BT/PR7866/AGR/

Genetic transformation of black
to confer resistance against vi

DBT-CP3

Final Report

2006-2011

Principal Investigator

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(Indian Council of Agricultural Research)

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RPF-III

PERFORMA FOR SUBMISSION OF FINAL REPORT OF RESEARCH PROJECTS

Part- I : General Information

800 Project Code : **Path. XII (813)**

8001 Institute Project Code No. : DBT-CP3

8002 ICAR Project Code No.

801 Name of the Institute and Division

8011 Name and address of Institute : Indian Institute of Spices Research, Calicut –673 012, Kerala

8012 Name of Division / Section : Division of Crop Protection

8013 Location of the Project : IISR, Calicut, Kerala, India

802 Project Title **:** Genetic transformation of black pepper to confer resistant against viruses

803 Priority Area : Crop Protection

8031 Research Approach :

Applied Res.	Basic Res.	Process or Tech. Dev.	Transfer of Tech.
	✓	✓	

804 Specific Area : Molecular Virology, Biotechnology

805 Duration of Project : 5 years

8051 Date of start : 29th September 2006

8052 Date of Completion : 28th September 2011

806 Total cost /Expenditure Incurred : Rs19.63704 lakhs
(Give reasons for variation, if any from original estimated cost)

807 Executive Summary

Two viruses namely *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) are associated with black pepper. None of the cultivars of black pepper grown is resistant to disease. Since there is no effective chemical to control viruses, it is necessary to impart resistance against viruses in agronomically superior cultivars.

Transgenic plants expressing coat protein or other viral sequences were shown to confer resistance against virus in many virus-host combinations. Similar approach can be used to transform existing cultivars of black pepper to get resistant plants through transformation using viral sequences. With this in view the project aimed to produce transgenic lines of black pepper using viral sequences as transgenes.

A portion of ORF III of PYMoV and coat protein gene of CMV amplified from infected plant through PCR was cloned in TA cloning vector and its nucleotide sequence determined. Selected clones having sense and antisense orientation of both CMV and PYMoV sequences were restricted and ligated into the binary vector, pBI121 through directional cloning. Presence of transgene sequences in pBI121 was confirmed by PCR and restriction digestion. Each of these identified clones was then mobilized into *A. tumefaciens* strain EHA 105 by triparental mating and presence of recombinant pBI121 (with PYMoV ORF III and CMV sequences) was further confirmed by isolating plasmid from *A. tumefaciens* and subjecting them to restriction analysis and PCR.

Initially leaf, internode and petiole were used as explants for transformation. But these explants failed to give consistent and high frequency regeneration. So as an alternative somatic embryogenesis from germinating seed was attempted. Protocol for direct somatic embryogenesis from germinating seeds of black pepper was established. Procedure for cyclic secondary somatic embryogenesis from primary somatic embryos and establishment of embryogenic mass was developed.

Effect of antibiotics such as cefotaxime and carbenicillin on somatic embryo induction, embryogenic mass proliferation and growth were studied. Cefotaxime at 100 µg/ml was found to be optimum for somatic embryo proliferation and prevention of *Agrobacterium* over growth. Optimum concentration of kanamycin for the selection of transformants at each explant stage was standardized. A step up concentration of kanamycin in three stages was found better for selection of transformants: initially, 25 µg/ml for 15 days followed by 50 µg/ml until plantlet development and at plantlet stage 100 µg/ml. This apparently reduced sudden shock to the explants and selection of maximum number of transformants. Embryogenic

mass was identified as ideal explant for transformation as it gave high frequency regeneration in presence of cefotaxime and carbenicillin.

Agrobacterium mediated transformation of embryogenic mass using a binary vector carrying GUS and npt II reporter genes was standardized and putative transgenic plants were established. GUS expression in putative transformants were determined by histochemical, PCR and Southern hybridization.

Embryogenic mass were transformed with a portion of *Piper yellow mottle virus* (PYMoV) sense (pBI121PYMoVS) and antisense (pBI121PYMoVAS) constructs and putative transformants were confirmed by PCR, dot blot assay and Southern hybridization for the presence of transgene. Similarly, embryogenic mass were also transformed with *Cucumber mosaic virus* coat protein (CMV-CP) sense (pBI121CMVS) construct and putative transformants were confirmed by PCR, dot blot assay and Southern hybridization for the presence of transgene. Transgenic plants carrying PYMoV ORF III region in sense and antisense orientation and CMV-CP in sense orientation was hardened and maintained under green house conditions. These putative transgenic plants obtained using different viral constructs need to be analyzed for resistance to CMV and PYMoV by challenge inoculation

808 Key words **:**Black pepper, *Cucumber mosaic virus*, *Piper yellow mottle virus*, *Agrobacterium* mediated transformation, transgenic plants, confirmation of transgenicity, PCR, dot blot, Southern hybridization

Part-II : Investigator Profile

(Please identify clearly changes, if any in Project personnel)

810 Principal Investigator :

8101 Name : Dr. A.Ishwara Bhat
8102 Designation : Senior Scientist

8103 Division/ Section : Crop Protection

8104 Location : Indian Institute of Spices Research

8105 Institute Address : Indian Institute of Spices Research
Calicut-673 012, Kerala, India

811 Co- Investigator:

8111 Name : Dr. R. Suseela Bhai

8112 Designation : Senior Scientist

8113 Division/ Section : Crop Protection

8114 Location : Indian Institute of Spices Research

8115 Institute Address : Calicut-673 012, Kerala, India

Par-III: Technical Details

820 Introduction and objectives

8201 Project Objectives:

(i) Preparation of gene constructs using viral sequences

(ii) *Agrobacterium* mediated genetic transformation of black pepper with viral gene constructs and their regeneration

(iii)Selection and screening of transformants.

(iv) Analysis of selected transformants.

8202 Background information and importance of the projects

Black pepper (*Piper nigrum* L.) 'the king of spices' is originated in the tropical evergreen forests of Western Ghats of India (Ravindran, 2000). It constitutes an important component of culinary seasoning of universal use and an essential ingredient of numerous commercial food stuffs (Pruthi, 1992). The productivity of the crop is considerably low due to many biotic stresses. Among the biotic stresses stunted disease caused by viruses occupy third position in the form of loss they cause on black pepper. Viral disease is a serious problem in all pepper growing areas including South East Asia and Brazil. The stunted disease is characterized by small, crinkled, brittle, leathery and chlorotic patches/streaks on leaves; in severe cases, the leaves become abnormally narrow and give sickle shaped appearance leading to

typical stunting of plants (Fig.1) (Wahid *et al.*, 1992; Lockhart *et.al.*, 1997; Sarma *et al*., 2001). In India incidence and severity is more at black pepper plantations located at high altitudes (Bhat *et al*., 2005c).

Association of two viruses namely *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) were reported with the disease (Lockhart *et al*., 1997; De Silva *et al*., 2002; Bhat *et al*., 2003; 2005a). CMV is the type species of the genus, *Cucumovirus* in the family Bromoviridae. Its genome consists of three plus single stranded RNAs plus a fourth sub genomic RNA. It has a broad host range and is readily transmitted in a non-persistent manner by more than 75 species of aphids (Palukaitis et al., 1992). PYMoV is a member of the genus *Badnavirus* in the family Caulimoviridae. Badnaviruses are plant pararetroviruses with non enveloped bacilliform particles having a dimension of 30 x 130-150 nm that contain a genome consisting of a circular, relaxed 7.5 – 8.0 kb double stranded DNA molecules (Lockhart, 1990; Bouhida *et al*., 1993., Qu *et al*., 1991). They have a narrow host range and shown to be transmitted through mealy bug (*Planococcus citri* and *Ferrisia virgata*) and black pepper lace bug (*Diconocoris distanti). (De* silva *et al.,* 2002; Bhat *et al*., 2003; Bhat *et al*., 2005 b).

The disease is known to be spread through the use of infected cuttings for planting and through insect vectors in the field. None of the cultivars of black pepper grown is resistant to disease. Since there is no effective chemical to control viruses, it is necessary to impart resistance against viruses in agronomically superior cultivars. Transgenic plants expressing coat protein or other viral sequences were shown to confer resistance against virus in many virus-host combinations. Similar approach can be used to transform existing cultivars of black pepper to get resistant plants through transformation using viral sequences. Coat protein gene of CMV and a portion of open reading frame I and III (ORF I & III) of PYMoV infecting black pepper have been cloned and their nucleotide sequence have been determined (Bhat *et al*., 2005; Hareesh and Bhat, 2008). Thus under this project development of transgenic black pepper carrying sequences from PYMoV and CMV was taken up.

821 Project Technical Profile

8211 Technical programme
(Indicate briefly plan of procedure, techniques, instruments and special materials, organisms, special environments etc.)

i). Isolation and maintenance of virus culture

This involved collection of isolates of stunted disease affected black pepper plants from different black pepper growing regions of Karnataka, Kerala and Tamil Nadu; isolation and maintenance of pure culture of the isolates under insect proof glass house conditions.

(ii). **Standardization of transformation and regeneration protocol for black pepper**

A known plant transformation vector containing marker such as GUS (pBI121) was used to develop a *Agrobacerium* mediated transformation protocol for black pepper. Explants such as leaf, nodal region, petiole and somatic embryo from germinating seeds were used. Ideal explant for transformation, optimum concentration of antibiotic required to kill *Agrobacterium* and selection of transformed tissue were detrermined.

(iii). **Preparation of gene construct**

Plant transformation gene constructs were prepared in pBI121 using coat protein gene of CMV (black pepper isolate) and portion of ORF III of PYMoV sequences. Presence of insert (transgene) confirmed through PCR and restriction analysis.

(iv) **Genetic transformation with viral gene construct and regeneration**

The above prepared gene constructs were used for *Agrobacterium* mediated genetic transformation of black pepper followed by regeneration of transformants in a selection medium containing step wise increase in the concentration of kanamycin.

(v) **Selection and screening of transformants**

The transformants were screened for the presence of transgene through PCR, dot blot and Southern hybridization.

(vi) Analysis of selected transformants

The above selected positive transformants were analysed for the copy number, through Southern hybridization.

8212 Total man months involvement of component project workers

a) Scientific 28
b) Technical 60
c) Supporting 12

822 Final Report on the Project

Detailed report containing all relevant data with a summary of results (not exceeding 2-5 pages)

8221 Achievements in terms of targets fixed for each activity

1. Maintenance of virus isolates

Isolates of stunted disease affected black pepper plants from different pepper growing regions of Kerala and Karnataka were maintained under insect proof glass house conditions through vegetative propagation (Fig.1).

Fig.1 Diseased isolates maintained in the glass house

2. Preparation of gene constructs

2.1 PYMoV in sense and antisense orientation.

A portion of ORF III of PYMoV amplified from infected plant through PCR was cloned in TA cloning vector. Selected recombinant clones identified by restriction digestion and a selected clone were sequenced. The sequenced portion contained 597 nucleotides potentially coding for 198 amino acids (Fig. 2). The cloned region of ORF III harboured a *Bam* H1 site at 409 bases from its 5′ end. A selected

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CTATATGAATGGCTTGTGATGCCATTTGGGCTTAAAAATGCACCTGCTGTGTTCCAAAGAAAAATGGACA   70
L  Y  E  W  L  V  M  P  F  G  L  K  N  A  P  A  V  F  Q  R  K  M  D  N   24
ACTGTTTCAAAGGTATGGAAGATTTTATAGCTGTCTACATTGATGATATTCTGGTGTTCTCAGAAAACAT  140
C  F  K  G  M  E  D  F  I  A  V  Y  I  D  D  I  L  V  F  S  E  N  M     47
GAGGGACCATGCCCAACACTTAGTCGCAATGCTGGAAGTATGCAAGAAGAATGGGCTTATCTTAAGCCCA   210
R  D  H  A  Q  H  L  V  A  M  L  E  V  C  K  K  N  G  L  I  L  S  P      70
ACAAAAATGAAAATTGGGCTTGGAACTATTGATTTCCTGGGAGCAACTATTGGAAACAGCAAGGTAAAGC   280
T  K  M  K  I  G  L  G  T  I  D  F  L  G  A  T  I  G  N  S  K  V  K  L    94
TACAAGAGCACATCGTTAAGAAAATCCTGGACTTCAATACTGATGGGCTCGAAGACAAGAAGAATCTCCG   350
Q  E  H  I  V  K  K  I  L  D  F  N  T  D  G  L  E  D  K  K  N  L  R     117
GAGTTGGCTGGGCATTCTCAACTATGCAAGGGCATACATACCAAACCTAGGAAGGATCCTAGGCCCATTA   420
S  W  L  G  I  L  N  Y  A  R  A  Y  I  P  N  L  G  R  I  L  G  P  L     140
TATGCCAAGGTAAGCCCAACTGGAGAAAGAAAGATGAATCAACAGGATTGGAGTATTGTGGCCCAAATCA   490
Y  A  K  V  S  P  T  G  E  R  K  M  N  Q  Q  D  W  S  I  V  A  Q  I  K   164
AGAAAATCATTCAGGAGTTGCCCGAATTAGAATTGCCGCCAGAAGACTGCTGCATCGTAATAAAAACTGA   560
K  I  I  Q  E  L  P  E  L  E  L  P  P  E  D  C  C  I  V  I  K  T  D     187
TGGCTGTATGAGTGGCTGGGGTGCCATCTGCAAGTGG   597
G  C  M  S  G  W  G  A  I  C  K  W     198
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Fig. 2 Nucleotide and deduced amino acid sequence of the cloned region of ORF III of PYMoV infecting black pepper

clone having sense orientation of the ORF III region was then restricted with *Bam* H1. The resulting 409 bp fragment of ORF III of PYMoV containing Bam H1 site at 3′ end and other *Bam* H1 site from the vector at 5′end was ligated to linearized pBI 121 at *Bam*H 1 site. Competent *E. coli* (DH 5α) was then transformed using standard procedures. Positive recombinants harbouring ORF III region were identified by restriction analysis using *Bam* H1 that released the insert (Fig. 3). Plasmid DNA isolated from positive recombinants was then subjected to PCR analysis to identify sense and antisense oriented clones. The sense orientation in the pBI121 was identified by PCR using ORF III forward primer as forward primer and primer designed from Gus region as reverse primer that gave an expected band at ~1000 bp (Fig. 4). Similarly antisense clone in pBI 121 was identified by PCR using primer designed for the CaMV 35S promoter region as forward primer and forward primer of ORF III region as reverse primer that gave an expected size of 539 bp (Fig. 5). Each

of the identified sense and antisense clones were then mobilized into *A. tumefaciens* strain EHA 105 by triparental mating with the help of helper plasmid pRK 2014. The presence of recombinant pBI121 with PYMoV ORF III was further confirmed by isolating plasmid from *A. tumefaciens*

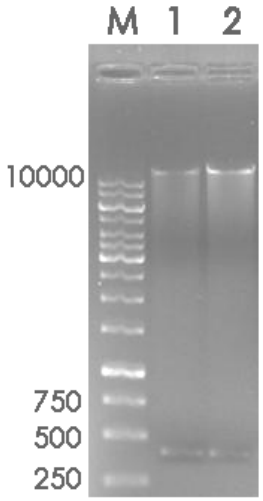

Fig. 3 Confirmation of recombinant pBI 121 plasmid containing PYMoV ORF III insert in *E. coli* by restriction analysis. Lane M: 1Kb ladder, Lane 1 & 2: Restriction digestion with *Bam* H1showing insert released at 400 bp

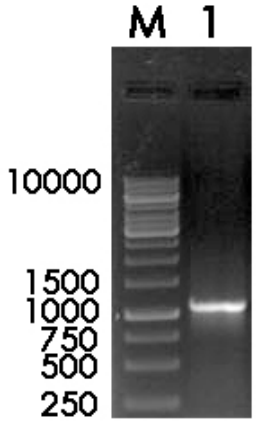

Fig. 4 Confirmation of recombinant pBI 121 plasmid containing PYMoV ORF III insert (sense orientation) in *E. coli* by PCR. Lane M: 1Kb ladder, Lane 1: Amplification obtained at 1000 bp with primers from the GUS and ORF III region.

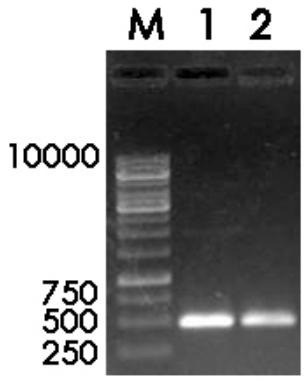

Fig. 5 Confirmation of recombinant pBI 121 plasmid containing PYMoV ORF III insert (antisense orientation) in *E. coli* by PCR. Lane M: 1Kb ladder, Lane 1 and 2: Amplification obtained at 530 bp with primers from the CaMV 35S promoter and ORF III region.

and subjecting them to restriction analysis and PCR (Fig. 6). Map of PYMOV ORF-III gene construct in pBI 121 is shown in Fig. 7. The sense and antisense constructs were designated as pBI121PYMoVS and pBI121PYMoVAS respectively.

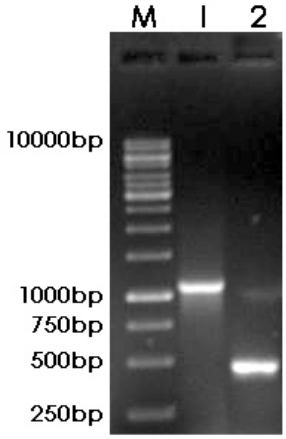

Fig. 6 Confirmation of recombinant plasmid pBI 121containing PYMoV ORF III insert in *Agrobacterium* strain EHA105 by PCR. Lane M: 1Kb ladder, Lane1: Amplification at 1000 bp with gus and ORF III region primer (sense orientation) and Lane 2: Amplification obtained at 539bp using CaMV 35S promoter and ORF III region primers (antisense orientation).

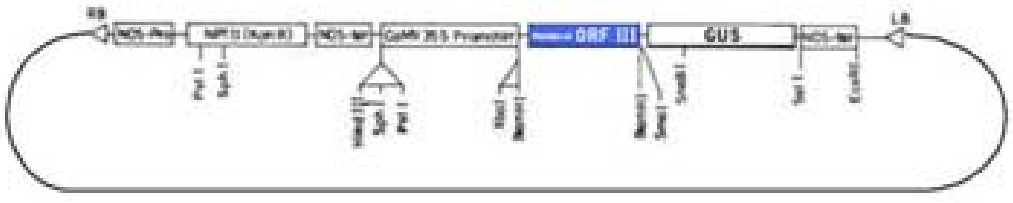

Fig. 7 Map of PYMoV ORF III gene construct in pBI 121

2.2.1 *Cucumber mosaic virus (*CMV) coat protein in antisense orientation

A 657 bp CMV coat protein gene sequence amplified through reverse transcription polymerase chain reaction (RT-PCR) using CMV CP specific primers was cloned in to a TA cloning vector. Positive recombinant clones were identified by restriction digestion and its nucleotide sequence determined by sequencing. The sequenced region contained a single ORF with 657 bases coding for a protein of 218 amino acids (Fig. 8). One selected clone where CMV CP got cloned in antisense orientation was restricted with *Bam* H1 and *Sac*1. The resulting fragment containing CMV CP region was isolated from the gel and ligated into pBI 121 restricted with same enzymes (*Bam*H1 and *Sac*I). Competent *E. coli* (strain DH5α) was used for

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ATGGACAAATCTGAATCAACCAGTGCTGGTCGCAACCGTCGACGTCGTCCGCGTCGCGGTTCCCGCTCCG      70
M  D  K  S  E  S  T  S  A  G  R  N  R  R  R  R  P  R  R  G  S  R  S  A      24

CTTCCTCCTCCGCGGATGCTACATTTAGAGTCCTGTCGCAACAGCTTTCGCGACTTAATAAGACGTTAGC     140
  S  S  S  A  D  A  T  F  R  V  L  S  Q  Q  L  S  R  L  N  K  T  L  A       47

AGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGCTGTAAACCTGGATACACGTTC     210
 A  G  R  P  T  I  N  H  P  T  F  V  G  S  E  R  C  K  P  G  Y  T  F       70

ACATCTATTACCCTGAAGCCTCCGAAAATAGACAAAGGGTCTTATTATGGTAAAAGGTTGTTACTTCCTG     280
T  S  I  T  L  K  P  P  K  I  D  K  G  S  Y  Y  G  K  R  L  L  L  P  D      94

ATTCAGTCACTGAGTTCGATAAGAAGCTTGTTTCGCGCATTCAAATTCGAGTTAATCCTTTGCCGAAATT     350
  S  V  T  E  F  D  K  K  L  V  S  R  I  Q  I  R  V  N  P  L  P  K  F     117

TGATTCTACTGTGTGGGTGACAGTCCGTAAAGTTCCTGCCTCCTCGGACTTGTCCGTTTCCGCCATCTCT     420
 D  S  T  V  W  V  T  V  R  K  V  P  A  S  S  D  L  S  V  S  A  I  S      140

GCCATGTTTGCGGACGGAGCCTCACCAGTACTGGTTTATCAGTATGCCGCGTCCGGAGTCCAAGCCAACA     490
A  M  F  A  D  G  A  S  P  V  L  V  Y  Q  Y  A  A  S  G  V  Q  A  N  N     164

ATAAATTGTTGTACGATCTTTCGGTGATGCGCGCTGATATTGGTGACATGAGAAAGTACGCCGTGCTCGT     560
  K  L  L  Y  D  L  S  V  M  R  A  D  I  G  D  M  R  K  Y  A  V  L  V      187

GTATTCAAAAGACGATGCGCTCGAGACGGATGAACTAGTACTTCATGTCGACATTGAGCACCAACGTATT     630
 Y  S  K  D  D  A  L  E  T  D  E  L  V  L  H  V  D  I  E  H  Q  R  I      210

CCCACTTCTGGGGTGCTCCCAGTTTGA     657
P  T  S  G  V  L  P  V  <       218
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Fig. 8 Coat protein gene sequence (shown as DNA) and deduced amino acid sequence of coat protein gene of *Cucumber mosaic virus* (CMV)

transformation. The presence of recombinant pBI 121 containing CMV CP gene was confirmed by isolating the plasmid and subjecting it to restriction analysis and PCR using primer designed for CaMV 35S promoter region as forward and CMV CP forward primer as reverse primer (Fig. 9). This was then mobilized into

Agrobacterium tumefaciens strain EHA 105 by freeze-thaw method. The transformants were selected on kanamycin and rifampicin plates. The presence of recombinant pBI 121 containing CMV CP gene in *A. tumefaciens* was confirmed by isolating the plasmid and subjecting it to restriction analysis and PCR (Fig. 10). The construct was designated as pBI121CMVAS.

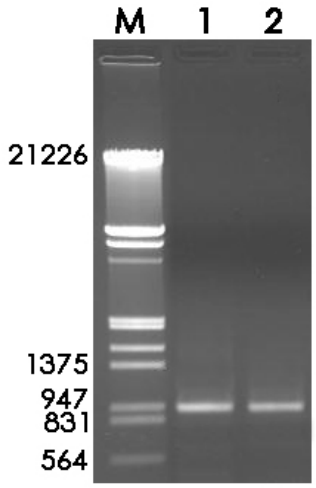


Fig. 9 Confirmation of recombinant pBI 121 plasmid containing CMV CP insert in *E. coli* by PCR. Lane M: DNA size marker; Lane 1 & 2 amplification using primer designed for CaMV 35S promoter and CMV CP region showing expected amplicon.

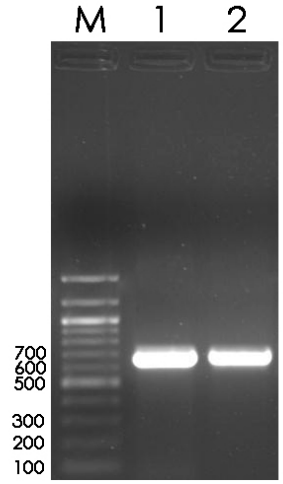


Fig. 10 Confirmation of recombinant pBI 121plasmid containing CMV CP insert in *Agrobacterium* strain EHA105 by PCR. Lane M: 100 bp DNA ladder, Lane 1& 2: amplification using CMV CP specific primers that gave expected band at 650 bp.

2.2.2 *Cucumber mosaic virus (*CMV) coat protein in sense orientation

A selected clone where CMV CP got cloned in sense orientation was restricted with *Bam* H1 and *Sac*1. The resulting fragment containing CMV CP region was isolated from the gel and ligated into pBI 121 restricted with same enzymes (*Bam* H1 and *Sac* 1). Competent *E. coli* (strain DH5α) was used for transformation. The presence of recombinant pBI 121 containing CMV CP gene was confirmed by isolating the plasmid and subjecting it to restriction analysis and PCR using primer designed for CaMV 35S promoter region as forward and CMV CP reverse primer (Fig. 11). This was then mobilized into *Agrobacterium tumefaciens* strain EHA 105 by triparental mating with the help of helper plasmid pRK 2014 and transformants were selected on kanamycin and rifampicin plates. The presence of recombinant pBI 121 containing CMV CP gene (sense orientation) in *A. tumefaciens* was confirmed by isolating the plasmid and subjecting it to restriction analysis and PCR (Fig. 12). The construct was designated as pBI121CMVS. The map of CMV CP gene construct in pBI121 is shown in Fig. 13

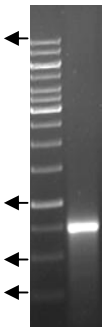


Fig. 11 Confirmation of recombinant pBI121 plasmid containing CMV CP insert in *E .coli* by PCR. Lane M: 1 Kb ladder; Lane 1: amplification using primer designed for CaMV 35S promoter region and CMV CP reverse showing expected amplicon.

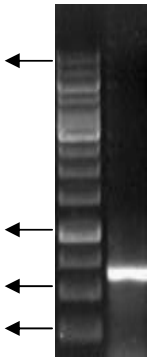


Fig.12 Confirmation of recombinant pBI121plasmid containing CMV CP insert in *Agrobacterium* strain EHA 105 by PCR. Lane M: 1 Kb DNA ladder, Lane 1: Amplification using CMV CP specific primers that gave expected band at 650 bp.

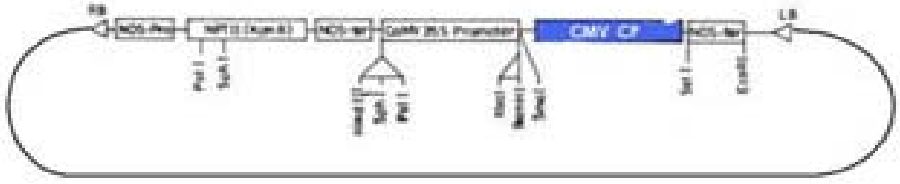


Fig. 13 CMV CP gene construct in pBI121

3. Transformation of black pepper

Based on reports from transformation experiments in black pepper and other crops, leaf, internode, petiole, embryo along with micropylar tissue and embryogenic mass were tested as explants for transformation.

3.1 Transformation using leaf, internode and petiole explants

3.1.1 Establishment of *in vitro* mother cultures of Black pepper

Nodal explants were collected from *Panniyur I* and *IISR Subhakara* varieties of black pepper. These explants were treated with 0.2% bavistin for 15-20 min and washed thoroughly with sterile distilled water to remove traces of fungicide. This was followed by treatment with 0.1% mercuric chloride for 4-6 min. After washing with sterile distilled water for 3-4 times the explants were directly inoculated in to basal Woody plant medium (WPM) and incubated at 26 ± 2^0C at light intensity of 1500 lux and 10 h photoperiod. Explants were periodically observed and contaminated ones were removed. Explants harvested from these fully grown *in vitro* plants were used for transformation (Fig. 14).

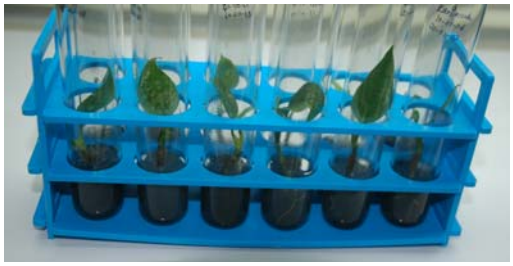


Fig. 14 *In vitro* mother cultures of black pepper

3.1.2 Transformation using PYMoV sense (pBI121PYMoVS) and antisense (pBI121PYMoVAS) constructs

PYMoV ORF III construct containing 409 bp region in sense and antisense orientation designated as pBI 121 PYMoVS and pBI 121 PYMoVAS respectively were used for transformation of different explants like leaf, internode and petiole. Explants harvested from *in vitro* grown plants were co-cultivated with *Agrobacterium* harbouring respective constructs for 15 min, blot dried and kept in basal MS medium at 28ºC for 48 h. Explants were then washed in cefotaxime and streptomycin (both at 100 µg/ml) and kept in basal MS containing cefotaxime and streptomycin (both 100 µg/ml) to kill *Agrobacterium* and other bacterial contaminants. Details of explants survived after co-cultivation with both the constructs is given in Table 1. Among the different explants, leaf explants showed better survival (61.5 and 44%) followed by petiole (53 and 26%) and internode (45 and 11%) in sense and antisense constructs. About 38.5 to 89% of explants either died or got contaminated within 15 days. The death of explants is mainly due to phenolic exudates from cut ends while contamination was mainly due to bacteria and in a few cases with fungi.

Table 1. Survival of explants up to 15 days after co cultivation

Construct	**No. of explants co-cultivated**			% explants survived upto 48 h			% explants survived after 15 days		
	Leaf	**Internode**	**Petiole**	**Leaf**	**Internode**	**Petiole**	**Leaf**	**Internode**	**Petiole**
PYMoV antisense	**813**	244	466	**74.8**	**77.9**	**79.2**	**61.5**	**45**	**53**
PYMoV sense	233	234	213	81	67	53	44	11	26

After 15 days the surviving explants were transferred to 16 different regeneration media containing different concentrations of BAP, NAA, TDZ and kinetin along with selective marker, kanamycin. The response of explants to different media is presented in Table 2. Callus formed readily from leaf, internode and petiole within 30 days when they were cultured both on SH and MS media supplemented with BAP, NAA and TDZ (Fig.15, 16). In the case of leaf explants, maximum callusing (92.8%) was seen in ½ SH medium containing 3 mg/l of BAP and 1 mg/l and NAA respectively followed by ½ SH medium containing 1 mg/l of BAP and 1

mg/l of NAA (76.5%) and MS medium containing 1, 1 and 0.1 mg/l of BAP, NAA and TDZ respectively (76.5%). Minimum callusing in leaf explant was seen in MS medium containing 1mg/l each of kinetin and NAA respectively (14.2 %). Types of callus observed in different phytohormone combinations were friable creamish, white powdery and hard callus (both green and creamy). Out of five greenish hard callus in BAP and NAA both at 1 mg/l in MS upon continuous subculture in the same media, one callus gave organogenesis (Fig. 17).Another two hard greenish callus gave shoot bud induction within one month in B5 medium containing kinetin 1.5mg/l and NAA 0.25mg/l (Fig.18a&18b).These shoot buds were transferred to different media combinations for complete plant development. Many of the creamish and white powdery callus with time turned brown and then black leading to death (Fig. 19a, b).

Fig. 15 Leaf explants with creamish callus showing creamish callus

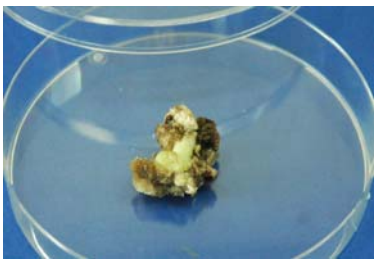


Fig. 16 Internode explants

Fig. 17 Greenish callus obtained from leaf tissue transformed with pBI121PYMoVAS showing organogenesis

a

b

Fig. 18. Transformed leaf explants with pBI121PYMoVAS showing shoot bud induction

a **b**

Fig. 19 Death of callus derived from leaf (a) and internode (b) due to browning

Use of kinetin (1 mg/l) along with NAA (1-2 mg/l) in both SH and MS medium induced rooting in the leaf explants within 15 days of culture (Table 2). Rooting as well as callusing was seen in leaf explants placed in MS medium added with both 3 and 1 mg/l of BAP and NAA or MS with 1mg/l each of kinetin and NAA. In regeneration media also death of explants were seen mainly due to browning and excess growth of *Agrobacterium*.

In the case of internode and petiole explants, 100% callusing was observed in SH media supplemented with 3 or 1 mg/l of BAP, 1 mg/l of NAA and 0.1mg/l of TDZ. While no response was seen SH medium supplemented with 2 mg/l of BAP and 1 mg/l of NAA (Table 2). In contrast, only 14-30% of petiole explants showed callusing in MS medium added with different hormones. Both internode and petiole explants produced two types of callus (creamish friable and white powdery) in the culture. A combination of NAA and kinetin each at 1 mg/l induced rooting in 14 to 100% of explants.

Table 2. Response of different explants of black pepper co cultivated with pBI121PYMoVAS construct to varying levels of phytohormones

Media	% explants showing response		
	Leaf	Internode	Petiole
SH_0 + 3 mg/l BAP+1 mg/l NAA	35.7 (C)	100 (C)	100 (C)
SH_0 + 1mg/l BAP +1 mg/l NAA	21.4 (C)	100 (C)	100 (C)

SH_0 + 1 mg/l BAP+1mg/l NAA+0.1mg/l TDZ	56 (C)	100 (C)	100 (C)
SH_0 + 2 mg/l BAP + 1 mg/l NAA	35.7 (C)	NR	NR
SH_0 + 1 mg/l kinetin + 1 mg/l NAA	71 (R)	30 (R)	100 (R)
SH_0 + 1 mg/l kinetin + 2 mg/l NAA	71 (R)	-	100 (R)
1/2 SH_0 + 3 mg/l BAP+1 mg/l NAA	92.8 (C)	NU	NU
1/2 SH_0 + 1mg/l BAP + 1 mg/l NAA	76.5 (C)	NU	NU
1/2 SH_0 + 1 mg/l BAP+1mg/l NAA+0.1mg/l TDZ	71.4 (C)	NU	NU
1/2 SH_0 + 1 mg/l kinetin + 1 mg/l NAA	35.7 (R)	NU	NU
1/2 SH_0 + 1 mg/l kinetin + 2 mg/l NAA	50 (R)	NU	NU
MS_0 + 3 mg/l BAP+1 mg/l NAA	64 (C), 21 (C+R)	-	30 (C)
MS_0 + 1mg/l BAP +1 mg/l NAA	35 (C)	-	30 (C)
MS_0 + 1 mg/l BAP+1mg/l NAA+0.1mg/ml TDZ	76.5 (C)	-	-
MS_0 + 1 mg/l kinetin + 1 mg/lNAA	35.7 (R) 14.2 (C+R)	-	14.2 (R)
MS_0 + 1 mg/l kinetin + 2 mg/l NAA	21.4 (R)	-	-

C, Callusing; R,Rooting; NU, Not used; NR, no response; -, lost due to death/contamination **.**

Transformation carried out using explants like leaf, internode and petiole gave organogenesis in very few cases and no further differentiation and plantlet production was seen. Hence, as an alternative, transformation of black pepper through somatic embryogenesis was attempted.

3.2 Transformation using embryogenic mass as explants

3.2.1 Direct somatic embryogenesis from germinating seeds of black pepper

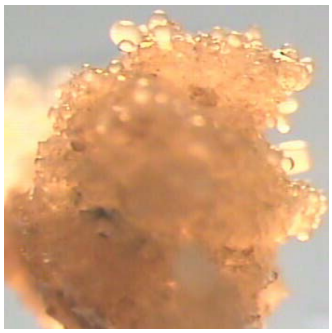
Mature seeds of black pepper (cvs.*Karimunda* and *Panniyur 1)* collected from plants grown in the germplasm repository at the Indian Institute of Spices Research, Calicut were used for establishing primary somatic embryogenic cultures. Embryo along with surrounding micropylar tissue scooped out from the surface sterilized seeds were cultured on agar gelled full-strength, Plant growth regulator (PGR) free SH (Schenk and Hildebrandt 1972) medium containing 3.0% (w/v) sucrose under darkness. Primary somatic embryos (PEs) derived from micropylar tissues of germinating seeds were seen after 90 days (Fig. 20). Somatic embryo induction was

higher in *Karimunda* (14%) than *Panniyur 1* (3%). Average number of primary somatic embryos/seed was 4-6 in *Karimunda* where as it was 1-3 in the case of *Panniyur 1*.

Fig. 20 Germinating seed showing primary somatic embryogenesis

3.2.2 Induction of cyclic secondary somatic embryogenesis and establishment of embryogenic mass

Primary somatic embryo clumps having pre-globular to torpedo shaped embryos (4–6 visible embryos per seed) were carefully detached and inoculated on full strength, PGR free SH medium containing 1.5% sucrose and gelled with 0.8% agar and incubated in complete darkness. Secondary embryos were visible from the root pole of the primary embryos within 10 days of culture. The brownish yellow tissue at the root pole of the primary embryo proliferated into a small mass of tissue (Fig. 21a) from which several secondary embryos emerged (Fig.21b). Embryogenic mass was maintained by sub culturing in to fresh medium of same composition in at monthly interval. However *Panniyur 1* did not show response to secondary embryogenesis. So all the further experiments were carried out with *karimunda* derived embryogenic mass



a

b

Fig. 21 Proliferation (a) and emergence of cluster of secondary embryos (b) from

embryogenic mass

3.2.3 Conversion of embryogenic mass in to fully developed plantlets

The proliferated embryogenic mass (250 mg) was transferred to basal SH (liquid) with 3% sucrose for further development of embryos into fully developed plantlets under dark with shaking at 110 rpm for 30 days. Medium was replenished in every 10 days. When the flask was crowded with well differentiated plantlets they were allowed to grow under 12 h day light. The plantlets were maintained in this condition until they grew and produced two to three green leaves (Fig. 22). These well developed plants were transferred to woody plant medium with 3% sucrose, 0.8% agar and 0.2% charcoal (Fig. 23). About 75-100 plantlets were obtained from every 250 mg embryogenic mass.

3.2.4 Selection of suitable explants for transformation and optimization of antibiotic concentration

3.2.4.1 Explants and their preparation

Two types of explants *i.e.* embryo along with surrounding micropylar tissue and embryogenic mass were used as explants.

Fig. 22 Plantlets in liquid medium

Fig. 23 Developed plantlets in WPM medium

3.2.4.2 Antibiotics used

Antibiotics used in the experiment were cefotaxime, carbenicillin and kanamycin.

3.2.4.3 Effect of carbenicillin and cefotaxime

3.2.4.3.1 Effect on embryo along with micropylar tissue

Embryo along with micropylar tissue was treated with five different concentrations (25, 50, 100, 250, 500 µg/ml) of cefotaxime and carbenicillin to test its effect on somatic embryo induction. Twenty five embryos were used in each treatment. Treatment without antibiotics served as control. Embryo along with micropylar tissue was placed on SH medium containing 3% sucrose gelled with 0.8% agar and antibiotics of respective concentration followed by incubation under darkness for 90 days. Number of embryos showing primary somatic embryo induction was recorded. Carbenicillin at all tested concentrations and cefotaxime >100 µg/ml inhibited primary somatic embryo induction. About 2-3% of embryos showed primary somatic embryo induction in presence of cefotaxime concentration ranging from 25-100 µg/ml while in control 14% of embryos showed primary somatic embryo induction.

3.2.4.3.2 Effect on embryogenic mass

Embryogenic mass was treated with different concentrations of antibiotics to test their effect on somatic embryo proliferation and growth of plantlets. Five different concentrations (25, 50, 100, 250, 500 µg/ml) of cefotaxime and carbenicillin were used. In order to get uniform number of embryos in all treatments, about three gram of embryogenic mass was suspended in 100 ml SH medium with 3% sucrose and kept under dark with shaking at 110 rpm for 2 days. From this 5 ml culture was added to 45 ml SH medium with 3% sucrose to make total volume 50 ml. Antibiotics of each concentration was added in to this and incubated under dark with shaking at 110 rpm for 30 days. Treatment without antibiotics served as control. Medium was replenished at every 15 days. After 30 days plantlets were allowed to grow under light for 15 days. Number of fully developed plants and length of plantlets were taken after 45days. The experiment was performed in three replications and results were analyzed using M stat C software. The effects of carbenicillin and cefotaxime on embryogenic mass was evaluated based on the number and growth of plantlets produced after 45 days of culturing of embryogenic mass on SH medium containing different antibiotic concentrations. Of the two antibiotics, carbenicillin was more inhibitory to number of plantlet produced. Maximum (108.3) and minimum (8.66) number of plantlets were produced in treatments containing 100 µg/ml of cefotaxime and 500 µg/ml of carbenillicin respectively (Table 3). Results clearly showed that cefotaxime at 100

µg/ml produced significantly higher number of plantlets (108.3) compared to control (62). In general addition of carbenillicin at all tested concentrations significantly reduced number of plantlets produced compared to corresponding concentrations of cefotaxime. In both antibiotics, number of plantlets produced increased with increasing concentration of antibiotics up to 100 µg/ml, thereafter decrease in number plantlets was seen. With regard to plantlet growth, maximum growth of 3.233 cm was seen when embryogenic mass was treated with carbenellicin at 100 µg/ml which was on par with control (3.5 cm) (Table 3). Minimum growth of plantlets (0.8 cm) was recorded with carbenicillin at 500 µg/ml. In general, increase in antibiotic concentration up to 100 µg/ml increased plant growth while further increase >100 µg/ml resulted in decreased growth. Both antibiotics at all concentrations (except carbenicillin at 100 µg/ml) retarded plantlet growth compared to control (Table 3).

3.2.4.4 Optimization of minimum lethal concentration of kanamycin for the selection of transformants

Embryo along with micropylar tissue, embryogenic mass and fully developed plantlets were used as explants to determine the optimum concentration of kanamycin required for the selection of transformants. The different concentrations of kanamycin used were 10, 25, 50, 100, 200 µg/ml.

Table 3. Effect of cefotaxime and carbenicillin on somatic embryo proliferation and plantlet growth

Antibiotic concentrat-ion (µg/ml)	Proliferation (No. of fully developed plantlets obtained)		Plant growth (Mean length of plants in cm)	
	Cefotaxime	Carbenicillin	Cefotaxime	Carbenicillin
25	54.33^{bc}	16.33^{fg}	2.367^{c}	2.300^{cd}
50	48.00^{c}	25.33^{ef}	2.533^{c}	2.867^{b}
100	108.3^{a}	43.67^{cd}	2.433^{c}	3.233^{a}
250	48.67^{c}	21.33^{f}	2.433^{c}	2.000^{d}
500	34.33^{de}	8.667^{g}	1.300^{e}	0.8000^{f}
Control	62.00^{b}		3.500^{a}	
LSD	11.45		0.3177	

3.2.4.4.1 Effect on embryo along with micropylar tissue

Embryo along with micropylar tissue was treated with different concentrations of kanamycin to test its effect on embryo germination and somatic embryo induction. Twenty five embryos were used in each treatment. Embryo along with micropylar tissue was placed on SH medium containing 3% sucrose gelled with 0.8% agar and different concentrations of kanamycin (10, 25, 50, 100, 200 µg/ml) followed by incubation under complete darkness. Percentage of zygotic embryo germination and somatic embryo induction was recorded after 90 days. The experiment was repeated three times. Results showed that kanamycin at all concentration tested inhibited the germination of zygotic embryo and primary somatic embryo induction.

3.2.4.4.2 Effect on embryogenic mass

Embryogenic mass was treated with different concentrations of kanamycin to test its effect on somatic embryo proliferation. Each treatment consisted 250 mg of embryogenic mass placed on SH medium containing different concentrations of kanamycin (10, 25, 50, 100, 200 µg/ml) along with 1.5% sucrose and gelled with 0.8% agar followed by incubation under complete darkness for 30 days. Number of somatic embryos produced in each treatment was recorded. The experiment was performed in three replications and results were analyzed using M stat C software. Embryogenic mass treated with kanamycin at lower concentrations (10 and 25 µg/ml) survived although there was significant reduction in number of somatic embryos produced (Table 4). At 50 µg/ml of kanamycin, blackening around embryogenic mass and at100 µg/ml complete blackening of embryogenic mass and only a few embryos produced (3.3). At 200 µg/ml of kanamycin, complete blackening of embryogenic mass and only 0.6 embryos were produced.

Table 4. Effect of kanamycin on somatic embryos proliferation

Kanamycin concentration in µg/ml	No. of somatic embryos produced
10	89.67 [b]
25	29.33 [c]
50	12.67 [cd]
100	3.333 [d]
200	0.6667 [d]
Control	112.0 [a]

LSD	20.65

3.2.4.4.3 Effect on fully developed plantlets

In order to test the effect of kanamycin on plantlets, non transformed plantlets of 2-3 leaf stage were treated with different concentrations of kanamycin. For each treatment, 10 plantlets were placed on woody plant medium containing kanamycin of respective concentration with light conditions of 13/11 h (light/dark) and the temperature of 25±2°C. Percentage of bleached plantlets was taken after 30 days and the experiment was repeated three times. Effect of kanamycin at plantlet stage showed that plantlet treated with kanamycin up to 50 µg/ml, survived without bleaching. Plantlets treated with 100 and 200 µg/ml of kanamycin started bleaching within 15 days and after 30 days, 60% and 100% of plantlets were bleached respectively.

3.2.5 Standardization of transformation using GUS construct and analysis of transformants

3.2.5.1 *A. tumefaciens strain* and plasmid

The *A. tumefaciens* strain EHA 105 (Hood et al. 1993) harboring a binary vector, pBI121was used in the present study. The T-DNA region of the binary vector pBI121 contained the neomycin phosphotransferase II (NPT II) gene under the control of the nopaline synthase (NOS) promoter, and the β-glucuronidase (GUS) gene with an intron (GUS-intron) fused to the CaMV35S promote. Bacterial colony was inoculated into liquid YEB medium (peptone 5 g/l, yeast extract 1 g/l, beef extract 5 g/l, sucrose 5 g/ l, magnesium sulphate hepta hydrate 0.24 g/l) containing kanamycin 50 mg/l (kanamycin monosulfate; Himedia, India) and rifampicin 50 mg/l (rifampicin; Himedia, India), and incubated for more than 18 h at 28°C in incubator shaker (160 cycles /min).

3.2.5.2 Optimization of carbenicillin and cefotaxime concentration to control *Agrobacterium* over growth

In order to find out the optimum concentration of antibiotics (cefotaxime and carbenicillin) to control *Agrobacterium* over growth from transformed tissue, different concentrations (25, 50, 100, 250, 500 µg/ml) of cefotaxime and carbenicillin were tested. About 250 mg of embryogenic mass was used in each treatment. Explants were infected (with intermittent vigorous shaking for 2 h) with $1/5^{th}$ diluted 18 h grown culture (OD_{600}-1.5) of *A. tumefaciens* EHA 105 containing the binary plasmid pBI121. After the agro-infection, the bacterial suspension was removed by passing the contents through a sterile filter paper (Whatmann No.2). The nearly dry explants were then co-cultured in dark on basal SH medium with 3% sucrose for 48 h. Then it was transferred to selection medium (SH basal +1.5% sucrose + antibiotics of respective concentration), and the plates were cultured for 3–5 weeks in darkness. *Agrobacterium* over growth in explants were recorded. Cefotaxime at 25 µg/ml and above or carbenicillin at 50 µg/ml and above were able to control the *Agrobacterium* growth from co cultivated embryogenic mass up to 30 days while carbenicillin at 25 µg/ml failed to control the *Agrobacterium* overgrowth.

Embryogenic mass showed cyclic secondary embryogenesis differentiation in to plantlets in presence of antibiotics indicating that embryogenic mass can be used as ideal explants for *Agrobacterium* mediated transformation experiments in black pepper. Embryogenic mass treated with cefotaxime at 100 µg/ml in addition to enhanced somatic embryo proliferation was also able to control *Agrobacterium* over growth and hence this concentration of cefotaxime was used for all further experiments.

3.2.5.3 Selection of transformants using kanamycin

Results indicated that kanamycin at 200 µg/ml would give complete selection of non transformed plants both at embryogenic mass and at plantlet stage. But we presume that such a high concentration at the initial stage itself may cause death and malformation of transformed tissue. So we adopted the following strategy for selection. Embryogenic mass after co-cultivation with *Agrobacterium* in the regeneration medium were subjected to selection in three stages with increasing concentration of antibiotic: initially at 25 µg/ml for 3-5 weeks followed by 50 µg/ml for next 30 days. At plantlet stage selection at 100 µg/ml of kanamycin was adopted.

3.2.5.4 Transformation and regeneration

About 1g of embryogenic mass was infected (Fig. 24) (with intermittent vigorous shaking for 2 h) with 1/5th diluted 18 h grown culture (OD_{600}-1.5, OD_{600}=1.0 corresponds to 1 X10^{8} cells/ ml) of *A. tumefaciens* EHA 105 containing the binary plasmid pBI121. The co-cultured embryogenic mass was transferred to selection medium (SH basal +1.5% sucrose +100 µg/ml cefotaxime +25 µg/ml kanamycin), and the plates were cultured for 3–5 weeks under darkness. Some vigorous growing points were observed in the first round of selection and growing points were removed onto the same medium with a higher kanamycin concentration (50 µg/ml) for another 30 days for further proliferation. The proliferated embryogenic mass was then transferred to basal SH (liquid) with 3% sucrose and 100 µg/ml cefotaxime (with out kanamycin) for further development of embryos into fully developed plantlets under dark with shaking at 110 rpm for 30 days. After 30 days plantlets were allowed to grow under light for 15 days. Fully developed plantlets were transferred to woody plant medium (WPM) with 3% sucrose, 0.8% agar, 100 µg/ml kanamycin and 0.2% charcoal. Plants started showing bleaching within 15 days and non bleached plants up to 30 days were transferred to same medium but without kanamycin. Surviving plants were hardened in sterile potting mixture (sand, soil, farmyard manure-1:1:1) in the green house and screened for the presence of transgene by PCR and Southern hybridization. Out of 130 plants regenerated, randomly selected 50 plants were used for transgene analysis.

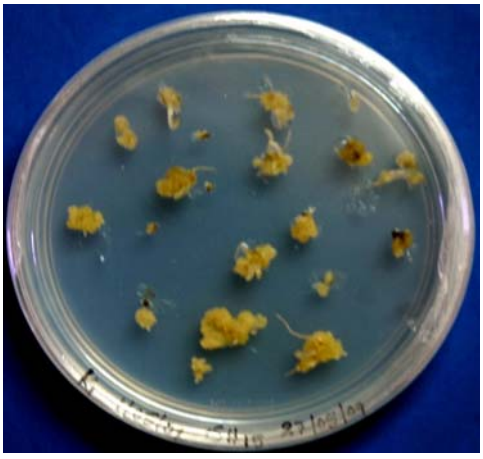
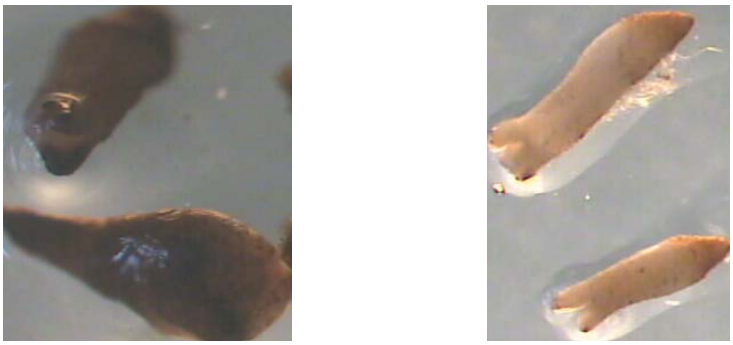


Fig. 24 Embryogenic mass used for transformation

3.2.5.5 Assay for GUS activity

The GUS expression in putative transformed embryogenic mass was determined histochemically by using 'β-Glucuronidase (GUS) Reporter Gene Staining Kit' (*Sigma*, Missouri, USA) by following the manufacturer's instructions. The possibility of endogenous GUS expression was tested by subjecting uninfected somatic embryos and tissues to the histochemical GUS assay. GUS expression was visually observed and photographed under a light stereo-zoom microscope (Nikon HF II). The β-glucoronidase enzyme catalyses conversion of colourless 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA) into blue coloured 5-bromo-4-chloro-3-indole. The results showed uniform blue colour in transformed embryogenic mass in contrast to the colourless negative control after overnight incubation with the substrate suggesting that the *gus*A gene is transcribed from the transformed embryogenic mass and dictates the synthesis of functional enzyme (Fig. 25). The colourless nature of non transformed embryogenic mass after staining indicate that there is no detectable endogenous GUS activity in non-transformed control embryogenic mass.



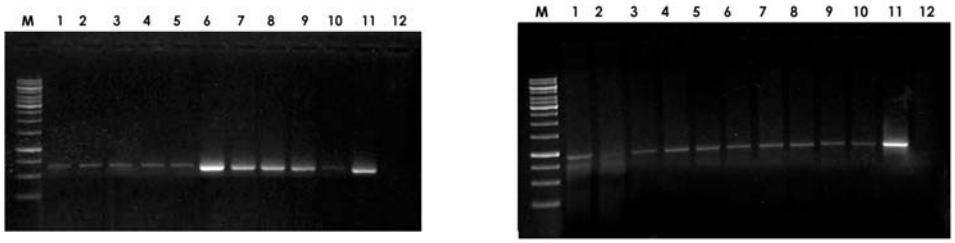
(a) **(b)**

Fig. 25 GUS histochemical assay of embryogenic mass (a) transformed embryo (b) non transformed embryo

3.2.5.6 PCR analysis of transformed plants

DNA isolated from young unfolded leaves (100 mg) of putative transformed and untransformed control plants using a CTAB procedure (Hareesh and Bhat, 2008) was subjected to PCR using primers specific to *npt -II* and *gus*A genes. A 980bp fragment of the *npt II* gene was amplified using the forward (5′CAACGTTGAAGGAGCCAC3′) and reverse (5′ ACGAGGAAGCGGTCAGC

3′) primers. Each 25 µl PCR reaction mixture contained 50 ng genomic DNA as template, 200 µM dNTPs, 1.5 mM $MgCl_2$, 15 pM each of forward and reverse primers, and 1 unit *Taq* DNA polymerase in 1x reaction buffer. The PCR conditions included 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 30 min. Similarly, a 660bp fragment of *gus A* was amplified with forward (5′GGGCATTCAGTCTGGATC3′) and reverse (5′ GTGCGGATTCACCACTTG 3′) primers using the same temperature profile as above. Intact pBI 121 plasmid DNA was used as positive control and the untransformed plantlets derived from embryogenic mass were used as negative control. After PCR, the amplified products were separated by electrophoresis in 0.8% agarose gels and photographed in Alpha imager (*Alpha Innotech Corporation*, California). Molecular sizes of the amplification products were estimated using a standard molecular weight ladder (*Fermentas*, Praha, Czech Republic). PCR analysis of 50 independent kanamycin-resistant lines revealed the specific predicted amplification products of 940 bp and 660 bp with *npt* II- and *gus*A gene-specific primers, respectively while no such bands were visible in non-transformed plantlets (Fig. 26a, 26b). This indicated the presence of both the linked genes *npt* II and *gus*A as a single T-DNA in the transformed genome. Out of 50 plants analyzed 40 were found to be positive in PCR.



a **b**

Fig. 26 PCR analysis of transformed plants. (a) amplification of npt II (940 bp) and (b) amplification of GUS (660 bp) genes. Lane M-1 Kb ladder,Lane1-10: Transformed plantlets, Lane 11- Positive control, Lane 12: Negative control

3.2.5.7 Southern hybridization of transformed plants

Genomic DNA isolated from the leaf tissue (1g) by CTAB method (Hareesh and Bhat, 2008) was subjected to Southern blot hybridization analysis using standard protocols (Sambrook and Russell, 2001). Fifteen µg of sample DNA was restricted and size-fractionated in a 0.7% agarose gel by electrophoresis, subsequently

transferred to a nylon membrane (Porablot NY plus, *Macherey Nagel*, Germany) by capillary method (Sambrook and Russell, 2001). A 940 bp DNA fragment corresponding to the kanamycin gene was labeled with digoxigenin and used as a probe for hybridization according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Plasmid vector pBI121, after digestion with same restrtiction enzyme (250 ng) was used as a positive control while genomic DNA from a non-transformed plant served as a negative control. Hybridization procedures and incubation with chemiluminescence substrate CSPD were carried out in accordance with the manufacturer's instructions. The tracks of chemiluminescence were captured, developed and fixed in X-ray film (Kodak) as per standard procedures. Southern analysis confirmed the stable integration of the *npt II* gene in 4 of the putative transgenic plants out of 6 plants screened which apparently indicates that 66% of the PCR positive plants were positive in Southern as well. In all the southern positive plants only single copy of transgene was detected.

3.3 Transformation of embryogenic mass with *Piper yellow mottle virus* (PYMoV) sense and antisense constructs

A portion of *Piper yellow mottle virus* (PYMoV) open reading frame ORF III (409 bp) in sense and antisense orientation as transgenes in *A. tumefaciens* carrying the recombinant binary vector, pBI121 were used for transforming black pepper. PYMoV sense and antisense constructs were designated as pBI121PYMoVS and pBI121PYMoVAS respectively. Transformation protocol followed was same as mentioned in the previous section. All hardened and survived plants (Fig. 27) were subjected to PCR to confirm the transgene presence.

Fig. 27 Hardened transformed plants (pBI121PYMoVAS) in green house

3.3.1 PCR analysis of transformed plants

PCR was performed using two sets of primers. One set primer was specific for kanamycin region while the other set was primer designed for the CaMV 35S promoter region (as forward primer) and reverse primer of ORF III region (as reverse primer) in the case of pBI121PYMoVS transformed plants. CaMV 35S promoter region (as forward primer) and forward primer of ORF III region (as reverse primer) were used to test transformed plants using pBI121PYMoVAS construct. Kanamycin specific primers gave an amplicon of approximately 940bp (Fig. 28) in positive plants where as the other set primers gave an amplicon of 539bp (Fig. 29) in case of both sense and antisense constructs. In the case of pBI121PYMoVS construct all the 30 plants screened were positive in PCR where as in pBI121PYMoVAS construct out of 78 plants screened, 62 plants tested as positive in PCR (Table 4). Selected PCR positive plants were subjected to dot blot assay.

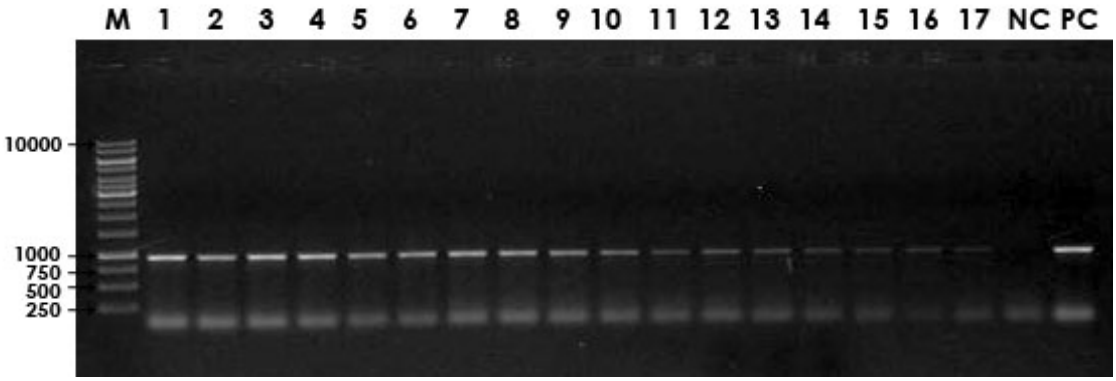


Fig. 28 Screening of pBI121PYMoVAS transformed putative transformants using npt-II specific primers. Lane M: 1 Kb ladder, Lane 1-17: transformed plantlets, Lane NC: Negative control, Lane PC: Positive control

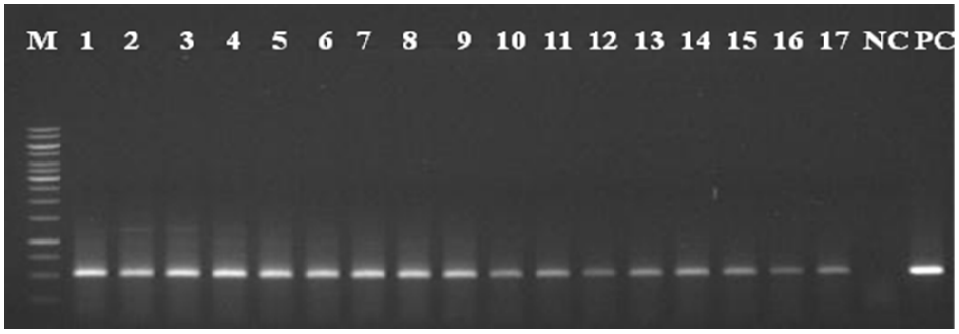


Fig. 29 Screening of pBI121PYMoVAS transformed putative transformants using primers specific for CaMV 35 S promoter region and PYMoV ORF III. Lane M: 1Kb ladder, Lane 1- 17: transformed plantlets, Lane NC: Negative control, Lane PC: Positive control

3.3.2 Dot blot hybridization to determine the presence of transgene

Total genomic DNA isolated from100 mg leaves of hardened plants were used for dot blot hybridization. About 1µg of DNA was dotted in to nitrocellulose membrane (porablot NY plus, Macherey Nagel, Germany) and fixed by heating at 80^0C for 2 h. A 940 bp DNA fragment corresponding to the kanamycin gene was labeled with digoxigenin and used as a probe for hybridization according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). The hybridized membrane was washed, subjected to chemilluminescent development using CSPD substrate, and then exposed to X-ray film. Plasmid pBI121 (250 ng) was used as a positive control while genomic DNA from a non-transformed plant served as a negative control. Presence of dark spots in X-ray film indicated positive samples (Fig. 30). In the case of pBI121PYMoVS construct out of 30 plants subjected to dot blot, 24 plants gave positive signal where as out of 44 plants in pBI121PYMoVAS construct screened, 38 were found to be positive. Plants that gave intense spots in dot blot assay were taken for Southern hybridization.

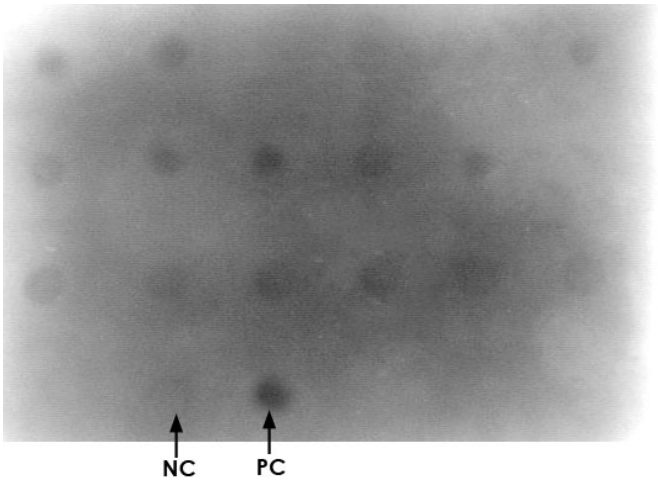


Fig. 30 Dot blot assay of selected PCR positive plants of PYMoV antisense construct (pBI121PYMoVAS). Dark spots indicate positive signals. NC- Negative control, PC- Positive control.

3.3.3 Southern hybridization

Ten microgram of total DNA was digested with *Hind* III, separated by electrophoresis in a 0.8% (w/v) agarose gel and subsequently transferred to a nylon membrane (Porablot NY plus, *Macherey Nagel*, Germany) by capillary method (Sambrook and Russell, 2001). A 940 bp DNA fragment corresponding to the kanamycin gene was labeled with digoxigenin and used as a probe for hybridization according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). The hybridized membrane was washed, subjected to chemilluminescent development using CSPD substrate, and then exposed to X-ray film. Plasmid pBI121 (250 ng) was used as a positive control while genomic DNA from a non-transformed plant served as a negative control. In pBI121PYMoVS construct, two plants showed positive signals out of 10 screened where as four out of seven tested turn to be positive in the case of pBI121PYMoVAS construct (Fig. 31).

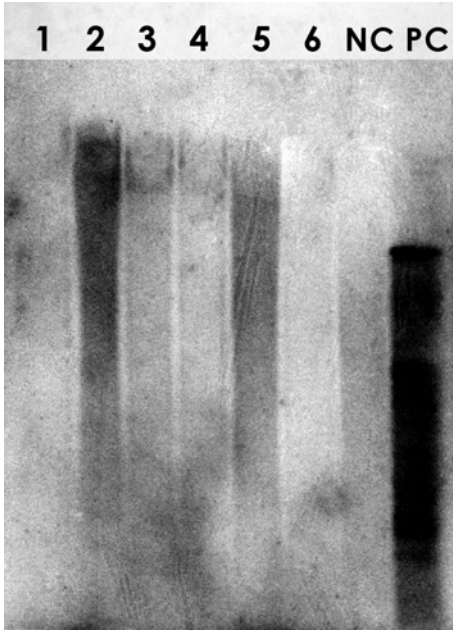


Fig. 31 Southern hybridization of putative transformants of PYMoV antisense construct (pBI121PYMoVAS). Lane 1-6: Putative transformants tested positive in dot blot assay, NC: Negative control, PC: Positive control

3.4 Transformation of black pepper explants with *Cucumber mosaic virus* coat protein (CMV-CP) sense construct and analysis of transformants

Viral construct consisting of sense sequence of *Cucumber mosaic virus* coat protein (CMV-CP) gene as transgene in *A. tumefaciens* carrying the recombinant binary vector, pBI121 were used for transforming black pepper. This CMV-CP construct was designated as pBI121CMVS. Protocol for transformation was same as mentioned in the previous section. All hardened survived (Fig. 32) plants were subjected to PCR using two sets of primers. One set primer was kanamycin specific and the other set was primer designed for the CaMV 35S promoter region as forward primer and reverse primer of CMVCP as reverse primer. Kanamycin specific primers gave an amplicon of around 940bp in positive plants where as the other set of primers gave an amplicon at 780bp (Fig. 33). Out of 109 plants screened, 104 plants found to be positive in PCR (Table 5). Selected PCR positive plants were subjected to dot blot assay as indicated above. Out of 80 plants subjected to dot blot, 43 plants gave positive signals (Fig. 34). Plants that gave intense spots in dot blot assay were taken for Southern hybridization. All the nine plants tested were found positive in Southern hybridization (Fig. 35).

Fig. 32 Hardened transformed plants (pBI121PYMoVAS) in green house

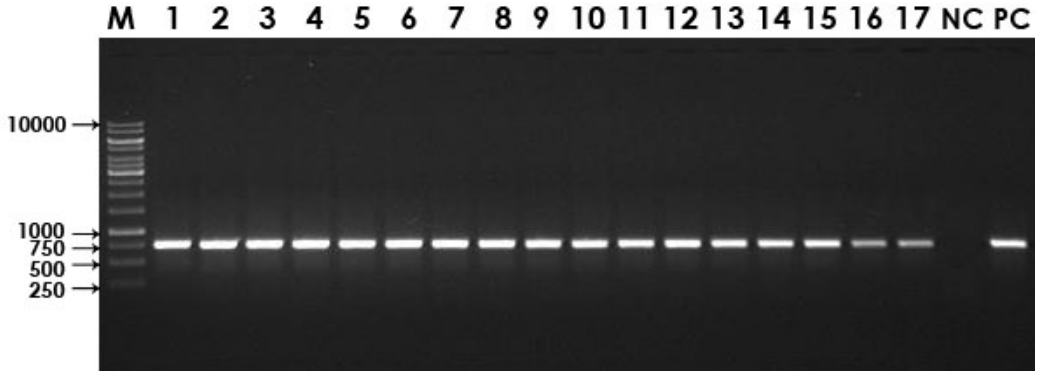


Fig.33 Screening of transformed putative transformants of CMV-CP sense construct (pBI121CMVS) using primers specific for CaMV 35S promoter region and CMV-CP. Lane M: 1Kb ladder, Lane 1- 17: transformed plantlets, Lane NC: Negative control, Lane PC: Positive control

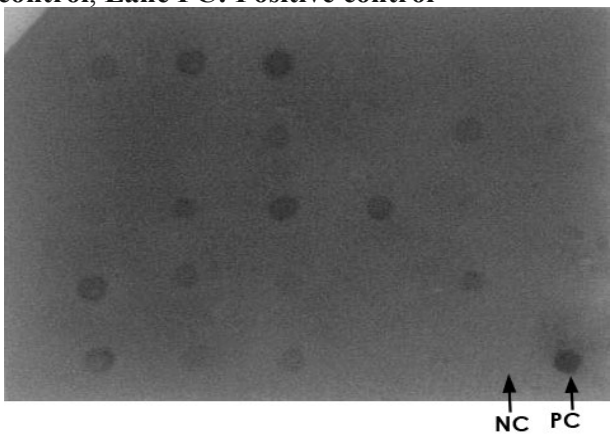


Fig.34 Dot blot assay of selected PCR positive plants of CMV-CP sense construct (pBI121CMVS). Dark spots indicate positive signals. NC- Negative control, PC- Positive control

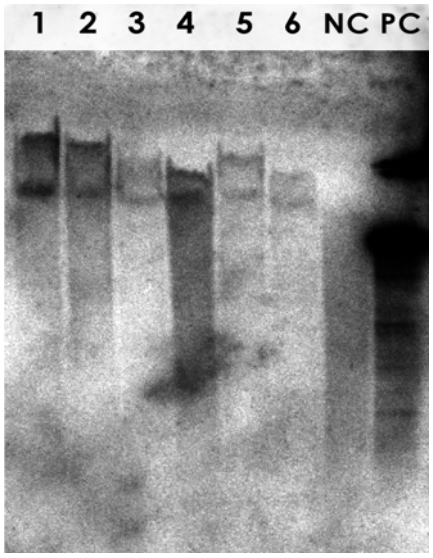


Fig.35 Southern hybridization of putative transformants (pBI121CMVS). Lane 1-6: Putative transformants tested positive in dot blot assay, Lane NC: Negative control, Lane PC: Positive control

Table 5. Number of putative transformants hardened and screened for the presence of transgene by PCR, dot blot and Southern hybridization in different constructs

Construct	No. of plants hardened	No. of plants survived	No. of plants positive in PCR	No. of plants positive in dot blot	No. of plants positive in southern
pBI121PYMoVS	50	30	30/30	24/30	2/10
pBI121PYMoVAS	228	106	62/78	38/44	4/7
pBI121CMVS	156	109	104/109	43/80	9/9

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8222 Questions- Answered

Developed an *Agrobacterium* mediated genetic transformation protocol for black pepper using embryogenic mass as explant.

Prepared plant transformation vector constructs using CMV and PYMoV sequences and using these constructs transgenic black pepper plants were produced. Presence of transgene sequence in the transgenic plants confirmed through molecular tests.

8223 Process/ Product/ Technology/ Developed

An efficient protocol for transformation of black pepper
Plant transformation vector constructs carrying CMV and PYMoV sequences
Transgenic black pepper plants carrying CMV and PYMoV sequences

8224 Practical Utility (not more than 150 words)

Viruses are systemic in nature and there is no effective chemical to control viruses. Hence best option to manage viruses is to develop resistant varieties. None of the currently grown cultivars is resistant to viruses. Hence it is necessary to impart resistance against viruses in agronomically superior cultivars. Transgenic plants expressing coat protein or other viral sequences were shown to confer resistance against virus in many virus-host combinations. In view of this, in the present project more than 200 transgenic black pepper plants carrying CMV and PYMoV sequences as transgenes were produced.

Constraints, if any: Nil

823 Publications and Material Development
(One copy each to be supplied with this proforma.)

8231 Research papers

Jiby, M.V. Bhat, A.I. 2011. An Efficient *Agrobacterium*-Mediated Transformation Protocol for Black Pepper (*Piper nigrum* L.) UsingEmbryogenic Mass as Explant. J. Crop Sci. Biotech. 14 (4): 163-171

Conference papers

M. V. Jiby and A. I. Bhat. 2009. Susceptibility of embryo and embryogenic mass of black pepper (*Piper nigrum L.)* to antibiotics used in *Agrobacterium*-mediated genetic transformation (Abstr). 5th International Conference on Plant Pathology in globalized era, IARI, New Delhi, November 10-13, 2009, P-406.

M. V. Jiby and A. I. Bhat. 2009. An efficient *Agrobacterium*-mediated transformation protocol for black pepper (*Piper nigrum* L.) using embryogenic mass to generate transgenic plants (Abstr). 5th International Conference on Plant Pathology in globalized era, IARI, New Delhi, November 10-13, 2009, P-407.

8232 Popular articles

Nil

8233 Reports
Nil

8234 Seminars, conferences and workshops (relevant to the project) in which the scientists have participated. (List abstracts forwarded)

5th International Conference on Plant Pathology in globalized era, IARI, New Delhi, November 10-13, 2009.

Peppertech meeting held during International Pepper Community conference, Cochin, 10th November 2010.

Changing plant disease scenario in relation to climate change, Indian Institute of Spices Research, Calicut, October 22-23, 2010

824 Infrastructural facilities developed

(Details of field, laboratory, note books and final material and their location)
Modification of the tissue culture lab of Pathology section (Photoperiodic simulation castor rack with timer and Air conditioner with stabilizer) was done.

825 Comments / Suggestions of Project Leader regarding possible future line of work that may be taken up arising out of this Project.

In the present project more than 200 putative transgenic black pepper plants were raised using CMV coat protein gene and PYMoV ORF III region as transgene. Putative transformants survived on the selection medium were hardened and subjected PCR, dot blot and Southern hybridization tests to confirm the presence of transgenes. But transgenic plants were not tested for their resistance against viruses. Hence the future line of work should involve screening of these transgenic plants for resistance against viruses and stability of the transgene.

Part-IV : Project Expenditure (2006 –11)
(Summary)

(in Lakhs)

Head	**Grant allotted**	**Total grant released**	**Grant utilized**	**Balance**
Non-recurring	3.49	3.08	3.08037	-0.00037
Manpower	4.21	4.15	5.36823	-1.21823
Recurring contingencies	1.75	1.42	1.37368	0.04632
Consumables	8.17	8.15	6.98659	1.16341
TA	1.05	1.07	0.82817	0.24183
Institutional charges	2.25	2.25	2.00000	0.25000
Total	**20.92**	**20.12**	**19.63704**	**0.48296**

Balance refunded		**0.48296**

Part-V : Declaration

This is to certify that the final report of the Project has been submitted in full consultation with the Project workers as per the approved objectives and technical programme and the relevant records, note-books, materials are available for the same.

Signature of the Project Investigator: A. Ishwara Bhat

Co-Investigator: R. Suseela Bhai

Signature & Comments of the Head
of the Division/ Section

Signature & Comments of the
Director

PROJECT EVALUATION AND IMPACT ASSESSMENT PROFORMA
~~ON-GOING/~~CLOSED PROJECT

1. Name of institute: Indian Institute of Spices Research
2. Name of project and project code: Genetic transformation of black pepper to confer resistant against viruses (DBT-CP3)
3. Name of Project Leader with designation: A. Ishwara Bhat, Senior Scientist
4. Name of Project Associates: Dr R.Suseela Bhai
5. Period covered in the project: 29-09-2006 to 28-09-2011
6. Date of the start of the project: 29-09-2006
7. Date of the completion of the project:28-09-2011
8. Budget sanctioned: 20.92 lakhs
9. Utilization of budget (%): 94 (Cumulative)
10. Type of the project (Please 'tick'):
    a. Institute Project □
    b. ICAR Project □
    c. Network Project □
    d. Grant-in-Aid Project□
    e. Consultancy Project □
    f. Sponsored Project □
    g. Others □ Please specify :

Date: (Signature of project leader)

The project leader will send the consolidated report to PME Cell after compiling the progress reports received from all the associates. The report should also list the constraints (if any) being faced by project associates so that remedial measures may be taken. **The format for the progress report should be standard RPF format only. However, an additional Executive Summary should be typed double-spaced on a separate page; not longer than one page.** The Executive Summary may include purpose of the project, the findings over the previous year, and how these findings contribute to the approved objectives and the nature of collaboration and training activities during the year. The content of the Executive Summary should be clear and concise.

PROJECT EVALUATION AND IMPACT ASSESSMENT PROFORMA
EXECUTIVE SUMMARY - ~~ON-GOING/~~CLOSED PROJECT

Name of project: Genetic transformation of black pepper to confer resistant against viruses

Two viruses namely *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) are associated with black pepper. None of the cultivars of black pepper grown is resistant to disease. Since there is no effective chemical to control viruses, it is necessary to impart resistance against viruses in agronomically superior cultivars. Transgenic plants expressing coat protein or other viral sequences were shown to confer resistance against virus in many virus-host combinations. Similar approach can be used to transform existing cultivars of black pepper to get resistant plants through transformation using viral sequences. With this in view the project aimed to produce transgenic lines of black pepper using viral sequences as transgenes. Three viral constructs carrying a portion of ORF III of PYMoV(in sense and antisense orientation) and coat protein gene of CMV (in sense orientation) were prepared in binary vector, pBI121 were used for transforming black pepper. A very efficient protocol for *Agrobacterium* mediated genetic transformation of black pepper was developed in the project using somatic embryos derived from the micropylar region of germinating seeds as explants. Embryogenic mass were transformed with above constructs and putative transformants were confirmed by PCR, dot blot assay and Southern hybridization for the presence of transgene. The confirmed transgenic plants obtained from all the three constructs were hardened and maintained under green house conditions. These putative transgenic plants obtained need to be analyzed for resistance to CMV and PYMoV by challenge inoculation

Date: (Signature of project leader)