the explant in the two treatments produced callus in the screening medium. The control with antibiotics showed no signs of callus induction and were dead. The control without antibiotics showed callus induction in 80 per cent of the leaf explants.

4.6.4.1.10 Leaf transformation-X

As revealed by the data presented in Table 26, all the explants in the two treatments showed bacterial overgrowth on withdrawal of cefotaxime. The controls

Table 23. Experimental details and results obtained in leaf transformation VII

Treatments	Details of treatments given								Results		
	Preculture (days)	Induction medium		Induction time (min)	Co-cultivation period (days)	Concentration of antibiotics in the screening medium		Day of withdrawal of cefo	Percentage of explant with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of explants
		Proline (mg l^{-1})	Bacterial density ($O.D_{560nm}$)			Cefo (mg l^{-1})	Kana (mg l^{-1})				
1	3	0	0.5	3	2	500	50	37th	Nil	Nil	All dead
2	3	300	1.4	3	2	500	50	37th	Nil	Nil	All dead
Control with antibiotics	3	300	-	3	2	500	50	37th	Nil	Nil	All dead
Control without antibiotics	3	300	-	3	2	-	-	-	-	60	60% explants callusing

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Table 24. Experimental details and results obtained in leaf transformation VIII

Treatments	Details of treatments given								Results		
	Preculture (days)	Induction medium		Induction time (min)	Co-cultivation period (days)	Concentration of antibiotics in the screening medium		Day of withdrawal of cefo	Percentage of explant with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of explants
		Proline (mg l^{-1})	Bacterial density ($O.D_{560nm}$)			Cefo (mg l^{-1})	Kana (mg l^{-1})				
1	32	0	1.0	5	2.5	500	50	52nd	100	Nil	Bacterial overgrowth in all explants
2	32	200	1.0	5	2.5	500	50	52nd	50	Nil	50% of explants without bacterial overgrowth were all dead
Control with antibiotics	32	200	-	5	2.5	500	50	52nd	Nil	Nil	All dead
Control without antibiotics	32	200	-	-	-	-	-	-	-	60	60% explants callusing

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Table 25. Experimental details and results obtained in leaf transformation IX

Treatments	Details of treatments given								Results		
	Preculture (days)	Induction medium		Induction time (min)	Co-cultivation period (days)	Concentration of antibiotics in the screening medium		Day of withdrawal of cefo	Percentage of explant with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of explants
		Proline (mg l^{-1})	Bacterial density ($O.D_{560nm}$)			Cefo (mg l^{-1})	Kana (mg l^{-1})				
1	4	0	0.98	3	3	500-1500	50	42nd	100	Nil	Bacterial overgrowth in all explants
2	4	400	1.25	3	3	500-1500	50	42nd	100	Nil	Bacterial overgrowth in all explants
Control with antibiotics	4	400	-	3	3	500-1500	50	42nd	Nil	Nil	All dead
Control without antibiotics	4	400	-	-	-	-	-	-	-	80	80% explants callusing

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Table 26. Experimental details and results obtained in leaf transformation X

Treatments	Details of treatments given								Results		
	Preculture (days)	Induction medium		Induction time (min)	Co-cultivation period (days)	Concentration of antibiotics in the screening medium		Day of withdrawal of cefo	Percentage of explant with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of explants
		Proline (mg l^{-1})	Bacterial density ($O.D_{560nm}$)			Cefo (mg l^{-1})	Kana (mg l^{-1})				
1	5	0	1.02	3	2	500	50	41st	100	Nil	Bacterial overgrowth in all explants
2	5	400	1.30	3	2	500	50	41st	100	Nil	Bacterial overgrowth in all explants
Control with antibiotics	5	400	-	3	2	500	50	41st	Nil	Nil	All dead
Control without antibiotics	5	400	-	3	2	-	-	-	-	100	All the explant showed callus initiation

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

were normal. The control with antibiotics had no callus induction, whereas, the control without antibiotics showed callus initiation in 100 per cent of the explants.

4.6.4.1.11 Leaf transformation-XI

All the explants in both the treatments developed bacterial overgrowth within two weeks of withdrawal of cefotaxime from the screening medium (Table 27). None of the explants in the two treatments produced callus in the screening medium. The control without antibiotics had 100 per cent callus initiation. The control with antibiotics did not produce callus in any of the explants.

4.6.4.2 Callus transformation experiments

4.6.4.2.1 Callus transformation-I

The results of callus transformation-I are given in Table 28. Very high phenol exudation was seen in all the four treatments, compared to the two controls, for the first six days in the screening medium (Plate 11a and 11b). On withdrawal of cefotaxime from screening medium on the 42nd day, heavy overgrowth of bacteria was seen in calli of treatment-1 and treatment-3 (61 and 53 per cent, respectively). Moderate bacterial overgrowth of 13 and 23 per cent was also seen in treatment-2 and treatment-4, respectively. However, none of the calli, which did not show bacterial over growth, produced fresh callus in the screening medium in any of the four treatments (Plate 12). The control with antibiotics did not produce fresh callus. In the control without antibiotics, 66 per cent of the calli produced fresh growth.

4.6.4.2.2 Callus transformation-II

Heavy bacterial overgrowth was observed in this experiment (Table 29). The bacterial overgrowth on withdrawal of cefotaxime from screening media was almost 100 per cent for treatment 3 and 4, followed by 81 per cent in treatment-2 and 40 per cent in treatment-1. One of the calli in treatment-4 had put forth fresh callus growth in the screening medium. However, later it died due to fungal

Table 27. Experimental details and results obtained in leaf transformation XI

Treatments	Details of treatments given								Results		
	Preculture (days)	Induction medium		Induction time (min)	Co-cultivation period (days)	Concentration of antibiotics in the screening medium		Day of withdrawal of cefo	Percentage of explant with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of explants
		Proline (mg l^{-1})	Bacterial density ($O.D_{560nm}$)			Cefo (mg l^{-1})	Kana (mg l^{-1})				
1	32	0	1.0	3	2	500/ 1000	50	40th	100	Nil	Bacterial overgrowth in all explants
2	32	500	1.3	3	2	500/ 1000	50	40th	100	Nil	Bacterial overgrowth in all explants
Control with antibiotics	32	500	-	3	2	500/ 1000	50	40th	Nil	Nil	All dead
Control without antibiotics	32	500	-	-	-	-	-	-	-	100	All explants with signs of callus initiation

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Table 28. Experimental details and results obtained in callus transformation I

Treatments	Details of treatments given										Results		
	Preculture (days)	Induction medium		Induction time (min)	Co-cultivation period (days)	Concentration of antibiotics in the screening medium			Phenol exudation during first six days in screening medium	Day of withdrawal of cefo	Percentage of explants with bacterial overgrowth on withdrawal of cefo	Percentage of explants producing callus	Nature of explants
		Proline (mg l^{-1})	Bacterial density ($O.D_{560nm}$)			Cefo (mg l^{-1})	Kana (mg l^{-1})	Hygro (mg l^{-1})					
1	3	0	0.75	5	3	500	50	20	Very high	42nd	61.5	Nil	All dead
2	3	0	0.75	15	3	500	50	20	Very high	42nd	13.3	Nil	All dead
3	3	500	0.80	5	3	500	50	20	Very high	42nd	53.3	Nil	All dead
4	3	500	0.80	15	3	500	50	20	Very high	42nd	23.07	Nil	All dead
Control with antibiotics	3	500	-	-	-	500	50	20	Normal	42nd	Nil	Nil	All dead
Control without antibiotics	3	500	-	-	-	-	-	-	Normal	-	-	66	66% calli with fresh growth

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Table 29. Experimental details and results obtained in callus transformation - II

Treatments	Details of treatments given										Results		
	Induction medium		Induction time (min)	Co-cultivation medium	Co-cultivation period (days)	Concentration of antibiotics in screening medium			Phenol exudation during first six days in screening medium	Day of withdrawal of cefo	Percentage of calli with bacterial overgrowth on withdrawal of cefo	Percentage of calli producing fresh growth	Nature of calli
	Proline (mg l^{-1})	Bacterial density (OD_{560nm})		Proline (mg l^{-1})		Cefo (mg l^{-1})	Kana (mg l^{-1})	Hygro (mg l^{-1})					
1	200	1.43	5	200	3	500	50	20	Very high	38^{th}	40	6	1 out of 15 calli showed fresh growth, which did not grow further. Others all dead
2	200	1.43	30	200	3	500	50	20	Very high	38^{th}	81	Nil	All dead
3	300	1.45	5	200	3	500	50	20	Very high	38^{th}	100	Nil	100% bacterial overgrowth
4	300	1.45	30	200	3	500	50	20	Very high	38^{th}	100	5	1 out of 17 calli showed fresh growth before withdrawal of cefo. Later it was contaminated by fungus and died. On withdrawal of cefo all the explant showed bacterial overgrowth.
Control with antibiotics	300	-	-	-	-	500	50	20	Normal	38^{th}	Nil	Nil	All dead
Control without antibiotics	300	-	-	-	-	-	-	-	Normal	-	-	100	All calli grew profusely

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

contamination. Fresh growth was also seen in one of the calli in treatment-1 which failed to grow further. The control with antibiotics did not show any fresh growth. In the control without antibiotics, all the calli grew profusely.

4.6.4.2.3 Callus transformation-III

The data on callus transformation-III is given in Table 30. When the calli were cultured in screening medium with 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime, one out of five calli (20%) in control with antibiotics showed fresh growth by 14th day. Hence, the level of kanamycin in screening medium was raised to 100 mg l^{-1}. Treatment-2 was contaminated by fungus growth and removed. Cefotaxime was withdrawn from screening medium on 58th day. By 65th day bacterial overgrowth was seen in all the calli of treatment-3 and 4. Treatment-1 did not show bacterial overgrowth. However, all the calli in treatment-I were dead and no fresh growth noticed. The control with antibiotics showed no fresh growth once the level of kanamycin was raised to 100 mg l^{-1}. The control without antibiotics showed fresh growth in 60 per cent of the calli used.

4.6.4.2.4 Callus transformation-IV

The results of this experiment is given in Table 31. The control with antibiotics did not show fresh growth in any of the calli. The control without antibiotics showed fresh growth in 75 per cent of the calli. No fresh growth was seen in any of the four treatments, including treatment-4, where calli after infection were cultured in regeneration medium containing cefotaxime alone. The increase of concentration of cefotaxime in the screening medium on 26th day from 500 mg l^{-1} to 1000 mg l^{-1} led to the formation of a thick bacterial scum on top of all calli in treatment-3 and on some calli in treatment-1 and 2. However, bacterial growth was not seen visually in treatment-4. This indicated the presence of live bacteria in the callus tissues, though they did not proliferate in the medium containing cefotaxime. The calli in all the four treatments were completely dark

Table 30. Experimental details and results obtained in callus transformation III

Treatments	Details of treatments given									Results		
	Wounded/Unwounded (W/UW)	Induction medium		Induction time (min)	Co-cultivation period (days	Concentration of antibiotics in the screening medium		Percentage of calli showing fresh growth on 14th day	Concentration of Kanamycin in screening medium increased to 100 mg l^{-1} from 14th day onwards	Day of withdrawal of cefo	Observation on 65th day		
		Proline (mg l^{-1})	Bacterial density (OD_{560nm})			Cefo (mg l^{-1})	Kana (mg l^{-1})				Percentage of calli with bacterial overgrowth on withdrawal of cefo	Percentage of calli producing fresh growth	Nature of calli
1	W	100	0.5	5	4	500	50	Nil		58th	Nil	Nil	All dead
2	UW	100	0.5	5	4	500	50	Contaminated by fungus		-	-	-	-
3	W	400	0.5	5	4	500	50	Nil		58th	100	Nil	100% of calli with bacterial overgrowth
4	UW	400	0.5	5	4	500	50	Nil		58th	100	Nil	100% of calli with bacterial overgrowth
Control with antibiotics	W	400	-	-	-	500	50	20		58th	Nil	Nil	All dead
Control without antibiotics	W	400	-	-	-	-	-	60		-	-	60	60% of calli grew profusely

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Table 31. Experimental details and results obtained in callus transformation IV

Treatments	Details of treatments given								Results	
	Wounded/ Unwounded (W/UW)	Induction medium		Induction time (min)	Co-cultivation period (days)	Concentration of antibiotics in the screening medium		Day on which concentration of cefo was increased from 500 mg l^{-1} to 1000 mg l^{-1}	Observation on 28th day	
		Proline (mg l^{-1})	Bacterial density ($O.D_{560nm}$)			Cefo (mg l^{-1})	Kana (mg l^{-1})		Bacterial presence	Percentage of calli with fresh growth
1	W	500	1.5	5	2	500	75	26th	Bacterial presence seen on top of few calli	Nil
2	W	0	1.0	5	2	500	75	26th	Bacterial presence seen on top of callus	Nil
3	UW	0	1.0	5	2	500	75	26th	Thick bacterial scum seen on top of all calli	Nil
4	W	0	1.0	5	2	500	0	26th	Bacterial presence not seen	Nil
Control with antibiotics	W	0	-	-	-	500	75	26th	-	Nil
Control without antibiotics	W	0	-	-	-	-	-	-	-	75% calli with fresh growth

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

resembling dead ones, except, in treatment-3, where, few calli had light coloured central portion and were producing excessive phenols.

4.6.4.2.5 Callus transformation-V

Callus transformation-V was laid to attempt complete elimination of *Agrobacterium* from callus tissues after co-cultivation, using high doses of cefotaxime. The results are given in Table 32. The control with antibiotics did not show any fresh growth, whereas, in control without antibiotics, 80 per cent of the calli produced fresh growth. Fresh growth was seen in 25 per cent of the calli in treatment-5 and 12.5 per cent in treatment-6 where screening with kanamycin was not done (Plate 13). The growth was seen before the withdrawal of cefotaxime. In treatment 1, 2, 3, and 4 where screening with kanamycin was resorted to, fresh growth was not noticed in any of the calli. All the experiments showed bacterial overgrowth on withdrawal of cefotaxime.

Table 32. Experimental details and results obtained in callus transformation V

Treatments	Details of treatments given									Results		
	Induction medium		Induction time (min)	Coculture period (days)	Air drying after co-cultivation (min)	Pre-selection period (days)	Concentration of antibiotics in the screening medium		Day of withdrawal of cefo	Percentage of calli with bacterial overgrowth on withdrawal of cefo	Percentage of calli producing fresh growth	Nature of calli
	Proline (mg l^{-1})	Bacterial density (OD_{560nm})					Cefo (mg l^{-1})	Kana (mg l^{-1})				
1	500	1.2	3	1	Nil	1	1500	100	19th	100	Nil	100% calli with bacterial overgrowth
2	500	1.2	3	2	30	Nil	1000	100	15th	100	Nil	100% calli with bacterial overgrowth
3	0	1.0	3	2	Nil	Nil	1500	100	18th	100	Nil	100% calli with bacterial overgrowth
4	0	1.0	3	1	Nil	1	1000	100	16th	100	Nil	100% calli with bacterial overgrowth
5	0	1.0	3	2	30	-	1500	Nil	15th	100	25	25% calli showed fresh growth before withdrawal of cefo. After withdrawal of cefo, bacterial overgrowth was seen in all the calli
6	0	1.0	3	1	Nil	-	1000	Nil	13th	100	12.5	12.5% calli showed fresh growth before withdrawal of cefo. After withdrawal of cefo, bacterial overgrowth seen in all the calli
Control with antibiotics	0	-	-	-	-	-	1000	100	19th	-	Nil	No fresh growth
Control without antibiotics	0	-	-	-	-	-	-	-	-	-	80	Fresh growth seen in 80% of the calli

Kana – Kanamycin; Cefo – Cefotaxime; Carb – Carbenicillin; Hygro - Hygromycin

Discussion

5. DISCUSSION

The conventional breeding programmes in black pepper has not yielded much in the development of resistant/tolerant lines mainly because of the lack of resistance in the available cultivars. However, related wild species like *Piper colubrinum* and *Piper obliqum* have been reported to have certain desirable traits especially resistance to disease like foot rot disease (Purseglove *et al.*, 1981). Since there are limitations in utilizing the resistance found in these related wild species by the conventional methods of hybridization and selection, genetic engineering techniques could be made use of.

Genetic transformation is defined as the transfer of foreign genes isolated from plants, viruses, bacteria or animals into a new genetic background.

Of the different gene delivery systems available, *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. Eady, *et al.*, 2000). High frequency of transformation, broad host range and high rate of expression of inserted genes have made *Agrobacterium* based gene transfer system the most popular one.

Panniyur-1 is one of the most popular and high yielding varieties of black pepper cultivated in Kerala. In the present study, an attempt was made to standardize the process of *Agrobacterium tumefaciens* mediated genetic transformation in this variety of black pepper, with *A. tumefaciens* strain AGL-1; 1303 using selectable marker genes (*npt II* and *hpt IV*) and reporter genes (*GUS* and *GFP*). The results of the studies conducted are discussed in this chapter.

5.1 Production of axenic seedlings for transformation

Axenic seedlings were raised by germinating seeds collected from ripe berries under *in vitro* conditions to generate the explants, both cotyledonary leaves

and callus, with reduced contamination for use in transformation works and related studies.

Germination of seeds was quicker in sterilized moist sand compared to the other media tested. Percentage recovery of seedlings was, however, much less than the percentage germination in all the three germination media. In moist sand, which gave the highest percentage of germination (73.89%), only 57.26 per cent of seedlings were recovered (Fig. 2). This was because of the fungal contamination which persisted in the sand even after double sterilization. Recovery of seedlings in liquid SH medium and semisolid modified MS medium (9.25 and 1.6%, respectively) was also very much less compared to the seeds germinated. In both the media poor growth of the seedlings resulted in low recovery of good seedlings. The germinating seeds in the two media often showed symptoms of drying at various stages of growth. The poor germination of seeds and recovery of seedlings noticed in the three tissue culture media could be due to the poor absorption of water by seeds / germinating seeds which is related to the difference in osmotic potential. The comparatively better performance seen in liquid SH medium than the other two agar solidified tissue culture media corroborates the observation. The relatively higher salt concentration in semisolid SH medium in comparison to half MS medium may be the reason for the lack of seed germination in the former. Sterilized moist sand on the contrary, seems to provide the most favourable conditions for seed germination and further growth of seedlings in *P. nigrum* L. probably due to availability of sufficient moisture and aeration. However, normal seed germination in tissue culture medium has been reported in *Hopea parviflora* (Sunilkumar *et al.*, 2000).

5.2 Callus induction and growth rate

Diverse plant tissues have been used for *Agrobacterium* mediated genetic transformation works. In fact, one of the distinct advantages of

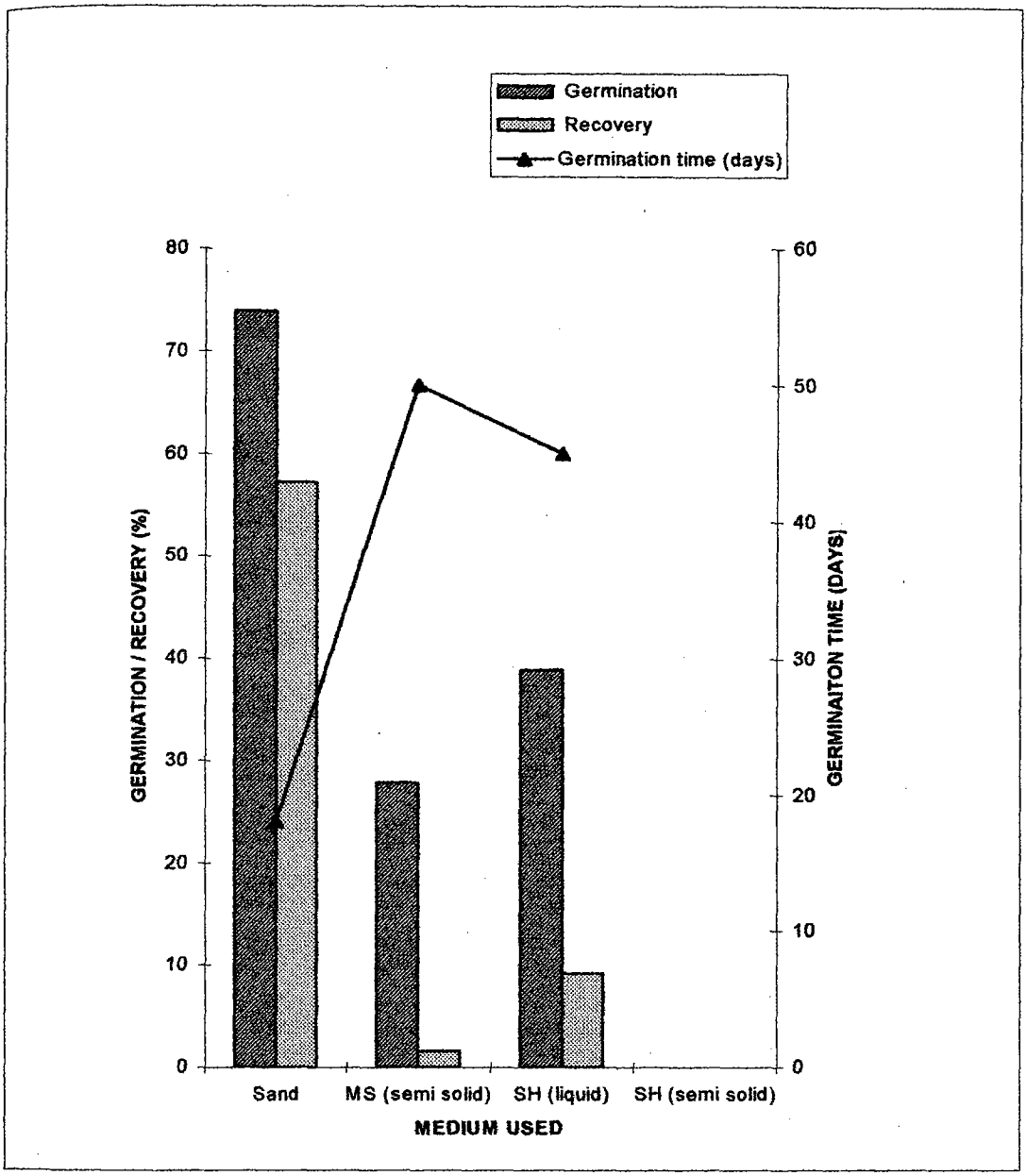


FIG. 2 GERMINATION OF SEEDS AND RECOVERY OF SEEDLINGS OF *PIPER NIGRUM* UNDER *IN VITRO* CONDITION

Agrobacterium tumefaciens transformation system, is the wide spectrum of explants that can be used for transformation.

Calli have been used for transformation work in a number of crops like *Heavea* (Arokiaraj *et al.*, 1996), *Phaseolus acutifolius* A Gray (Dillen *et al.*, 1997) and *Phalaenopsis* (Belarmino and Mii, 2000). In the present study also callus was used as one of the explants for transformation because of its availability throughout the year.

For the production of callus during the present study, leaf cotyledonary explants from axenic seedlings were cultured in half MS medium supplemented with two different combinations of IAA and BA, viz., 1.5 mgl^{-1} IAA + 1.0 mg l^{-1} BA and 1.0 mg l^{-1} each of IAA and BA. Percentage of callusing was much higher in the growth regulator combination of 1.0 mgl^{-1} each of IAA and BA (34.3%) compared to the other (24.3%). Similarly, the callus index, which is an indicator of callus growth, was much higher (97.7%) under this combination.

Variation in induction and growth of callus in black pepper cultured in half MS medium with varying combinations of IAA and BA has been reported by Nazeem *et al.* (1990) and Philip *et al.* (1995). They have observed that the combination of 1.0 mgl^{-1} each of IAA and BA is the best.

5.3 Multiplication of callus (Secondary callus)

The primary callus produced by culturing leaf explants, were cut and cultured in their respective media (half MS supplemented with 1.5 mgl^{-1} IAA + 1.0 mg l^{-1} BA or 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA) and multiplied for use in transformation and related works.

The response of cultured tissue depends on both the concentration ratio and absolute concentration of auxins and cytokinins. The concentration of auxins and cytokinins are equally as important as their ratio. Callus initiation generally

occurs when the ratio of auxins to cytokinins is high (Torres, 1989). However, the interaction is much more complicated and it is not known as to how exactly the plant cells perceive auxins:cytokinin ratio.

In the present study, establishment of the cut callus in both the media was 100 per cent i.e. all the calli cut and cultured in their respective media showed growth (Table 6). But the rate of growth was higher in half MS supplemented with 1.0 mg l^{-1} each of IAA and BA compared to supplementation with 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA. This is indicated by the callus index which is 276 for the former and 208.3 for the latter combination.

5.4 Regeneration

The ratio of auxins to cytokinins influences the balance between root and shoot organogenesis from cultured tissues. Generally, a high auxin relative to cytokinin favours root formation and the converse situation favours shoot formation (Warren, 1991).

MS medium supplemented with the auxin, IAA and the cytokinin, BA at 1.0 mg l^{-1} each was found to be most effective for both callus induction from cotyledonary leaves as well as callus mediated regeneration in black pepper (Nazeem *et al.*, 1990; Shylaja, 1996). In this study, regeneration to the extent of 3.0 per cent was seen in the treatment combination where callus cultured in half MS medium supplemented with 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA were transferred to half MS medium supplemented with 1.0 mg l^{-1} each of IAA and BA. The regenerating calli when cut and cultured in the same media, retained the organogenetic potential and produced new shoots. Regeneration was not noticed in any other treatment combinations tried (Table 7).

The failure in obtaining regeneration from callus in half MS supplemented with 1.0 mg l^{-1} each of IAA and BA as reported by Nazeem *et al.*

(1990) and Shylaja (1996) to the extent of 30 per cent during third subculture may be due to subtle differences in cultural conditions.

Philip *et al.* (1995) had reported that regeneration from callus was best when it was cultured in half MS supplemented with 1.0 mg l^{-1} IAA + 2.0 mg l^{-1} BA. They had used 13 genotypes which did not include Panniyur-1. The lack of morphogenetic response noticed in this study in the treatment combination reported by Philip *et al.* (1995) can possibly be attributed to the genotypic variation of the material. Variation in culture response exhibited by different genotypes within a species has been reported in *Avena* spp. (Gana *et al.*, 1995).

Callus establishment also varied considerably depending on the growth regulator combinations. The three combinations containing 0.5 mgl^{-1} kinetin along with different levels of IAA and BA gave a poor establishment of callus (0 to 75%). All other growth regulator combinations lacking kinetin gave almost 100 per cent establishment, revealing that kinetin did not favour callus growth and regeneration in this species.

5.5 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 chaemeric plants is avoided. Secondly, transformed embryogenic calli, if obtained, can be multiplied rapidly and thousands of transformed plants can be raised within a short period of time.

Attempt was made to generate embryogenic calli by culturing zygotic embryo in liquid basal SH medium as reported by Joseph *et al.* (1996). Certain modifications in cultural conditions were also tried. A total of 1012 static/shake cultures using three different explant in different media combinations were

incubated in light and dark condition for almost an year with bimonthly subculturing.

Embryo with endosperm cultured in liquid SH medium in 60 ml test tubes supported by filter paper bridge gave almost similar germination percentage when incubated in light and in dark conditions (70.45 and 69%, respectively). Embryo with endosperm in semisolid basal SH medium exhibited similar response whether incubated in dark or in light but the growth in semisolid SH medium was less compared to the growth in liquid SH medium. Leaf production was seen only when embryos with endosperm were cultured in liquid basal SH medium and incubated in light, probably because chlorophyll development takes place in light which in turn sustains the growth and development of leaves. In all the above cases germination was seen in 14 days period.

Embryo along with endosperm and a part of perisperm (about 1/3 of the seed) failed to germinate in liquid basal SH medium under light conditions whereas germination (55.5%) was noticed when inoculated in dark conditions. Evidences of light inhibiting germination are rarely seen. A possible explanation to this observation could not be given.

When embryo alone, taken out of the endosperm, was cultured in liquid basal SH medium and incubated in dark, a very low percentage of germination (11.11%) was noticed probably because of the lack of nourishment support from the endosperm and desiccation of the explants.

The time taken for germination was around 25 days in both the cases where 1/3rd seed or when embryo alone was cultured.

2,4-D either alone or in combination with other growth regulator is generally used in the media to induce rapid cell proliferation, callus production, somatic embryogenesis and organogenesis. It was successfully used in somatic embryogenesis in *Pennisetum glaucum* (Lambe *et al.*, 1999) and *Manihot*

esculenta Crantz (Raemakers *et al.*, 1993). However, in the present study, 2,4-D only hampered the germination. Except the lowest concentration of 0.5 mg l^{-1} 2,4-D, all the other doses above 0.5 mg l^{-1} did not induce germination. In all the cases of 2,4-D application, the endosperm tissue seemed to multiply forming big callus mass which dried later. There was no sign of embryogenic calli development in any of the 2,4-D combinations attempted.

When embryos with endosperm were cultured on rotary shaker in liquid basal SH medium, one of the young seedlings subsequently gave rise to seven embryo-like structures from the junction of root and shoot, one after the other, each of which developed into a normal seedling. However, embryogenic calli failed to appear. The production of multiple seedlings from the root – shoot junction of a single seedling germinated from an embryo with endosperm and the nature of its development raises suspicion of them being produced from somatic embryos.

Joseph *et al.* (1996) had reported obtaining embryogenic calli in black pepper, when zygotic embryos were cultured in liquid SH medium and incubated in dark under static condition. He had obtained embryogenic calli in black pepper variety Karimunda (personal communication). In the present study, only variety Panniyur–1 was tried. The study showed that embryos with endosperm had a positive response to liquid basal SH medium and the suspected somatic embryos were even produced when embryos were cultured in liquid SH medium on rotary shaker. The failure in obtaining embryogenic calli as reported by Joseph *et al.* (1996) could be due to the genotypic variation present in the different varieties and also some subtle differences in cultural conditions which cannot be defined.

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. Col22 to nil in other lines. Similarly, in *Arachis hypogea*, Mc Kently (1995) found wide variations in the

capacity to produce somatic embryos among different species and even among different lines in the same species.

5.6 *Agrobacterium* mediated genetic transformation

5.6.1 Sensitivity of host tissues to antibiotics

Selection of 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. There are antibiotics to which plant cells are susceptible and genes conferring resistance to these antibiotics are generally used as selectable markers. Once the tissue is transformed with the antibiotic resistance genes, it is grown in the medium containing the antibiotic concerned. The cells which are transformed with the antibiotic resistance gene would multiply and grow normally, whereas, the untransformed cells are killed.

Several factors affect the efficiency of the antibiotics used as selection agent. The selection agent must be toxic to plant cells, though not so toxic that products from dying, untransformed cells kill adjacent transformed cells. Thus the most effective selection agents are those which either inhibit growth or slowly kill the untransformed cells. Optimal selection pressure will use the lowest level of antibiotic needed to kill untransformed tissues. This necessitates the evaluation of sensitivity of plant tissue to antibiotics and the minimum concentration of the antibiotics required to inhibit growth.

In the present study, sensitivity of leaf and callus of *Piper nigrum* L. variety Panniyur–1 to different antibiotics were evaluated. The evaluation was carried out with different doses of the antibiotics and varying methods of application.

Initially, callus sensitivity to single application of five antibiotics at different doses were tested. The antibiotic stock solutions were prepared earlier and stored under frozen condition. None of the antibiotics at the doses tested could

completely suppress the callus growth (Table 9). Only hygromycin at increased doses of 40 and 50 mg l^{-1} could partially suppress callus growth and to some extent by ampicillin at 200 and 500 mg l^{-1} concentration. Kanamycin, cefotaxime and rifampicin did not show any effect on callus growth (Fig. 3).

The suppression of callus growth with 10 mg l^{-1} hygromycin and 50 mg l^{-1} kanamycin when subcultured at two weeks interval was comparatively better (Table 10) than single application tried earlier. This may be because the antibiotic looses its activity with time in the medium and the tissue need to be subcultured on to fresh medium to realize its action.

When freshly prepared antibiotic stocks were used for treating the callus and also for supplementing the medium during subsequent subculturing, the sensitivity response of the calli was different from the earlier observations. About 30 per cent of the calli turned completely dark (and possibly dead) in the treatment with 50 mg l^{-1} kanamycin, within a short period of three weeks. This elicited the effectiveness of freshly prepared antibiotic stocks over the use of antibiotic stock stored under frozen condition.

Describing the method of storage of antibiotic stock, Barry (1976) brings to notice that antibiotic stocks can be stored at –20°C for a period of six months. However, he cautioned that the frozen stock once melted should be used immediately and that it should never be re-frozen. In our present circumstances, where there are regular power failures, the antibiotic stock kept under frozen condition may undergo many cycles of freezing and melting, which may go unnoticed, leading to the inactivity of the antibiotic stock. So, the longer the antibiotic stock is stored under frozen condition the more is its chances of being inactivated. Hence in the prevailing conditions it is better to use freshly prepared antibiotic stock unless uninterrupted power supply is assured for the freezing device.

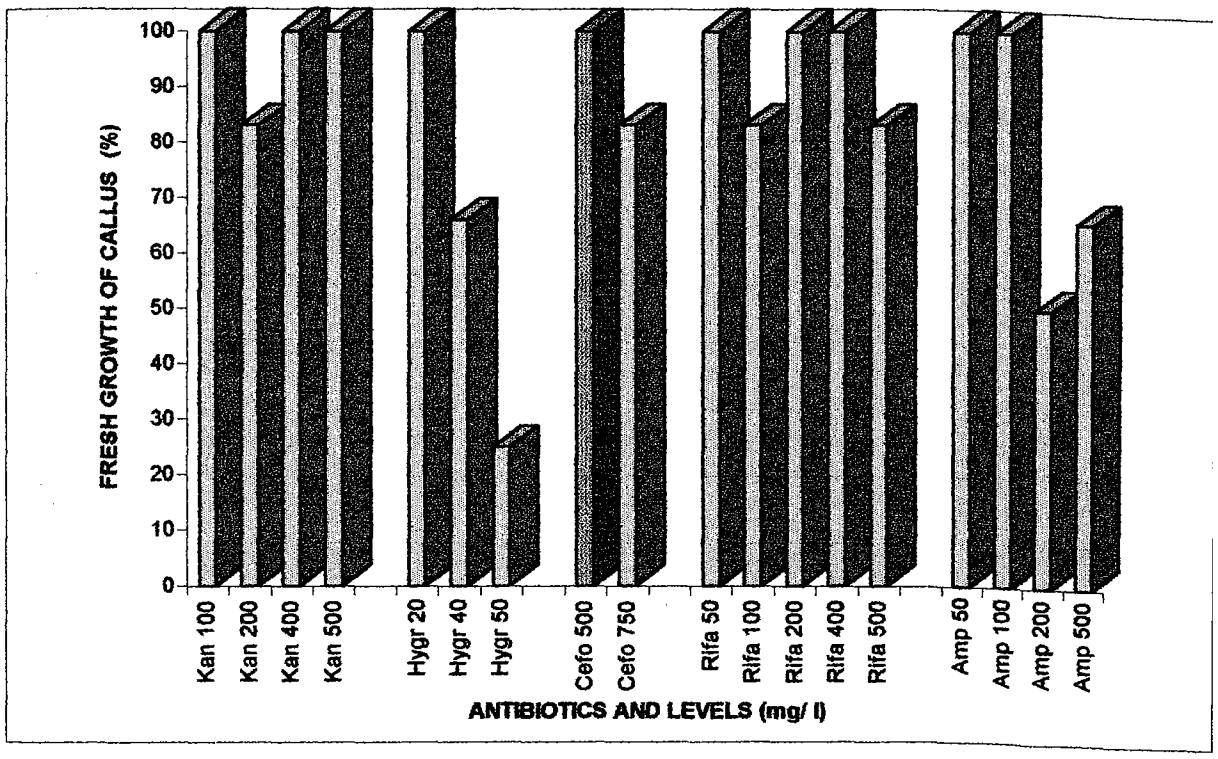


FIG. 3 SENSITIVITY OF BLACK PEPPER CALLUS TO VARYING LEVELS OF FIVE ANTIBIOTICS

Leaf and callus were found to show total inhibition of growth at different concentrations of hygromycin, when freshly prepared hygromycin was used and the tissue subcultured at an interval of two weeks. The leaves showed complete arrest of callus induction at a minimum dose of 10 mg l^{-1} hygromycin, whereas, callus showed complete arrest of fresh growth only at a minimum strength of 30 mg l^{-1} (Table 12, Fig. 4)). Since the genetic makeup of both leaf and callus tissues are the same, the differential dose requirement could be attributed to the fast dividing / multiplying state of the callus tissues as well as the difference in the cellular characteristics of differentiated (leaf) and undifferentiated (callus) tissues. Fast dividing cells may contain higher levels of vital enzymes which in turn may make the cells more resistant to antibiotic treatments.

The complete inhibition of callus induction on leaf at 10 mg l^{-1} hygromycin when freshly prepared antibiotic stock was used (Table 12) in comparison to 22.2 per cent leaf showing callus induction at the same dose of hygromycin stored under frozen condition (Table 10) clearly shows that activity of antibiotic was reduced on storage under frozen condition.

Similar to hygromycin, with kanamycin also callus required a higher dose than leaf for complete suppression of growth when freshly prepared stock solution was used and tissues subcultured at an interval of two weeks. Callus induction on leaf was completely inhibited at 50 mg l^{-1} kanamycin, whereas, 100 mg l^{-1} was required to prevent fresh growth of the callus (Table 13, Fig. 5).

When antibiotics hygromycin or kanamycin was administered in the medium, the fresh growth produced in callus was difficult to be recognized by colour as it readily turns brown. Fresh growth could be recognized only by the increase in size. Relatively lower percentage of callus showed growth at 10 mg l^{-1} hygromycin and 50 mg l^{-1} kanamycin when stocks stored under frozen condition were used (Table 10) compared to the callus growth with the same concentrations of the antibiotics when freshly prepared stocks were used (Table 12 and 13). This

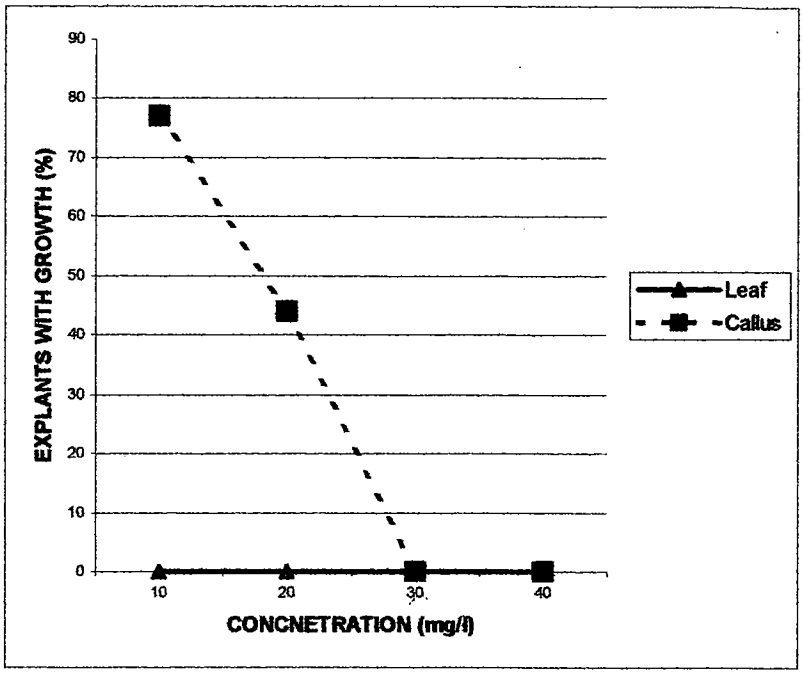


FIG. 4 SENSITYVITY OF LEAF AND CALLUS OF BLACK PEPPER TO FRESHLY PREPARED HYGROMYCIN

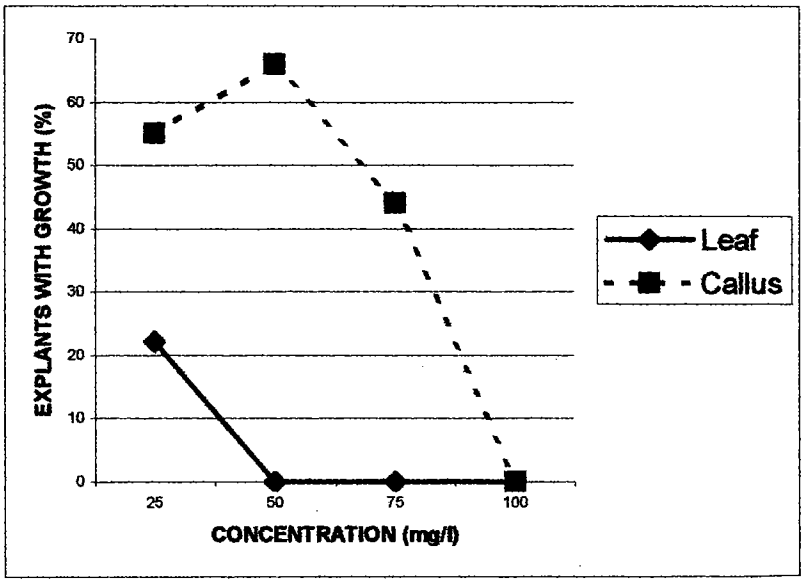


FIG. 5 SENSITIVITY OF LEAF AND CALLUS OF BLACK PEPPER TO FRESHLY PREPARED KANAMYCIN

could probably be because of the failure in recognition of fresh growth by size in the former case due to its short culture duration.

In order to confirm the response of callus to kanamycin, another trial was laid with 100 mg l^{-1} and above concentrations and reducing the subculturing interval to 8 days. No fresh growth was seen in any of the concentrations tried (Table 14) thereby confirming that a minimum dose of 100 mg l^{-1} kanamycin could suppress the callus growth completely.

Thus, the series of antibiotic sensitivity tests conducted under this study showed that black pepper tissues are susceptible to both hygromycin and kanamycin and the two can be used as selecting agent in the transformation experiments. Kanamycin may be added at a strength of 50 mgl^{-1} to the screening media when transformed leaf explants are screened, whereas, it should be added at a strength of 100 mgl^{-1} when transformed calli are screened. Hygromycin is required at a strength of 10 mg l^{-1} for screening transformed leaf explants, whereas, it will be required at a strength of 30 mg l^{-1} for screening transformed calli. In all the cases, the antibiotics should be prepared fresh and the explants should be subcultured at an interval of 14 days into the medium containing a fresh supply of the antibiotic.

Sasikumar and Veluthambi (1994) had reported that 50 mg l^{-1} kanamycin completely arrested callus induction on leaf segments of black pepper. This is in consensus with the findings in the present study. During successful 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. The present study also signifies the fact that the method/condition of storage of antibiotic stock is found to greatly influence its activity, thereby the threshold concentration.

Different strengths of the antibiotics have been used for selection in different crops. Kanamycin was used at a strength of 20 mg l^{-1} for selection during transformation in *Gerbera hybrida* (Nagaraju *et al.*, 1998) and at a strength of 200 mg l^{-1} in *Moricandia arvensis* (Rashid *et al.*, 1996). Similarly, hygromycin was used at a strength of 20 mg l^{-1} in *Robinia pseudoacacia* (Igasaki *et al.*, 2000) and at a strength of 256 mg l^{-1} in cowpea (Muthukumar *et al.*, 1996). This indicates that there is variation in the sensitivity / resistance to antibiotic depending on the genotype of the plants.

5.6.2 Test for bacteriocidal activity of cefotaxime

After co-cultivation of the plant tissue with the vector bacteria, a period during which the transfer of genes from bacteria to the plant tissue is expected to take place, the bacteria are to be killed. Further survival of the bacteria in the plant tissue may affect the growth and regeneration of the transformed tissue. Elimination of the *Agrobacterium* from the plant tissue is done using the optimum dose of an appropriate antibiotic. Hence, the antibiotic used for elimination of the bacteria and its concentration need to be standardized.

During this study, *Agrobacterium tumafaciens* strain AGL-1; 1303 was found to be effectively killed by cefotaxime at the strength of both 500 and 1000 mg l^{-1} when the antibiotic was added to pure bacterial cultures (Table 15). Hence, the lower strength of 500 mg l^{-1} was used for the elimination of the bacteria during transformation work to minimize the effect, if any, of the antibiotic on the growth and regeneration of the plant tissue.

Cefotaxime had been used successfully for elimination of *Agrobacterium* in transformation work in a number of crops. It was successfully used at a strength of 200 mg l^{-1} in eliminating the *Agrobacteruim* from the inoculated explant during transformation in white clover (Voisey *et al.*, 1994). In *Casuarina glauca* it was used at a strength of 250 mgl^{-1} for eliminating *Agrobacterium* (Le *et al.*, 1996). Cefotaxime was used at a concentration of 300

and 500 mg l^{-1} for eliminating *Agrobacterium* during transformation works in commercial melon (Valles and Lasa, 1994) and *Phalaenopsis* orchid (Belarmine and Mii, 2000), respectively.

5.6.3 Effect of proline as a stimulant to *Agrobacterium tumefaciens* strain AGL-1; 1303

Proline had been used possibly as an inducer during transformation works in rubber at RRII, Kottayam (personal communication). Hence, attempt was made to study the effect of proline in the transformation of black pepper by *Agrobacterium tumefaciens* strain AGL-1; 1303.

During the study it was found that addition of proline at the rate of 300 mg l^{-1} and above increased the rate of multiplication of the bacterium in liquid suspension compared to the absence of it (Table 16). The increase in the rate of multiplication in the presence of proline shows that proline has some favourable stimulating effect on the bacteria. However, the role of proline in improving the infectivity of the bacteria and the transfer of T-DNA from the bacteria to the plant genome is to be further studied.

5.6.4 Experiments on transformation

In the present study, *A. tumafaciens* strain AGL-1; 1303 was used for genetic transformation of black pepper with marker and reporter genes. The selectable marker genes present in the T-DNA are *npt II* (neomycin phospho-transferase II) gene and *hpt IV* (hygromycin phosphotransferase IV) gene which confers resistance to kanamycin and hygromycin, respectively. The reporter genes present are *GUS* (beta-glucuronidase) and *GFP* (green fluorescent protein) genes. Besides, the four genes mentioned above, the T-DNA also harbours a gene which confers resistance to carbenicillin and acts as a bacteria selection gene to prevent contamination of the bacterial strain. Once the T-DNA of the bacteria is transferred to the plant genome, the transformed plant cells are expected to develop resistance

to kanamycin and hygromycin and will be capable of normal growth in the medium containing these antibiotics, in contrast to the untransformed cells which are susceptible to these antibiotics. The transformed tissue thus developed will also show GUS activity which can be detected by various chemical tests and produce green fluorescence under ultra-violet light because of the activity of *GFP* gene.

During this experiment, the plant tissues after co-cultivation with *A. tumefaciens* were transferred to screening media, which consisted of regeneration media (Nazeem *et al.*, 1990) supplemented with cefotaxime, for elimination of the bacteria, kanamycin and hygromycin, for selection of transformed cells and in some cases, carbenicillin, to prevent contamination from other bacteria.

Initially, leaf transformation was carried out with low densities of *Agrobacterium* ranging from 0.19 to 0.54 ($O.D._{560nm}$). Cefotaxime was used at a concentration of 500 mg l^{-1} for elimination of the bacteria after co-cultivation. The explants were subcultured on to fresh screening medium after every two weeks. Heavy overgrowth of bacteria was seen ranging from 33 to 55 per cent, when cefotaxime was withdrawn from screening medium on 28th day (Table 18). When cefotaxime was again added to these cultures the bacterial overgrowth could be checked. However, the explants also died subsequently in this medium.

On maintaining cefotaxime in the screening medium for a longer period of 56 days, bacterial overgrowth could be completely checked. But the leaf explants were all dead by this time. When cefotaxime was withdrawn on 42nd day, eventhough the initial bacterial innoculum was low ($O.D._{560nm}$ = 0.25), bacterial overgrowth to the extent of 31.7 per cent was seen (Table 17).

In all the cases discussed above (Table 17 to 21) explants which did not show bacterial overgrowth, were all dead at the end of the experiment and did not

show induction of callus in the screening media. However, bacterial presence could not be seen in the medium when it contained cefotaxime.

These observations indicate that irrespective of the bacterial density (0.19-0.54), induction time (5-30 min) and co-cultivation period (2-4 days), the bacteria survived in the leaf tissue even up to 42 days when cefotaxime was administered at a strength of 500 mg l^{-1} and sub cultured once in two weeks, with fresh supply of antibiotics. In treatments where, bacterial elimination was noticed, the explants were dead and did not produce callus in the screening medium. It could be either because transformation was not taking place at all or that the presence of bacteria for such a long period as 42 days was disrupting cell multiplication and callus formation in transformed cells. The control with antibiotics in all the above cases did not show callus induction on leaves. This indicates, that screening medium containing 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime was sufficient to arrest the growth of untransformed cells in the leaf explants. The control cultures with no antibiotics in the medium were normal and showed callus induction on leaf explants.

The possibility of eliminating the bacteria from leaf explants by subculturing the explants in the screening media at shorter intervals was looked into during further leaf transformation experiments.

Braun (1975) had reported that actively dividing cells are more susceptible to *Agrobacterium* infection. Since in black pepper the leaf explants takes 3-5 weeks for initiation of callus in regeneration medium, transformation was attempted using leaf explants precultured for 30-40 days and explants subcultured in the screening media at shorter intervals.

When explants precultured for 40 days were used for transformation with a bacterial density of $O.D._{560nm}$ = 1.2; induction time of 5 min and co-cultivation period of four days, bacterial overgrowth could be seen to the extent of

100 per cent, even when the explants were maintained in the screening media containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin for 60 days with frequent subculturing (5-8 days interval for first 25 days) (Table 22). This may be because the explants had initiated callus formation and the bacteria had better chances of survival in this tissue.

Leaf transformation carried out with explants precultured for 32 days (bacterial density - 1.0; induction time - 5 min; co-cultivation period - 2.5 days) which were maintained in the screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin for 52 days and subcultured frequently (2-13 days interval) developed bacterial overgrowth ranging from 50 to 100 per cent, the remaining explants being dead (Table 24).

In another instance, when leaf segments precultured for 32 days were transformed (bacterial density - 1.0/1.3; induction time - 3 min; co-cultivation period - 2 days) the cefotaxime concentration in the screening medium was increased during the first subculture (3 days after co-cultivation) from 500 mg l^{-1} to 1000 mg l^{-1} and continued with 1000 mg l^{-1} in subsequent subculture. Here also bacterial overgrowth was noticed to the extent of 100 per cent when cefotaxime was withdrawn on 40th day. Thus the bacteria was not controlled even with 1000 mg l^{-1} cefotaxime when subcultured between 11-14 days.

Leaf transformation carried out with explants precultured for five days, using bacterial density of 1.02 to 1.3 ($O.D._{560nm}$), induction time of 3 min and co-cultivation period of two days, produced bacterial overgrowth in 100 per cent of the explant despite being subcultured at an interval of 6 to 13 days in the screening media containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin (Table 26).

Subsequently, the initial bacterial density in the induction medium was increased to as high as $O.D._{560nm}$ = 1.4 (induction time - 2 min; co-cultivation period - 2 days) and cefotaxime administered at a strength of 500 mg l^{-1} in the

screening medium. Subculturing was done at five to seven days interval. The cultures did not show any bacterial overgrowth when cefotaxime was withdrawn on 37th day (Table 23). However, none of the explants exhibited symptoms of growth at this stage.

Increasing the cefotaxime concentration from 500 mg l^{-1} to 1000 mg l^{-1} and again to 1500 mg l^{-1} in successive subclture also did not help in attaining complete elimination of bacteria (Table 25).

The heavy bacterial overgrowth seen when explants precultured for 40 days were used for transformation, even after maintaining them for 60 days in screening medium containing 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin with frequent subculturing (Table 22) and complete elimination of the bacteria by 37th day when explants precultured for three days were transformed and handled in the similar way (Table 23), suggests that the bacteria survived in the tissue so long as the tissue is alive and is killed with the death of the tissue as it cannot survive in the medium containing cefotaxime.

The test for bacteriocidal activity of cefotaxime had clearly shown that cefotaxime at a concentration of 500 mg l^{-1} kills the bacteria effectively in pure bacterial suspension culture with an $O.D._{560nm}$ = 1.0. The inability of cefotaxime at a concentration of 500 mg l^{-1} and even at 1000 mg l^{-1} to eliminate the bacteria from plant tissues during *in vitro* culture, suggests that eventhough cefotaxime is added to the medium at a strength of 500 mg l^{-1}, the bacteria in the plant tissue may not be subjected to the same concentration of the antibiotic.

Different reasons can be attributed to such a phenomenon. Firstly, the cefotaxime may be loosing its lethal activity at a short interval of time in the culture medium under the culture conditions followed (especially light), which necessitates subculturing even at a shorter interval of time to get the right effect of the antibiotic. Secondly, cefotaxime may not be absorbed uniformly by the

explants in the regions at the concentration applied in the medium, so that some of the bacteria especially in the inner region escape the lethal dose and develop resistance. Here, an increase of dosage may solve the problem. Lack or restricted entry of the antibiotic into the explant tissues was also noticed by Hammerschlag (1997) in apple. They could succeed in eliminating the *Agrobacterium* only by resorting to vacuum infiltration of cefotaxime at a concentration of 2000 mg l^{-1} for 30 minutes. Thirdly, cefotaxime may be metabolised into non-toxic form by the black pepper tissues, thereby helping the bacteria to escape the lethal dose and develop resistance. Here again, increasing the dosage may serve the objective.

In all the leaf transformation work carried out under this study, none of the explants produced callus in the screening media, as against the control where 100 per cent cultures showed callus initiation and proliferation. The explants either showed bacterial overgrowth or were dead at the end of each experiment. The failure to obtain transformed tissue in the above leaf transformation experiments may not be due to lack of transformation but can also be because of the failure of transformed cells to multiply due to the presence of the bacteria which disrupts callus formation from transformed cells of the leaf segments.

Ineffective elimination of bacteria after co-cultivation was a problem with callus transformation also. In the initial callus transformation experiments, the callus after co-cultivation with the bacteria, were placed in screening medium containing 500 mg l^{-1} cefotaxime, 50 mg l^{-1} kanamycin and 20 mg l^{-1} hygromycin and were subcultured once in two days and even daily for a period of eight to ten days. Heavy phenol exudation was seen in all the experiments compared to the two controls. The heavy phenol exudation can be considered indicative of the plant defense system against the invasion of the bacteria. Heavy overgrowth of bacteria (ranging from 13-100 per cent) was seen when cefotaxime was withdrawn after a period of 38 to 41 days. Bacterial overgrowth was comparatively less (13-61%) when small calli were used (Table 28) than when bigger calli were used (40-100%) (Table 29). This could be due to poor penetration of cefotaxime to the interior of

bigger callus. The fresh growth seen in screening media (Table 29) did not grow further and could be escapes which could not survive the action of selection agent during subsequent subculturing. In the two experiments, the control with antibiotics did not show growth indicating that growth of untransformed cells can be checked with 50 mg l^{-1} kanamycin and 20 mg l^{-1} hygromycin. But such a combination of selection agents can find use only in a hypothetical situation where higher doses of kanamycin and hygromycin when applied singly are hampering regeneration of transformed tissue and the bacterial strain used has the selectable marker genes for both the antibiotics. However, this result has also to be confirmed with further studies.

Cefotaxime at a concentration of 500 mg l^{-1} could not eliminate the bacteria even when the calli were subcultured into fresh screening media every one to three days for a period of 58 days (Table 30). This conclusively proved that cefotaxime at this concentration cannot eliminate the bacteria in the calli after co-cultivation no matter how frequently it is subcultured. Unwounded calli were quicker in showing bacterial overgrowth than the wounded ones probably because of poor penetration of cefotaxime to the interior of the calli in the former situation. The few calli which did not show bacterial presence were all dead.

The fact that kanamycin at 50 mg l^{-1} can partially suppress growth when subcultured at two weeks interval was noted earlier (Table 13). The observation of growth in control with antibiotics with 500 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin when subcultured daily, reveals that kanamycin at 50 mg l^{-1} cannot suppress callus growth completely even if it is subcultured daily (Table 30). It also gives an indication that cefotaxime at 500 mg l^{-1} may not affect normal callus growth.

Screening media used in one of the callus transformations contained 500 mg l^{-1} cefotaxime and 75 mg l^{-1} kanamycin (Table 31). Subculturing was done once in one to three days for almost 26 days. The control with antibiotic did not

show callus growth. However, the period of experiment was short and cannot be concluded that 75 mg l^{-1} kanamycin could completely suppress callus growth when subcultured at shorter interval of time.

In one instance, wounded calli after co-cultivation with the bacteria was grown in regeneration media containing 500 mg l^{-1} cefotaxime alone (Table 31). Here, cefotaxime was not withdrawn from the medium at all. Though bacterial presence could not be seen visually in the presence of cefotaxime, the untransformed cells did not show growth in the absence of kanamycin. The failure of untransformed cells to grow in the absence of kanamycin, could either be due to the presence of live bacteria (an observation based on earlier experiments) or due to a hypersensitive response of the callus to the invading pathogen and to some extent to wounding. If the former is the reason for failure of the untransformed cells to grow, effective elimination of the bacteria with higher doses of cefotaxime or a change of the antibiotic could solve the problem. If the latter is the reason, then only very low bacterial densities could help in avoiding the self destructive defense response of the callus.

Humara *et al.* (1999) have reported such a hypersensitive response from the leaf explants in *Pinus pinea*. The transient GUS expression after seven days of co-cultivation showed very high transformation events when bacterial density was 1.0 ($O.D._{600nm}$). But none of the transformed cells survived due to the hypersensitive response of the plant defense against pathogen. However, when bacterial density was 0.01 ($O.D._{600nm}$) transformed buds were recovered at a frequency of four per cent.

The hypersensitive response of the callus could also have been compounded by the characteristic of the super virulent AGL-1 strain of *Agrobacterium tumefaciens* used in this experiment. Samac (1995) have reported that AGL-1 strain had a detrimental effect on leaf explant tissue during his transformation experiment in alfalfa (*Medicago sativa*). Majority of the leaf

explants belonging to nine germplasm that were co-cultivated with this strain had reduced amounts of callus and a greater degree of tissue browning than the explants co-cultivated with strain LBA 4404. However, Chabaud *et al.* (1988) had reported that strain A 281, from which AGL-1 is derived, gave better transformation than LBA 4404 in *Medicago varia*.

The experiments with black pepper callus has shown that the phenol exudation in response to cutting prevents growth at the cut end for a long time. Wound healing response of black pepper is generally poor. In callus transformation works, the calli used for control without antibiotic were also wounded. The calli showing fresh growth after wounding varied from 60 to 100 per cent (Table 28-32). The poor recovery of callus after wounding poses a question as to how far wounding could be effective in transformation works. Potrykus (1991) had suggested that only plants with an appropriate wound response develops large population of wound adjacent competent cells for regeneration and transformation. Further investigation can reveal whether wounding of callus is detrimental or beneficial for transformation in black pepper.

Detrimental effect of wounding has been reported in a number of crops. Confalonieri *et al.* (1995) have reported that they could not find any marked difference in transformation when punctured and unpunctured cotyledon were used in black poplar clones. Instead a reduction in regeneration of transformed explant was noticed. In *Pinus pinea* L., Humara *et al.* (1999) tried different methods of wounding during transformation work. They found that only unwounded explant, with only excision wound, alone produced transgenic buds. Whereas, cotyledon wounded by ultra sound, particle gun or scraped did not produce buds. Similarly, transformation was reduced in *Casuarina glauca* when explants were wounded by microprojectile bambardments (Le *et al.*, 1996).

Ineffective elimination of the bacteria after co-cultivation was identified as the major problem in this study both with leaf and callus transformation work.

Failure in obtaining growth of transformed tissue in screening media in both callus and leaf transformation work, may not be due to the failure of transformation of cells but can be due to the failure of transformed cells to multiply either due to the presence of bacteria and/or the hypersensitive response of the plant tissue.

Similar situation of ineffective elimination of a super virulent strain causing a major problem in transformation work was reported in apple by Hammerschlag *et al.* (1997). The team tried 100 mg l^{-1} of carbenicillin, cefotaxime and cefoxitin singly and in combination to eliminate the super virulent *A. tumefaciens* strain EHA 101 during their transformation work in apple but failed. They could not succeed even by short term (1-18 hrs) vacuum infilteration of the above antibiotics at a concentration of 500 mg l^{-1}. Finally success was obtained only when cefotaxime at a strength of 2000 mg l^{-1} was vacuum infilterated for 30 minutes.

Attempt was made in this study to eliminate the bacteria from the callus after co-cultivation using higher doses of cefotaxime in combination with other measures like restricting the co-cultivation period to one day and air drying of the callus after co-cultivation. However, the increased doses of 1000 and 1500 mg l^{-1} of cefotaxime in combination with other measures could not eliminate the bacteria completely eventhough subculturing onto the fresh screening media was done once in two to three days. The seemingly fresh growth seen in some of the calli cultured in regeneration medium containing 1500 and 1000 mg l^{-1} cefotaxime and no kanamycin, respectively, was an indication that the bacterial population had been restricted to the inner regions of callus to the extent that the outer cells showed signs of growth. It also indicated that cefotaxime at 1000 or 1500 mg l^{-1} did not interfere with the callus growth.

The various observations in the present study has revealed that when hygromycin or kanamycin are used for elimination of untransformed cells during transformation work 30 mg l^{-1} and 100 mg l^{-1}, respectively, are the minimum dose effective for callus transformation while 10 mg l^{-1} and 50 mg l^{-1}, respectively, are

effective for leaf transformation. The lack of proliferation of untransformed cells in the absence of selection pressure indicates that the cell growth is hindered after infection with *Agrobacterium*. This could probably be due to ineffective elimination of the *Agrobacterium* and/or hypersensitivity of cells. Therefore, proliferation of transformed cells could be achieved only if these constraints are overcome.

Thus the future work in transformation of black pepper using *Agrobacterium tumefaciens* strain AGL-1; 1303 may be directed to find an effective method for elimination of the bacteria after co-cultivation either by trying higher doses of cefotaxime or adopting better methods of infiltration say, vacuum infiltration or by a change of the antibiotic. Different antibiotics are reported to be effectively used for elimination of bacteria in transformation works. Ling *et al.* (1998) have reported ticarcillin potassium clavulanate to be much more effective than cefotaxime in elimination of *Agrobacterium* during transformation of tomato. Similarly, Aliev *et al.* (1997) studied six different antibiotics on their role in eliminating bacteria during their transformation work in tobacco. They found ampiox to be the best among the six.

The hypersensitive response of the callus should be effectively dealt with by using lower bacterial density and restrictive wounding.

During this study interaction between a single strain of the bacteria and a single variety of black pepper was studied. Bacterial strain and crop genotype specificity are reported to be very pronounced in many crops. Desgagnes *et al.* (1995) used three different strains of *A. tumefaciens* with a wide host range and transformed three highly regenerative genotypes of alfalfa belonging to three different breeding lines. They found that the interaction between bacterial strains and plant genotypes were highly significant. Such reports are seen in *Robinia pseudoacacia* (Igasaki *et al.*, 2000), *Pinus pinea* L. (Humara *et al.*, 1999) and even in black pepper (Sasikumar and Veluthambi, 1996). Hence transformation in black pepper with different strains and with other genotypes may also be attempted.

Summary

6. SUMMARY

Investigations on *Agrobacterium* mediated genetic transformation in black pepper (*Piper nigrum* L.) were carried out in the Plant Tissue Culture Laboratory, College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur during the period from November, 1997 to August 2000. The objective of the study was to develop a procedure for transfer of genes into black pepper, variety Panniyur-1 through *Agrobacterium tumefaciens* strain AGL-1; 1303 using antibiotic resistance genes (*npt II* and *hpt IV*) as selectable markers and beta-glucuronidase (*GUS*) and green fluorescent protein (*GFP*) genes as reporter genes.

Axenic seedlings of black pepper, variety Panniyur-1, were raised under *in vitro* condition to generate explants, both cotyledonary leaf and callus, with reduced contamination for transformation and related works. Of the different media used for raising the axenic seedlings, double sterilised moist sand gave the highest percentage of seed germination and recovery of normal seedlings.

Callus was generated by culturing cotyledonary leaf explants in half MS supplemented with two different combinations of IAA and BA, viz, 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA and 1.0 mg l^{-1} IAA + 1.0 mg l^{-1} BA. Number of explants callusing and callus growth was found to be better in half MS supplemented with 1.0 mg l^{-1} each of IAA and BA.

Callus raised in half MS under two different combination of IAA and BA, viz., 1.5 mg l^{-1} + 1.0 mg l^{-1} and 1.0 mg l^{-1} IAA and 1.0 mg l^{-1} BA were used for regeneration trial. The callus from the two sources were cultured in half MS supplemented with nine different combinations of IAA (1.0 mg l^{-1}), BA (1.0, 1.5, 2.0 mg l^{-1}) and kinetin (0.1, 0.5 mg l^{-1}). Thus of the eighteen treatment combinations tried regeneration was to the extend of three per cent only in one of them, where callus cultured in half MS supplemented with 1.5 mg l^{-1} IAA +

1.0 mg l^{-1} BA were transferred to half MS supplemented with 1.0 mg l^{-1} + each of IAA and BA. Kinetin was found not to favour callus growth and regeneration.

Attempt was made to generate embryogenic calli for transformation work by culturing zygotic embryos in SH medium with and without 2,4-D, under varying cultural conditions. Germination of zygotic embryos was hampered by 2,4-D. Zygotic embryos germinated in most of the treatments without 2,4-D but embryogenic calli failed to appear. However, upto seven seedlings were produced from a single zygotic embryo when they were incubated in SH liquid shake cultures which indicated that somatic embryogenesis could have occurred.

Leaf and callus sensitivity to different antibiotics at varying doses and methods of application were tested. Under the present circumstances, antibiotic stock solutions stored under frozen condition was found to loose their activity, probably because of the frequent power failure leading to repeated cycles of melting and freezing of the antibiotic stock solution. When freshly prepared antibiotic stocks were used along with subculturing at two week interval, black pepper tissues were found to be susceptible to both kanamycin and hygromycin. Callus induction on leaf was completely suppressed with 50 mg l^{-1} of kanamycin, whereas callus growth was suppressed only with 100 mg l^{-1} of kanamycin. Similarly, hygromycin at 10 mg l^{-1} concentration completely suppressed callus induction on leaf, whereas, it was required at a strength of 30 mg l^{-1} to suppress the growth of callus completely.

The bacteriocidal activity of cefotaxime on *Agrobacterium tumefaciens* strain AGL-1; 1303 was studied. It was found that cefotaxime at 500 mg l^{-1} effectively killed the *Agrobacterium* in pure bacterial suspension culture with a density of 1.0 ($O.D._{560nm}$).

Stimulatory effect of proline on *A. tumefaciens* strain AGL-1; 1303 was studied. It was found that proline increased the rate of multiplication of the bacterium. However, it's role in transformation is to be further studied.

Leaf transformation experiments were carried out with varying the factors, like, preculture of explants (2-40 days), attractants added to induction medium (Proline: 0-500 mg l^{-1}; Acetosyringone: 0-30 mg l^{-1}), bacterial density of induction media ($O.D._{560nm}$ = 0.19-1.4), induction time (3-30 mins), attractants in co-culture medium (Proline: 0-100 mg l^{-1}), coculture period (2-4 days) and subculturing interval in screening medium. None of the explants showed callus induction in the screening media. The explants were either dead or showed bacterial overgrowth on withdrawal of cefotaxime from screening media. Cefotaxime at strenths of 500 and 1000 mg l^{-1} could not eliminate *Agrobacterium* effectively from the leaf tissue. Prolonged survival of the *Agrobacterium* could be the reason for non-multiplication of transformed cells.

Callus transformation experiments were carried out with varying factors, like, attractants added in induction media (Proline: 0-500 mg l^{-1}), bacterial density ($O.D._{560nm}$ = 0.5-1.5), induction time (3-30 min), attractants in co-cultivation medium (Proline: 0-200 mg l^{-1}), co-culture period (1-4 days) and subculturing interval in screening medium.

Heavy phenol exudation was seen during first few days in the screening medium compared to the control treatments. Fresh growth of callus was not obtained in the screening media in any of the experiments. Heavy bacterial overgrowth was seen on withdrawal of cefotaxime from screening media. Cefotaxime at a strength of 500 mg l^{-1} failed to eliminate the *Agrobacterium*, no matter at what interval it was subcultured. *Agrobacterium* could not be completely eliminated even at 1000 and 1500 mg l^{-1} of cefatoxime. Ineffective elimination of *Agrobacterium and /or* the super-virulent character of the strain leading to the hypersensitive response of callus to bacterial infection could be the reason for

failure of transformed cells to multiply. Therefore, proliferation of transformed cells could be achieved only if these constraints are overcome.

Future work in transformation of black pepper with *Agrobacterium tumefaciens* strain AGL-1; 1303 should concentrate on effective elimination of the bacterium from explants after co-cultivation. Transformation work may also be tried with different strains of *Agrobacterium* and different cultivars of black pepper.

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Plates

Plate I. *Agrobacterium tumefaciens* strain AGL–1; 1303 colony in YEP medium

Plate II. Axenic seedling of black pepper variety Panniyur – 1 raised in moist sand

Plate III. Heavy exudation of phenol seen in callus culture 10 days after subculturing in comparison to freshly subcultured callus

Plate IV. Regeneration of plant from callus of black pepper cultured in half MS supplemented with 1.0 mg l^{-1} each of IAA and BA.

Plate V. Zygotic embryo emerging from the endosperm during development and growth under *in vitro* condition.

Plate VI. Development of radicle and from the zygotic embryo under *in vitro* condition

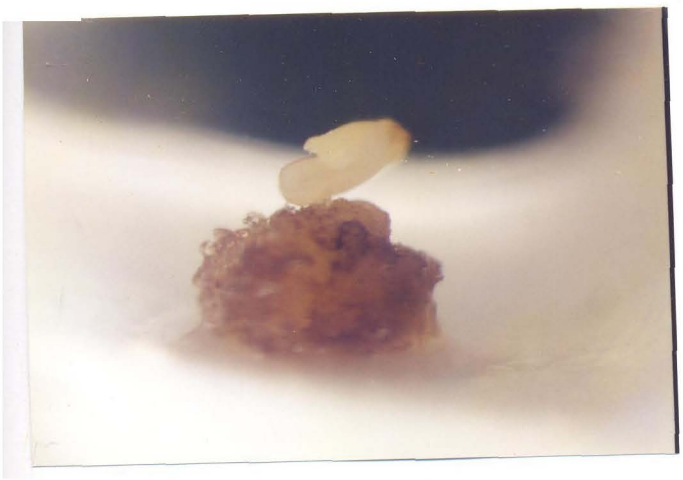


Plate VII. Differentiation of root hairs in the germinating zygotic embryo under *in vitro* condition

Plate VIII. Formation of seedling from zygotic embryo under *in vitro* condition

a) embryo was incubated under light
b) embryo was incubated under dark

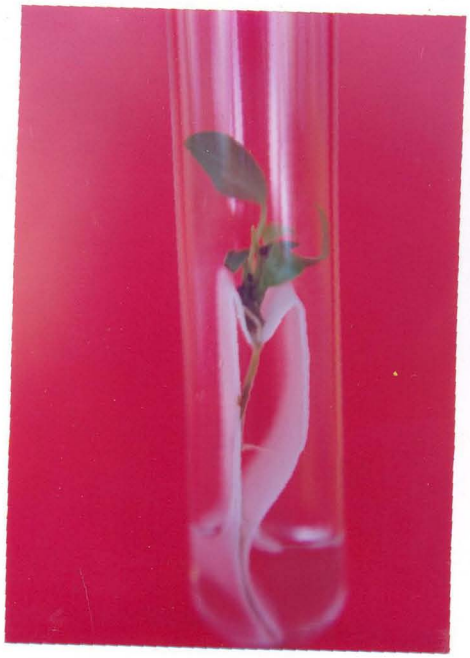


Plate IX. Development of somatic embryo from the junction of root and shoot in a seedling developed from zygotic embryo under *in vitro* condition

Plate X. Callus of black pepper cultured in regeneration media supplemented with kanamycin

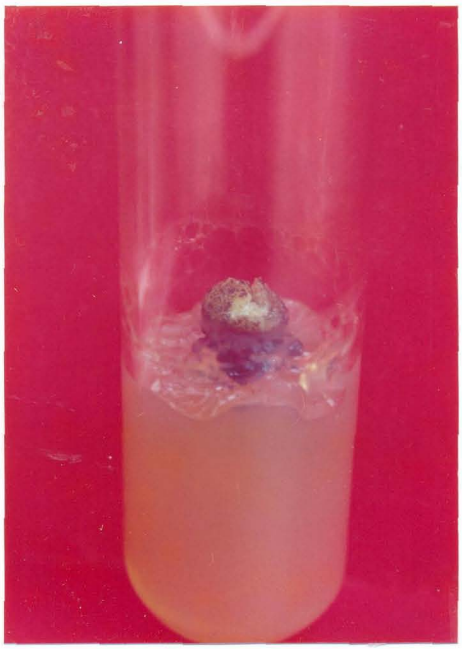
a) Fresh growth in medium containing 50 mg l^{-1} kanamycin
b) Lack of fresh growth in medium containing 100 mg l^{-1} kanamycin

Plate XI. Callus cultured in regeneration medium containing antibiotics

a) Heavy phenol exudation in callus cultures after co-cultivation with A. tumefaciens
b) Callus cultured without co-cultivation

Plate XII. Death of callus cultured in screening media after co-cultivation with *Agrobacterium*

Plate XIII. Fresh growth in callus of black pepper cultured in regeneration medium containing 1500 mg l-1 cefotaxime after co-cultivation.



Appendices

APPENDIX-I

Chemical composition of media used for culturing different explants of black pepper

Constituents	MS ($mg\ l^{-1}$)	SH ($mg\ l^{-1}$)
Inorganic constituents		
NH_4NO_3	1650	-
$(NH_4)H_2PO_4$	-	300
KNO_3	1900	2500
$CaCl_2.2H_2O$	440	200
$MgSO_4.7H_2O$	370	400
KH_2PO_4	170	-
$FeSO_4.7H_2O$	27.8	15
Na_2 EDTA	33.6	20
$MnSO_4.4H_2O$	22.3	-
$MnSO_4.H_2O$	-	10
$ZnSO_4.7H_2O$	8.6	1.0
H_3BO_3	6.2	5.0
KI	0.83	-
$Na_2MoO_4.2H_2O$	0.25	0.1
$CuSO_4.5H_2O$	0.025	0.2
$CoCL_2.6H_2O$	0.025	0.1
Organic constituents		
Myoinositol	100	1000
Sucrose	30000	30000
Glycine	2.000	-
Nicotinic acid	0.500	5.000
Pyridoxine HCl	0.500	0.500
Thiamine HCl	0.100	5.000
pH	5.7	5.8

Half MS represents 50 per cent concentration of inorganic constituents

APPENDIX-II

Chemical composition of Yeast Extract Peptone (YEP) medium used for culturing *Agrobacterium tumefaciens*

Yeast Extract	- 100 $g\ l^{-1}$
Peptone	- 10 $g\ l^{-1}$
Sodium chloride	- 5 $g\ l^{-1}$
pH	- 7

AGROBACTERIUM - MEDIATED GENETIC TRANSFORMATION IN BLACK PEPPER

(Piper nigrum L.)

By

HOMEY CHERIYAN

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy in Agriculture

Faculty of Agriculture
Kerala Agricultural University

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2000

ABSTRACT

Investigations on genetic transformation in black pepper (*Piper nigrum* L.), variety Panniyur-1, using *Agrobacterium tumefaciens* strain AGL-1; 1303 harbouring antibiotic resistant selectable marker genes (*npt II* and *hpt IV*) and *GUS* and *GFP* reporter genes were carried out in Plant Tissue Culture Laboratory, College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur, during the period from November, 1997 to August, 2000.

For the collection of explants for transformation work and related studies, axenic seedlings of black pepper were raised. Of the different media used for this, double sterilised moist sand was found to be the best.

Callus induction on cotyledonary leaf explants and callus growth was found to be maximum in half MS medium supplemented with 1.0 mg l^{-1} each of IAA and BA compared to other combinations of IAA, BA and kinetin tried.

Of the different growth regulator combinations tried in half MS medium for regeneration, only when callus cultured in half MS supplemented with 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} BA were transferred to half MS supplemented with 1.0 mg l^{-1} each of IAA and BA produced regeneration to the extent of three per cent.

In order to produce embryogenic calli for somatic embryogenesis, zygotic embryos were cultured in liquid basal SH medium under different cultural conditions. Somatic embryos were suspected to be produced when embryo with endosperm were cultured in liquid basal SH medium on rotary shaker. However, embryogenic calli were not generated.

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 week interval. Callus induction on leaf was completely inhibited at 50 mg l^{-1} of 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}.

Cefotaxime at a strength of 500 mg l^{-1} effectively killed the *Agrobacterium tumefaciens* strain AGL-1; 1303 in pure bacterial suspension cultures with an $O.D._{560nm} = 1.0$.

Proline was found to increase the rate of multiplication of *A. tumefaciens* strain AGL-1; 1303.

Leaf transformation was carried out by varying different factors affecting transformation. None of the leaf explants showed callus induction in screening media. Cefotaxime at strengths of 500 and 1000 mg l^{-1} could not eliminate *Agrobacterium* effectively from the leaf tissue. Prolonged survival of the *Agrobacterium* in the tissues could be the reason for non-multiplication of transformed cells.

Callus transformation was carried out varying different factors affecting transformation. Excessive exudation of phenols was noticed in all the treatments compared to the controls. Heavy overgrowth of *Agrobacterium* was seen on withdrawal of cefotaxime from the screening media. Cefotaxime at 500, 1000 and 1500 mg l^{-1} could not eliminate the *Agrobacterium* effectively from callus tissues. Ineffective elimination of *Agrobacterium*, the super virulent character of the strain and the hypersensitive response of the callus to bacterial infection and wounding could be the reasons for failure of transformed cells to multiply.