BT/PR14813/AGR/02/761/2010

Testing transgenic black pepper for resistance to viruses

DBT- CP 5

Final Report (RPP III)

Principal Investigator
A. Ishwara Bhat

Co-Principal Investigator
D. Prasath

ICAR-INDIAN INSTITUTE OF SPICES RESEARCH
(Indian Council of Agricultural Research)
Kozhikode- 673012, Kerala

ICAR-INDIAN INSTITUTE OF SPICES RESEARCH
KOZHIKODE-673-012, KERALA
(Indian Council of Agricultural Research)

FINAL RESEARCH PROJECT REPORT (RPP- III)

1. Institute Project Code : DBT CP5

2. Project Title : Testing transgenic black pepper for resistance to viruses

3. Key Words : Cucumber mosaic virus, Piper yellow mottle virus, black pepper, transgenic plants, somatic embryos, transformation, challenge inoculation, PCR, Southern hybridization, northern hybridization, loop-mediated isothermal amplification, SSR profiling, virus resistance

4. (a) Name of Lead Institute : ICAR-Indian Institute of Spices Research

(b) Name of Division/Regional Center/Section : Division of Crop Protection

5. (a) Name of the Collaborating Institute(s) : NA

(b) Name of Division/Regional Center/Section of Collaborating Institute(s) : NA

6. Project Team (Name(s) and designation of PI, CC-PI and Co-PIs, with time spent :

S. No.	Name, designation and institute	Status in the project (PI/CC-PI/	Time spent (%)	Work components assigned to individual scientist
1	A Ishwara Bhat, Principal Scientist, ICAR-IISR, Kozhikode	PI	80	Production of somatic embryos of different varieties of black pepper; Production of putative transgenic black pepper plants through *Agrobacterium* mediated transformation using three viral constructs; Confirmation of transgenicity of plants by PCR and Southern hybridization tests, Challenge inoculation of transgenic plants with viruses and analysis of challenged plants.
2	D. Prasath, Senior Scientist, ICAR-IISR, Kozhikode	Co-PI	20	Production of somatic embryos of different varieties of black pepper; Challenge inoculation of transgenic plants with viruses and analysis of challenged plants.

7. Priority Area : Disease management

8. Project Duration: Date of Start : 4th November 2011; Date of Completion: 3rd November 2014

(a) Objectives :

(i) Production of transgenic black pepper using coat protein gene of *Cucumber mosaic virus* and portion of ORF III of *Piper yellow mottle virus* as transgene.

(ii) Testing of transgenic black pepper lines for viral resistance in the green house.

(b) Practical Utility :

- Developed protocols for *Agrobacterium* mediated transformation of different varieties of black pepper
- Produced transgenic plants of black pepper harbouring PYMoV and CMV sequences
- Obtained tow CMV resistant trasnsgenic black pepper plants while four transgenic plants of PYMoV showed symptom remission.

10. Final Report of the Project

(a) Materials and Methods :

(i). **Production of somatic embryos**

Somatic embryos were obtained from the micropylar region of germinating seeds of different black pepper varieties in growth regulator free SH medium using the protocol of Nair and Gupta (2003; 2006). Mature berries of black pepper collected from nine varieties (IISR Thevam, IISR Girimunda, IISR Shakthi, IISR Malabar Excel, Pournami, Subhakara, Sreekara, Karimunda and Panniyur-1) were used for establishing primary somatic embryogenic cultures. The procedure included soaking the harvested matured berries in tap water overnight and outer fleshy mesocarp of the berries were removed by slight rubbing before surface sterilization. Seeds were surface sterilized with 0.1% mercuric chloride solution for 5 minutes, followed by repeated washing (3 - 4 times) with sterile double distilled water. Seeds were allowed to dry on sterile filter paper in laminar air flow for 30 min. Embryo along with surrounding micropylar tissue scooped out from the surface sterilized seeds were cultured on agar gelled full strength, plant growth regulator-free SH (Schenk and Hildebrandt, 1972) medium containing 3.0% (w/v) sucrose under darkness. The primary somatic embryo clumps having pre-globular to torpedo shaped embryos 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 compete darkness. The brownish yellow tissue at the root pole of the primary embryo proliferated into a small mass of tissue from

which several secondary embryos emerged.

(ii) Regeneration of plants from somatic embryos and genetic fidelity testing

The embryogenic mass was transferred to SH (liquid) medium with 3% to 4.5% sucrose for development of embryos into fully developed plantlets under dark with shaking at 110 rpm for 30 days. When the well differentiated plantlets started appear, they were allowed to grow under 12 h day light until they grew further and produced 2-3 leaves. The well developed plants were then transferred to woody plant medium with 4.5% sucrose, 0.8% agar and 0.2% charcoal. The well rooted plantlet was then hardened using sterile potting mixture under green house condition. Genetic fidelity testing of somatic embryo derived plants with corresponding mother plant was done using SSR primers.

(iii) Production of transgenic plants

Three constructs (*Cucumber mosaic virus* coat protein gene in sense orientation, portion of open reading frame III of *Piper yellow mottle virus* in sense and antisense orientations) prepared in binary vector, pBI121 were used for transforming four varieties of black pepper (Panniyur 1, IISR Malabar Excel, IISR Shakthi and Subhakra) using *Agrobacterium* mediated transformation. About 100 mg of embryogenic mass was infected (with intermittent vigorous shaking for 30 min) with $1/5^{th}$ diluted 18 h grown culture (OD_{600}-0.5-0.8, OD_{600}=0.5 corresponds to 1 $x10^4$ cells/ ml) of *A. tumefaciens* EHA 105 containing the construct. After agro-infection, the bacterial suspension was removed by passing the contents through a sterile filter pepper (Whatmann # 2). The nearly dry explants were then co-cultured in dark on basal SH medium with 1.5% sucrose for 48 h. 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 dark (concentration of sucrose was changed according to variety). The vigorous growing points were observed in the first round of selection while non-transformed embryos turned black indicating their death. These 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 3.5% sucrose for further development of embryos into 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 3.5% sucrose, 0.8% agar, 100

µg/ml kanamycin and 0.2% charcoal. The non-bleached plants (up to 30 days) were hardened in sterile potting mixture in the green house.

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

The transformants were screened for the presence of transgene through standard methods such as PCR, Southern hybridization and northern hybridization methods. A new alternate method namely loop-mediated isothermal amplification method was also developed for quick screening of transgenic plants.

(v) Challenge inoculation of transgenic plants with viruses

The short listed transgenic plants were challenge inoculated with respective viruses using graft method. Challenge inoculated plants were observed for symptom development and analyzed for the presence of virus by PCR.

(b) Results and Discussion :

(i) Production of somatic embryos

The number of scooped out embryo along with micropylar tissue inoculated in different varieties of black pepper is given in Table 1; Fig. 1. Primary somatic embryos (PEs) derived from micropylar tissues of germinating seeds were seen after 45 to 80 days in different varieties (Fig. 2). Secondary embryos were visible from root pole region of primary embryos within 65 to 90 days in different days in black pepper varieties (Table 1; Fig. 2). Somatic embryos induction was maximum in Panniyur-I variety while IISR Girimunda variety showed the minimum. Secondary somatic embryo gave rise to cyclic secondary somatic embryos within 10 days. The rate of cyclic secondary somatic embryogenesis also varied from variety to variety. The variety such as IISR Shakthi, IISR Malarbar Excel showed higher proliferation compared to variety such as IISR Girimunda. The embryogenic mass was multiplied and maintained in SH medium containing sucrose under dark. The concentration of sucrose played an important role in the proliferation of embryogenic mass. The optimum sucrose concentration was found to be 1% in the case of IISR Shakthi, Subhakara and Panniyur-1 whereas it was 1.5% in the case of IISR Malabar Excel and Karimunda. These embryogenic mass were used as explants for *Agrobacterium* mediated transformation using viral constructs.

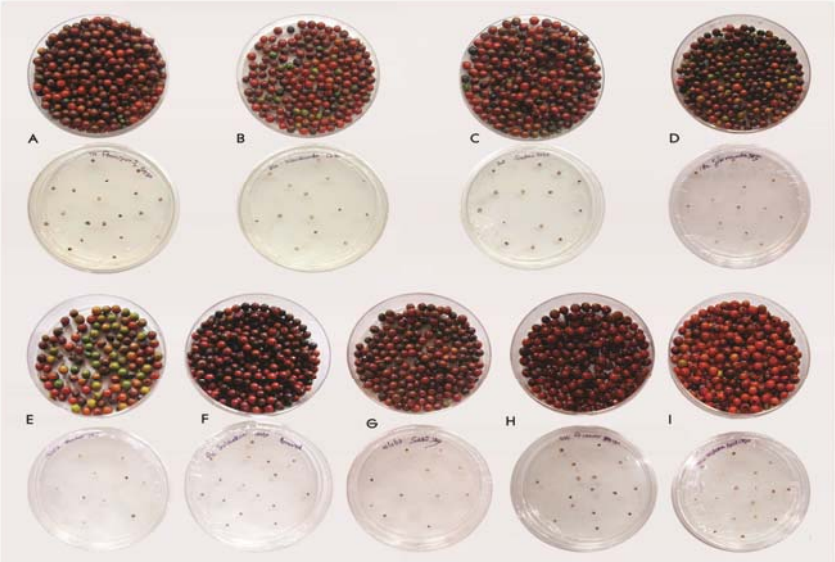

Fig. 1 Matured berries and corresponding scooped out embryo along with micropylar tissue inoculated for somatic embryogenesis in SH medium of different varieties of black pepper such as A. Panniyur-1, B. Karimunda, C. Sreekara, D. IISR Girimunda, E. IISR Thevam, F. Subhakara, G. IISR Shakthi, H. Pournami, I. Malabar Excel.

Table 1 Somatic embryogenesis from germinating seeds in different varieties of black pepper

Variety	No. of embryo inoculated	No. of germinated embryos	Percent germinating seeds showing PE	Days taken for PE production	Days taken for SE production	Percent PE showing SE
Panniyur I	1178	303	62.37	80	90	13.22
Sreekara	397	144	86.11	65	70	4.03
Subhakara	253	42	45.23	65	70	26.31
Karimunda	381	150	56.00	65	80	7.14
Pournami	135	102	53.92	45	65	5.45
IISR Thevam	132	45	77.77	45	90	5.71
IISR Shakthi	313	75	44.00	45	90	12.12
IISR Malabar Excel	1302	395	69.36	45	90	4.74
IISR Girimunda	602	112	74.10	80	80	1.20

PE, primary somatic embryo; SE, secondary embryo

Figure 2 Somatic embryogenesis as seen under stereomicroscope. (A) Scooped out micropylar region with embryo (B-D) Germinating embryo (E) Germinated embryo showing primary somatic embryogenesis (F) Secondary somatic embryo giving rise to proembryogenic mass (G) Differentiated plantlets in SH liquid medium (H) Plantlets in WPM (I) Hardened plantlets

1.2 Regeneration of embryogenic mass into plantlets

In order to obtain plantlets, embryogenic mass was transferred to SH (liquid) medium with 3% to 3.5% sucrose under dark with shaking at 110 rpm for 30 days. As sucrose concentration was increased from 3 to 4.5%, the rate of conversion of embryo into plantlet was higher and faster till 3.5% . When it was increased >3.5%, it caused reduction in number of plantlet production. Thus optimum sucrose concentration for conversion of somatic embryo to plantlets was found to be 3.5% for most of varieties. Medium was replenished at every 10 days. When the well differentiated plantlets started to appear, they were allowed to grow under 12 h day light until they produced 2-3 leaves. The well developed plants were then transferred to WPM with 3.5% sucrose, 0.8% agar and 0.2% charcoal (Fig. 3). The well rooted plantlet was then hardened using sterile potting mixture under green house condition.

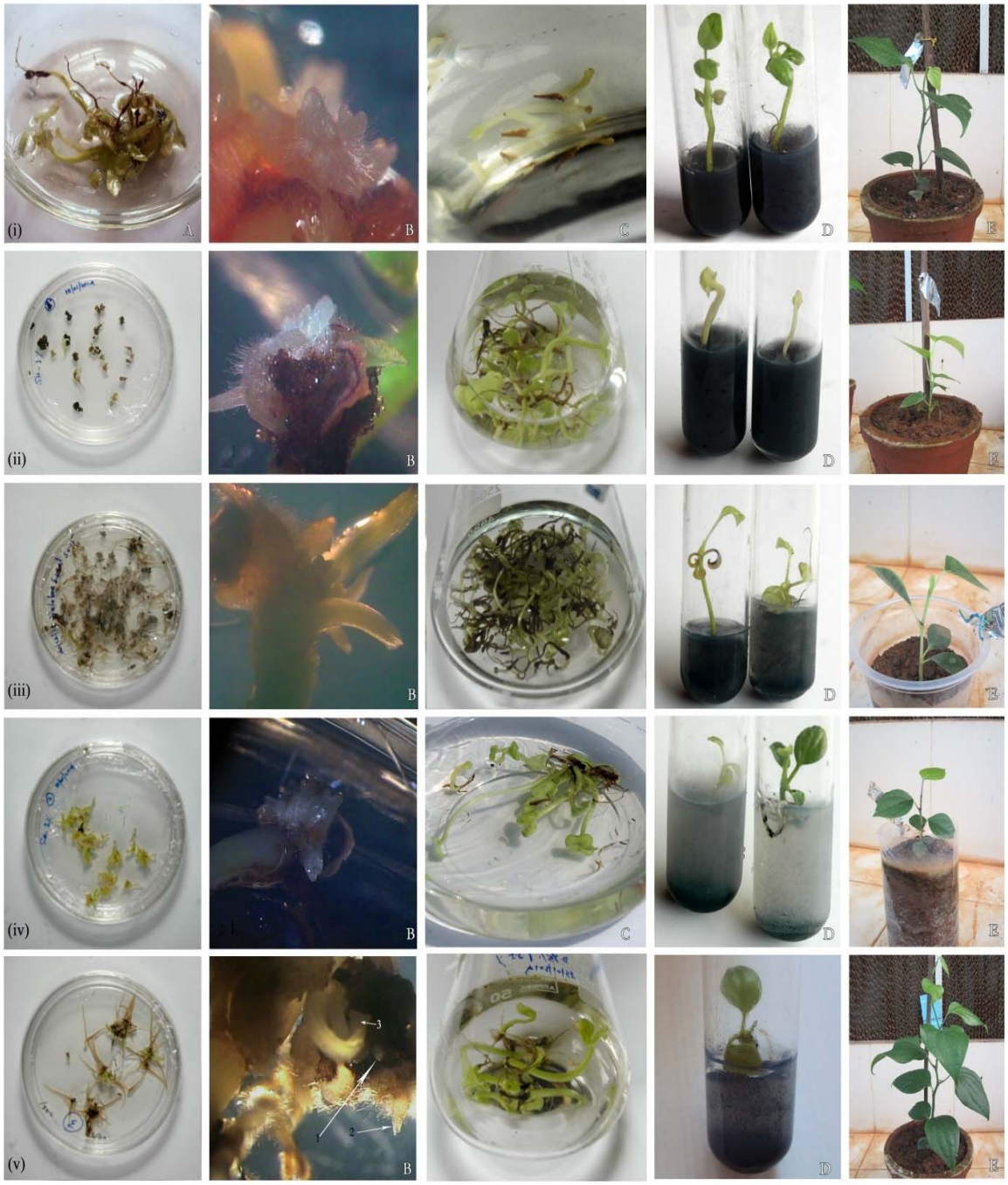

Fig. 3 Regeneration of plantlets from embryogenic mass in different varieties (i) Pournami (ii) IISR Shakthi (iii) IISR Malabar Excel (iv) Subhakara (v) Panniyur 1. Embryogenic mass in SH medium (A & B); Differentiated plantelts in liquid SH medium (C); Plantlets in WPM (D); Hardened plantlets (E).

1.3 Genetic fidelity testing of regenerated plants

Genetic fidelity testing of somatic embryo derived plants with their mother plants was done using SSR markers developed by Menezes et al. (2009) (Table 2). Ten plants

regenerated from somatic embryos of each variety were tested along with corresponding mother plant. Another related species of black pepper namely *Piper colubrinum* was also used to compare the profile. Total DNA isolated from each of the plants was subjected to PCR to amplify SSR regions using six pairs of SSR primers (Table 2). CTAB procedure described by Hareesh and Bhat (2008) was used to isolate the total DNA. The PCR reaction contained 1x PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 ng each of forward and reverse primers, 1.5 units of *Taq* polymerase, 1 µl of template DNA and sterile water to a final volume of 25 µl.

The thermal cycler was programmed for initial denaturation at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 52-57°C (depending on the primer combination, Table 2) for 1 min, synthesis at 72 °C for 1 min and a final extension for 10 min at 72 °C. The PCR products when analyzed on 1% agarose gel showed an amplicon ranging from 164 bp to 298 bp in different primer combination (Fig. 4). The banding pattern among mother and somatic embryo derived black pepper plants were same across different varieties while *P. colubrinum* either showed band of different size or no band depending on the primer used. A variety specific band was observed in the variety, Pournami which gave two bands with primer PN D10 while the same primer in other varieties gave single band. The mother plant and all tested somatic embryo derived plants of five varieties of black pepper showed bands of same size for all SSR loci indicating genetic uniformity of plants.

Table 2: Primers used for genetic fidelity testing through SSR profiling

Locus	Forward primer (5'….3')	Reverse primer (5'….. 3')	Annealing Temp (C)	Expected Size range (bp)
PN A5	CTTCCAGACCAATAA TCAACTT	ATCCCAAAATACACAA CAATTC	53	164-194
PN E3	TTTGTGTCCTCTCCCT CTCC	AAGACTAAAATAGGC AAGGCAAA	57	260-298
PN F1	ACTTCAGTGCTATTTT TATCTTCC	CCAACGCCCACTCTCA T	55	110-152
PN G11	TTACTAGTGTCCACCC CCACT	TCGATGGAAAGTCACC CTCT	55	210-238
PN H8a	TGTGTCTTTTATATTT TTGATG	TATTAGTAGTTCTCCC TTTTGA	55	266-288
PN D10	GTGTTACCTTTGGGGC ATTCA	TGTGTCAGGGCATCAA ACC	55	216-296

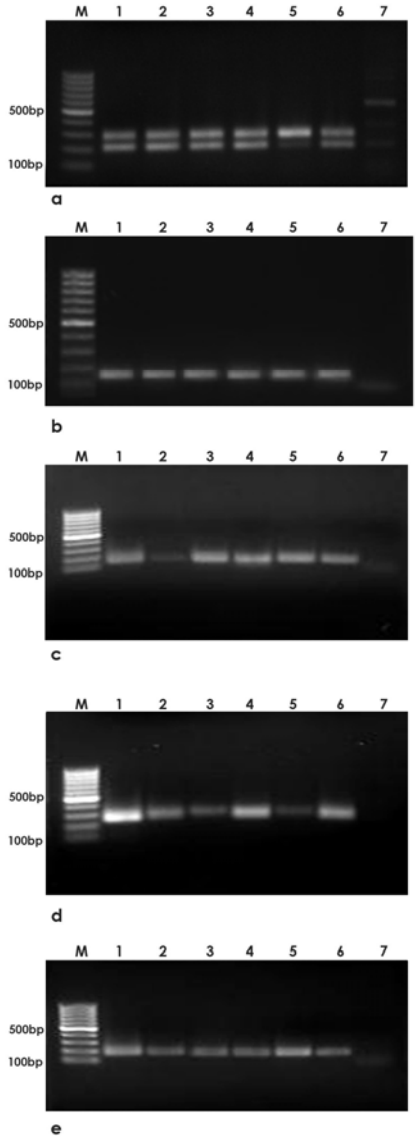

Fig. 4 Genetic fidelity testing of var. Pourami using SSR makers. Lane M shows Marker DNA ladder, Lane 1-5 shows somatic embryo derived plants, Lane 6 shows corresponding mother plant and Lane 7 shows *Piper colubrinum* (a) with primer PN D10, (b) with primer PN F1 (c) with primer PN E3 (d) with primer PN H8a, and (e) with primer PN G11.

1.4 *Agrobacterium* mediated transformation and regeneration

The vigorous growing points were observed in the first round of selection while non-transformed embryos turned black indicating their death. These 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 3.5% sucrose for further development of embryos into plantlets under dark with

shaking at 110 rpm for 30 days (Fig. 5). After 30 days plantlets were allowed to grow under light for 15 days. Fully developed plantlets were transferred to woody plant medium (WPM) with 3.5% sucrose, 0.8% agar, 100 µg/ml kanamycin and 0.2% charcoal. The non-bleached plants (up to 30 days) were hardened in sterile potting mixture in the green house.

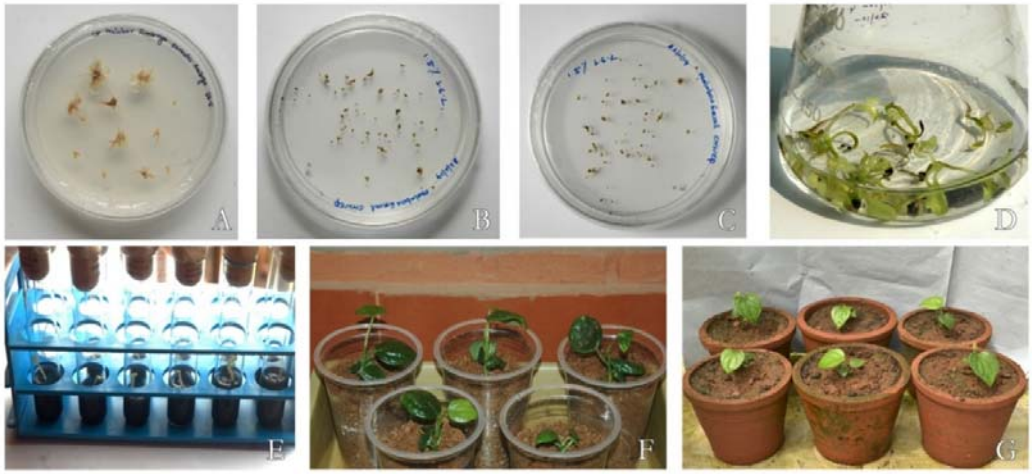

Fig. 5. Transformation and regeneration. (A) Embryogenic mass (explants) (B) Co-cultivation with *Agrobacterium* (C) Growing points observed after 4 weeks after co-cultivation (D) Prolifierated embryogenic mass in SH liquid medium (E) Plantlets in WPM medium (F& G) Hardened plantlets

2. Development of loop-mediated isothermal amplification (LAMP) and real-time LAMP assays for identification of transgenic black pepper plants

An alternative method to PCR, called loop-mediated isothermal amplification (LAMP) developed by Notomi et al. (2000) has been used by a few workers for the detection of transgenic plants with high sensitivity and speed under isothermal conditions. The method uses four to six primers and a DNA polymerase with strand-displacing activity to generate amplification products and the products can be detected by agarose gel electrophoresis or visually by turbidity or can be detected on real-time through real-time LAMP instrument. The products of LAMP are of different lengths and contain alternately inverted repeats of the target sequence, visible as ladder-like patterns on a gel. As the reactions are performed at a single temperature, LAMP assays can be performed very quickly since there is no separate denaturation and do not require thermal cyclers. In the present study, LAMP and real-time LAMP assays were developed for the quick and sensitive screening of transgenic black pepper plants using primers based on the DNA sequence of the recombinant construct inserted into the black pepper genome.

Transgenic plants of black pepper plants produced using the vector, pBI121 that carry Cauliflower mosaic virus (CaMV) 35 S promoter and kanamycin marker gene were used. Non transgenic plants of black pepper were used as negative control. The status of the plants were confirmed by subjecting total DNA isolated from putative transgenic plants by PCR using CaMV 35 S promoter and kanamycin marker gene specific primers. Initial standardization of the LAMP and real-time LAMP assays was performed with one plant each of transgenic and non-transgenic black pepper, whereas the validation was done on 20 putative transgenic black pepper plants. The total DNA of the test plants was isolated from 50 mg leaf sample using CTAB procedure as described previously (Hareesh and Bhat 2008).

Primers for LAMP and real-time LAMP assays were designed (Table 3) for the kanamycin marker gene and CaMV 35S promoter region sequences using a software package, namely Primer Explorer version 4 (http://primer explorer.jp/e/). Two external primers, F3 and B3, two internal primers FIP and BIP, and the loop primers (B-loop and F-loop), were designed. Desalted primers were custom synthesized by Integrated DNA Technologies (Coralville, Iowa, USA).

Table 3: Primers used for loop-mediated isothermal amplification (LAMP) and real-time LAMP

Target region	Primer	Sequences
Kanamycin	F3	CTGTTCGCCAGGCTCAAG
	B3	CGCCAAGCTCTTCAGCAATA
	FIP	GAAAAGCGGCCATTTTCCACCAGCGATGATCTCGTCGTGAC
	BIP	GGATTCATCGACTGTGGCCGGGGTAGCCAACGCTATGTCC
	BL	GTGTGGCGGACCGCTAT
	FL	TTCGGCAAGCGGCATCGC
CaMV 35 S promoter	F3	CTCCTCGGATTCCATTGC
	B3	GTCTTGCGAAGGATAGTGG
	FIP	GGCAGAGGCATCTTCAACGAGGAAGGTGGCTCCTACAA
	BIP	CACGAGGAGCATCGTGGAAACGTCAGTGGAGATATCACATC
	FL	TTTCCTTTATCGCAATGATGGC
	FL	AGAAGACGTTCCAACCACG

Reaction components for the LAMP assays were optimized in 0.2 ml tubes using total nucleic acids extracted from the transgenic (the positive control) and non- transgenic black pepper plant (the negative control). The third control was the 'water control' (a negative control, in which water was used instead of nucleic acids). The LAMP reaction mixture (25 µl) contained 1 µl (about 60 ng) of the template nucleic acids, 2× thermopol buffer (New

England Bio Labs, Ipswich, Massachusetts, USA), 1.4 mM each of dNTPs, 0–14 mM $MgSO_4$ and 0.4–2.4 M betaine (Sigma Chemicals, Bengaluru, India), 0.2 μM each of the external primers F3 and B3, 2 μM each of the internal primers FIP and BIP, 1 μM of the each loop primer (B-loop and F-loop) and 8 U of *Bst* polymerase (New England Bio Labs) (Table 4). The reaction was carried in an incubator (Thermo Scientific, USA) at 65°C for 60 min followed by incubation at 80°C for 5 min to inactivate the *Bst* polymerase. In determining the optimum concentration of $MgSO_4$, betaine was maintained at 0.8 M whereas in determining the optimum concentration of betaine, $MgSO_4$ was maintained at 6 mM for both target regions. To find out the optimum duration, the reaction was carried out at 65ºC for different durations (30 min, 45 min and 60 min). LAMP results were analysed through agarose gel electrophoresis. Reaction components for the real-time LAMP assay was optimized in 0.2 ml strips (Optigene, UK) using same nucleic acid templates used in the LAMP assay (Table 4). The real-time LAMP reaction mixture contained 1 μl of the template nucleic acids, 15 μl isothermal master mix (Optigene) and primers as indicated in the LAMP assay. The reaction was carried out in a real-time LAMP instrument (Genie II, Optigene) held at 65°C for 60 min. Real-time LAMP results were analysed in terms of Tp values (Tp is the time taken to generate a positive result based on the fluorescence). Annealing/melting temperature analysis from 98ºC to 80ºC was used to validate the authenticity of the LAMP products. Initially the specificity of the real-time LAMP product was also confirmed through gel electrophoresis.

The degree of sensitivity of the LAMP and real-time LAMP assays were tested using serially diluted (10^0 to 10^{-10}) total DNA isolated from transgenic black pepper plant. The PCR was carried out using 1 μl of each of the above dilutions and the respective F3 and B3 primers as described by Jiby and Bhat (2011). In order to validate the LAMP and real-time LAMP assays for the detection of transgenic plants, total DNA isolated from 20 putative transgenic plants were initially amplified through PCR using the specific primer and subsequently subjected to LAMP and real-time LAMP assays along with negative control and positive control using primers for both target regions.

Products of LAMP obtained using two sets of primers corresponding to CaMV 35S promoter region, kanamycin gene and 1μl of the nucleic acid from the transgenic black pepper plant showed a typical ladder-like pattern in agarose gel electrophoresis while no amplification was observed in the negative controls (Fig. 6a). The optimum concentration of $MgSO_4$ was found to be 6 mM and that of betaine was found to be 0.4 M for both target regions.

Table 4: Components of the optimized LAMP and real-time LAMP assays for the detection of different target regions

Component	Stock conc	Required concentration		Required volume per reaction	
		LAMP	Real-time LAMP	LAMP	Real-time LAMP
Thermopol reaction buffer	10x	2x	1x*	5.0	15.0
$MgSO_4$	50mM	6mM**	-	3.0**	-
dNTPs	10mM	1.4mM	-	3.5	-
F3 primers	10µM	0.2µM	0.2µM	0.5	0.5
B3 primer	10µM	0.2µM	0.2µM	0.5	0.5
FIP	100µM	2µM	2µM	0.5	0.5
BIP	100µM	2µM	2µM	0.5	0.5
BL primers	100µM	1µM	1µM	0.25	0.25
FL primers	100µM	1µM	1µM	0.25	0.25
Betaine	5M	0.4M**	-	2.0**	-
Bst polymerase	8U/µl	8U	-	-	-
Sterile Water	-	-	-	8.0	6.5
Template nucleic acid	60ng/µl	-	-	1.0	1.0
Total reaction volume				25	25

*Isothermal master mix (Optigene, UK) containis $MgSO_4$, dNTPs and Bst polymerase
** vary depending on the target regions

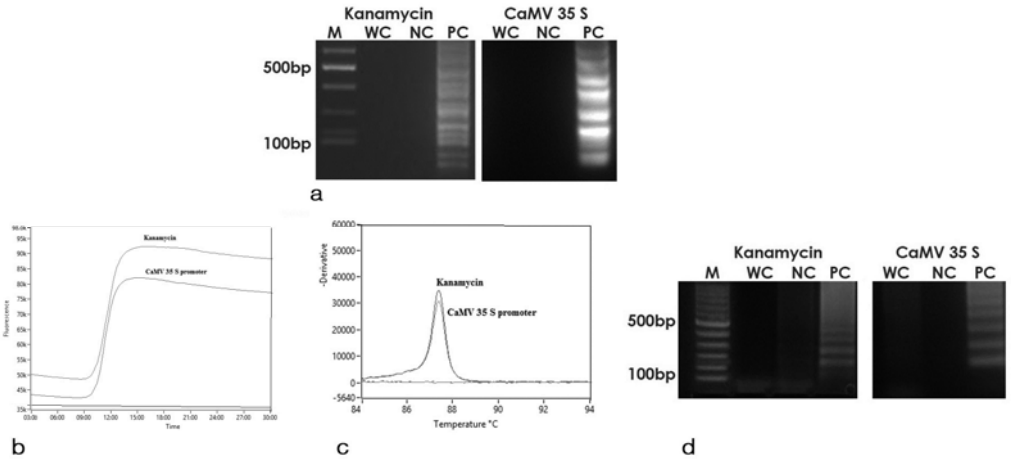

Fig. 6 Standardization of loop-mediated isothermal amplification (LAMP) and real-time LAMP for the identification of transgenic black pepper plants by amplifying kanamycin and Cauliflower mosaic virus 35S promoter (CaMV 35S) specific sequences present in transgenic black pepper plant. Lane M: 100 bp DNA ladder, Lane WC: water control, Lane NC: negative control, PC: positive control. (a) LAMP, (b) real-time LAMP, (c) Anneal curve of real-time LAMP product, and (d) agarose gel electrophoresis of real-time LAMP products.

Amplification was not optimum at 30 min and 45 min but good at 60 min (in terms of number and intensity of bands). Real-time LAMP detected both CaMV 35 S promoter and kanamycin target regions only in transgenic plant but not in non-transgenic plant and water control (Fig. 6b). Each assay was specific for its target region, and amplification was observed within 10 min. The specificity of real-time LAMP product was confirmed through anneal curve that showed a single peak at 89ºC and 88ºC for kanamycin and CaMV 35S promoter region respectively (Fig. 6c). Further, specificity of the product was confirmed by gel electrophoresis that showed typical ladder like bands only in transgenic plant (Fig. 6d).

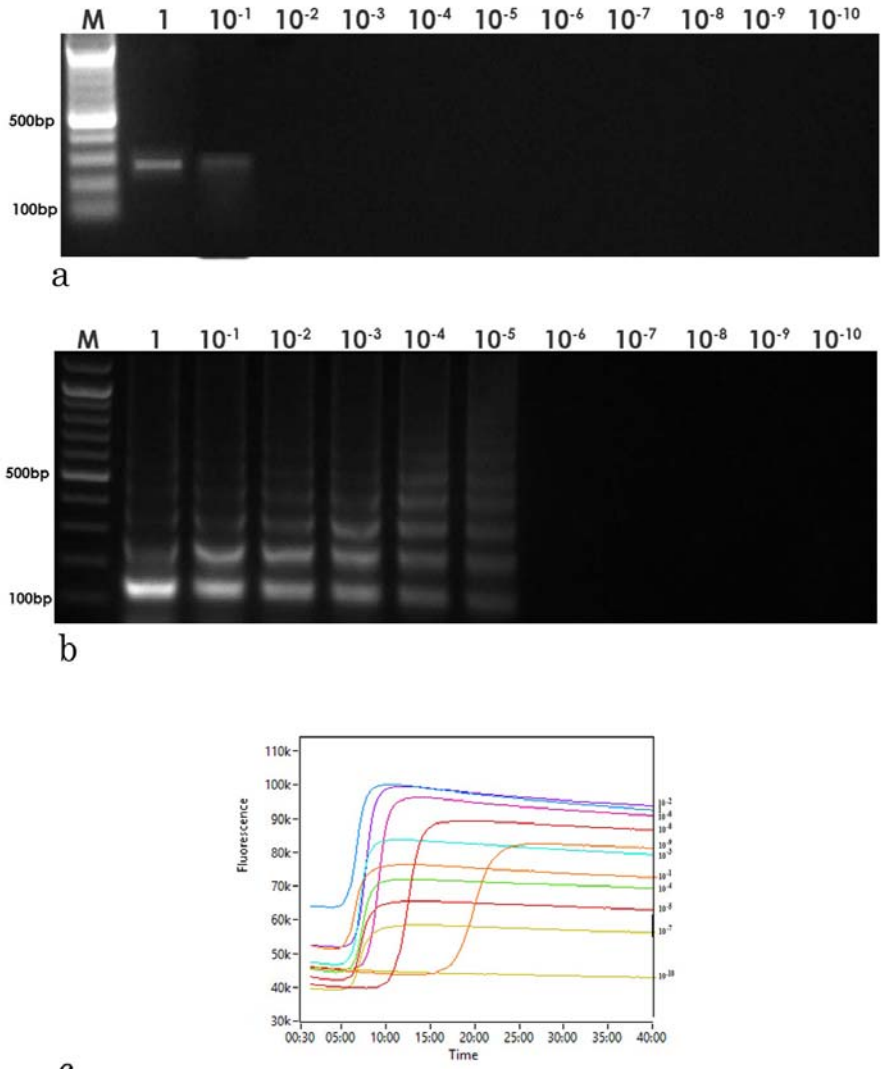

Fig. 7 Comparison of sensitivity of detection of transgenic plants by PCR, loop-mediated isothermal amplification (LAMP) and real-time LAMP by amplifying CaMV 35S promoter gene present in transgenic plant. (a) PCR, (b) LAMP, (c) real-time LAMP. Lanes 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, $10^{-9}$and 10^{-10} show different dilutions of the original extract of total nucleic acids; Lane M shows a 100 bp DNA ladder.

The detection limits for both kanamycin and CaMV 35S promoter target regions were 10^{-5} using LAMP and 10^{-8} using real-time LAMP while it was only 10^{-1} using PCR (Fig. 7). The 20 putative transgenic black pepper plants were used for validation of the LAMP and real-time LAMP assays. Initially when total DNA from these plants were subjected to PCR using F3 and B3 primers specific for each target regions, 12 plants showed positive reaction indicating that they are true transgenics (Fig. 8a). Non amplification of remaining plants indicate that they are non- transgenics. Similar results were obtained when these samples were subjected to LAMP and real-time LAMP assays confirming their accuracy in the detection of transgenic black pepper plants, whereas no amplification was observed in the non-transgenic control (Fig. 8b; 8c). The time taken for the detection in the real-time LAMP assay varied from 6 to 15 min in different samples probably indicating variation in the copy number of the transgene in the plants.

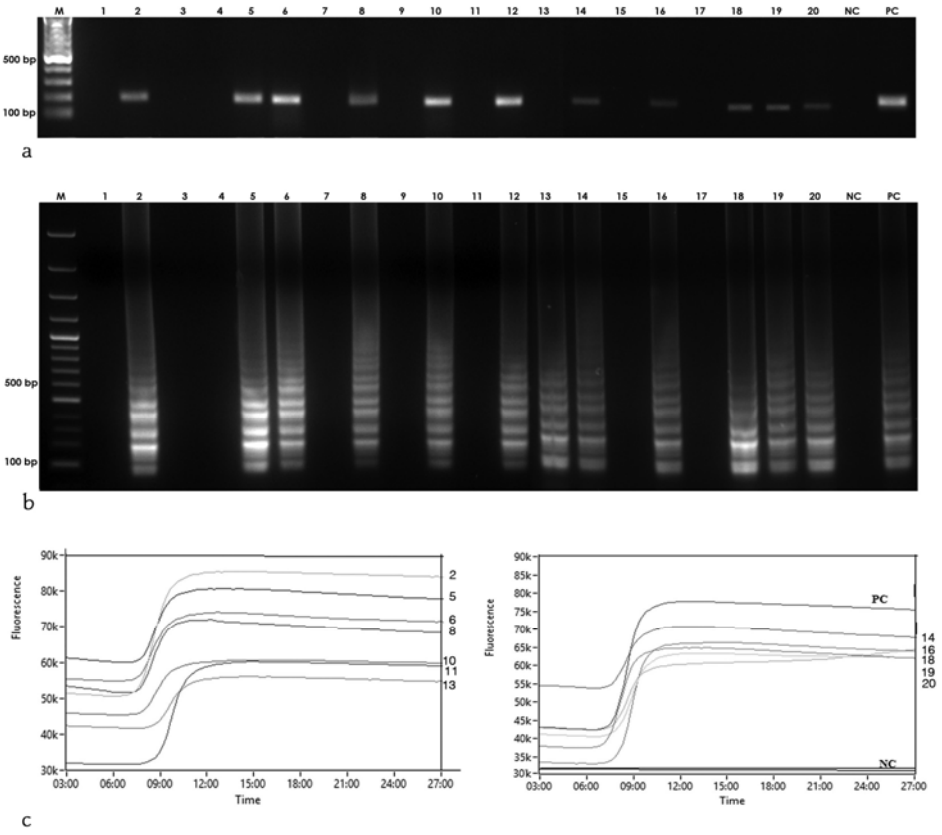

Fig. 8 Validation of LAMP and real-time LAMP assays for the detection of transgenic black pepper plants by amplifying kanamycin sequences inserted into the transgenic plants. Lane M: molecular size markers; Lane 1-20: test samples of putative transgenic black pepper; Lane NC: negative control; Lane PC: positive control. (a) PCR, (b) LAMP, (c) real-time LAMP.

3.0 Testing transgenic plants of PYMoV sense and antisense constructs

3.1 PCR

Sixty seven transgenic lines carrying the PYMoV sequence in sense and antisense orientation were used. The total DNA isolated from putative transgenic plants were subjected to PCR using transgene (PYMoV) (AIB 1, 2; AIB 104, 105; Table 5) and marker (kanamycin) specific primers (AIB 119, 120; Table 5). Non transgenic black pepper plants were used as negative control. The PCR reaction contained 1x PCR buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 50 ng each of forward and reverse primers, 1.5 units of *Taq* polymerase, 1 µl of template DNA and sterile water to a final volume of 25 µl. The thermal cycler was programmed for initial denaturation at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 52-57°C (depending on the primer combination) for 1 min, synthesis at 72 °C for 1 min and a final extension for 10 min at 72 °C. The PCR products when analyzed showed presence of expected size band indicating presence of inserted DNA sequence in the transgenic plants (Fig. 9).

Table 5. Sequence of primers used for PCR, RT-PCR and real-time PCR and real-time RT-PCR

Primer	Sequence	Amplicon size (bp)	Region	Remarks
AIB 104	CTATATGAATGGCTAGTGATG	400	ORF III of PYMoV (transgene)	Testing presence of transgene
AIB 105	TTCCTAGGTTTGGTATGTATG			
AIB1	ATG GACAAATCTGAATCAAC	657	Coat protein gene of CMV (transgene)	Testing presence of transgene
AIB2	TCAAACTGGGAGCACCC			
AIB119	CAACGTTGAAGGAGCCAC	950	3' end of NOS promoter and 5' end of Kananmycin gene	Testing presence of kanamycin gene in transgenic plant
AIB 120	ACGAGGAAGCGGTCAGC			
AIB 183	GTCGTCAGTGCGAATTGA	426	2b region CMV	Testing presence of CMV in transgenic plants
AIB 184	TTCAAAACGCACCTTCCG			

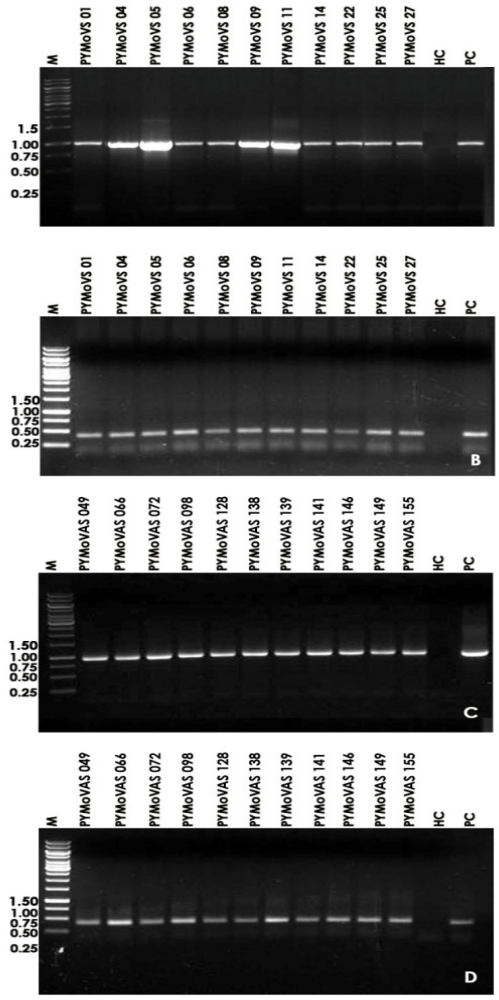

Fig 9. Testing transgenic lines for presence of trangene by PCR. (A) and (B) Transgenic lines of PYMoV sense construct using primers specific for kanamycin and transgene region respectively (C) and (D) Transgenic lines of PYMoV antisense constructs using primers specific for kanamycin and transgene region respectively. Lane M: 1Kb ladder, Lane 1-11: transgenic plants, Lane HC: Healthy control (untransformed), Lane PC: Positive control

3.2 Southern analysis

About 20 µg of genomic DNA from transgenic plants was digested with restriction enzyme *Hind* III (which has a unique restriction site in the construct), separated by electrophoresis in a 0.8% agarose gel and subsequently transferred to a nylon membrane (Porablot NY plus, *Macherey Nagel*, Germany) by capillary method. A 550 bp DNA fragment corresponding to the transgene (ORF III region of PYMoV) was labeled with

digoxigenin and used as a probe for hybridization according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). The minimum size of hybridized fragment expected when *Hind* III was used was 1.5 kb while size of the linearized recombinant plasmid used as positive control would give a 13 kb band. The hybridized membrane was washed, subjected to chemilluminescent development using CSPD substrate, and then exposed to X-ray film. Linearized recombinant plasmid pBI121with the same restriction enzyme was used as a positive control while genomic DNA from a non-transformed plant served as a negative control. A total of 42 plants including the plants produced previous years were subjected to southern hybridization. Of these five plants showed positive reaction with single band in each plant indicating single copy of the transgene (Fig. 10).

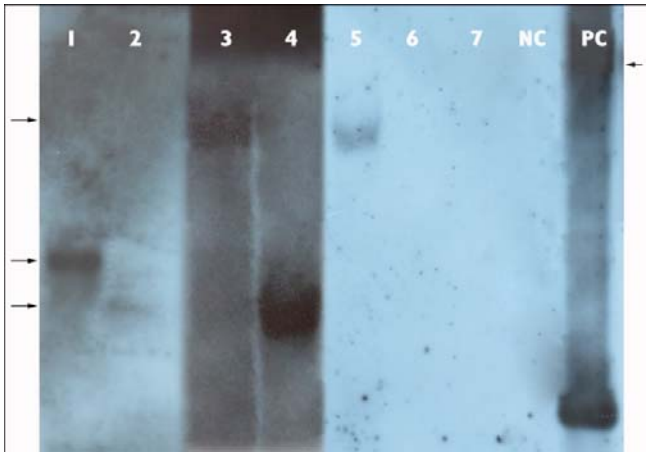

Fig. 10. Testing transgenic plants carrying portion of ORFIII of PYMoV by Southern hybridization. Lane 1-7: transgenic plants; NC: non-transformed control and PC: positive control

4.0 Testing transgenic black pepper lines for *Piper yellow mottle virus* (PYMoV) resistance in the green house

Short listed transgenic lines based on PCR test of both sense (11 plants) and antisense constructs (15 plants) were subjected to challenge inoculation with PYMoV. Challenge inoculation was carried out using mealybug and graft inoculation (Fig. 11). The mealybug transmission was carried out as described in the previous year by giving 12 h each of acquisition access and inoculation access time respectively. Mealybug inoculation to a non-transgenic somatic embryo derived healthy black pepper served as positive control for transmission while mock inoculation onto a non transgenic somatic embryo derived healthy

black pepper served as negative control. Wedge graft inoculation was carried out using PYMoV infected black pepper as root stock while transgenic plants were used as scion. Graft inoculation to a non-transgenic healthy black pepper served as positive control while graft inoculation using healthy root stock and healthy scion served as negative control. After 60 days of incubation, the plants were scored at fortnightly interval. When scored based on symptoms, three categories of plants could be discriminated: severe, moderate and mild. Severe symptoms included yellow mottling, deformation, curling and reduction in leaf size, moderate symptoms included yellow mottling and slight deformation and curling of leaves (Table 6; Fig. 12). Mild symptoms include yellow specs or mottle on leaves.

Fig. 11 Challenge inoculation of transgenic lines of PYMoV. (A) Rearing of healthy mealybug *in vitro* on pumpkin (B) acquisition access with PYMoV on infected black pepper leaves (C) inoculation access on transgenic plants (D) Inoculation through wedge grafting

Table 6 Analysis and testing of transgenic lines of *Piper yellow mottle virus* sense and antisense constructs

Construct	PCR analysis		RT-PCR analysis for transcript	Challenge inoculation by mealybug and grafting				
	Transgene primer	Kanamycin primer		PCR test	Symptom at 120th day*	Symptom at 180th day*	Symptom at 240th day	Symptom at 300th day
PYMoV sense construct (pBI121PYMoVS)								
PYMoVS 01	+	+	+	+	Mi	Mo	Mo	S
PYMoVS 04	+	+	+	+	Mo	S	Mo	Mo

PYMoVS 05	+	+	+	+	Mi	Mi	Mo	Mi
PYMoVS 06	+	+	+	+	S	S	S	S
PYMoVS 08	+	+	+	+	Mo	S	S	S
PYMoVS 09	+	+	+	+	S	Mo	S	S
PYMoVS 11	+	+	+	+	Mo	S	Mo	Mo
PYMoVS 14	+	+	+	+	Mi	Mi	Mi	Mi
PYMoV S 22	+	+	+	+	Mo	Mo	Mi	Mo
PYMoVS 25	+	+	+	+	S	Mi	Mi	Mo
PYMoVS 27	+	+	+	+	S	Mi	Mi	Mi
PYMoV antisense construct (pBI121PYMoVAS)								
PYMoVAS 49	+	+	+	+	S	Mi	Mi	Mi
PYMoVAS 66	+	+	+	+	Mi	Mi	Mi	Mo
PYMoVAS 72	+	+	+	+	Mo	Mo	Mi	Mo
PYMoVAS 98	+	+	+	+	Mo	Mo	Mo	Mo
PYMoVAS 128	+	+	+	+	Mo	Mo	S	S
PYMoVAS 138	+	+	+	+	S	S	S	S
PYMoVAS 139	+	+	+	+	S	Mi	Mi	Mo
PYMoVAS 141	+	+	+	+	Mi	Mi	Mi	Mo
PYMoVAS 146	+	+	+	+	S	S	S	S
PYMoVAS 149	+	+	+	+	S	S	S	S
PYMoVAS 155	+	+	+	+	Mi	Mi	Mi	S
PYMoVAS 160	+	+	+	+	Mo	Mo	Mo	S
PYMoVAS 171	+	+	+	+	Mo	Mo	Mo	S
PYMoVAS 182	+	+	+	+	Mi	Mi	Mi	S
PYMoVAS 184	+	+	+	+	Mo	Mo	Mo	S

***Mi, Mild; Mo, Moderate; S, Severe**

The positive control (wild type) plants of both mealybug and graft inoculated plants started showing mild symptoms in about 60 days and showed severe symptoms by 120 days. At 120 days, of the 11 plants of sense construct, three plants showed mild symptoms out of which 1 turned moderate at 180th day. Of the 4 plants that showed moderate symptom in 120 days, 3 turned severe in 180 days and out of the 4 plants that showed severe symptoms in 120 days 2 turned mild and 1 turned moderate by 180 days. Similarly, of the 15 plants of antisense construct challenged, four plants showed mild symptoms while 6 plants showed moderate and five plants showed severe symptoms. Out of the 5 plants that showed severe symptoms in 120 days 2 turned mild in 180 days while in all other plants there was no change in symptoms (Fig. 12). When the symptoms were recorded by 240th day 3 plants of sense construct and 2 of antisense construct continued to show mild symptoms (Table 6). As PYMoV infected plants are known to show severe symptoms when subjected to higher temperature, these plants were subjected to a temperature of 35° C and humidity of 70% in a controlled environment chamber for 30 days starting from 240th day. By the end of 270th day the plants

were still showing mild symptoms indicating that the symptoms were not influenced by temperature. By the end of 300th day out of these 5 plants, 1 turned severe while the other 4 remained mild (Fig. 13). All challenged irrespective of the kind of symptoms showed presence of the virus when tested through PCR (Table 6; Fig. 14). When results of southern hybridization was compared, three of symptom remission plants were positive in Southern as well.

Fig. 12 Symptoms observed on challenge inoculated transgenic plants. From left to right: apparently healthy (no symptoms), mild, moderate and severe.

Fig. 13 Transgenic plants that showed symptom remission 300 days after challenge inoculation.

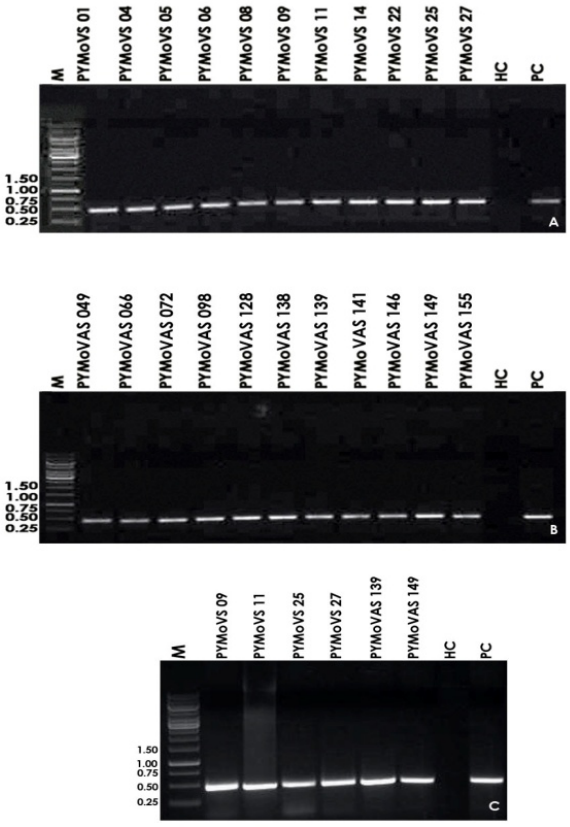

Fig. 14. Testing for the presence of PYMoV in challenge inoculated transgenic lines after 120 days. (A) Transgenic lines of sense construct challenge inoculated by mealybug (B) Transgenic lines of antisense construct by mealybug and (C) Transgenic lines of selected sense and antisense constructs challenged by grafting. Lane M: 1Kb ladder, Lane 1-11: transgenic plants, Lane HC: Healthy (non challenged) control, Lane PC: Positive control

4.1 siRNA analysis

Three plants of sense construct and one plant of antisense construct that showed mild symptoms till 300 days after challenge inoculation were subjected to siRNA analysis. Total RNA was extracted from 1 g of leaf sample using Tri reagent (Sigma) as indicated in the manufacturer's protocol. Briefly the sample was ground in 10 ml of Tri reagent and left at room temperature for 5 min. The homogenate was centrifuged at 12000g for 10 min at 4°C to precipitate the insoluble material. The clear supernatant was transferred to a fresh tube and allowed to stand for 5 min, 2 ml of chloroform was then added and shaken vigorously for 15 sec and allowed to stand at RT for 10 min. This was then centrifuged at 12000g for 15 min at 4°C. To the supernatant 5 ml of isopropanol was added, vortexed briefly and allowed to stand for 5 min at RT and then centrifuged at 12000g at 4°C for 10 min. The pellet was washed

with 75% ethanol and dissolved in 150 µl of DEPC treated water. This was run in 15% polyacrylamide gel containing urea (4.2 g of urea, 3ml RNase free water, 3.74 ml 40% polyacrylamide , 0.5 ml of 5x TBE, 100 µl 10% ammonium persulfate and 5 µl of TEMED).The RNA samples were mixed with equal volume of 2x loading dye, vortexed and heated at 90°C for 30 sec and loaded onto the gel along with miRNA marker. The gel was run for 1 h at 120 V and stained in SYBR gold (Invitrogen) for 10 min and viewed under UV transilluminator. Two bands were seen in the 21-25 nt region indicating the presence of small RNA (Fig. 15).

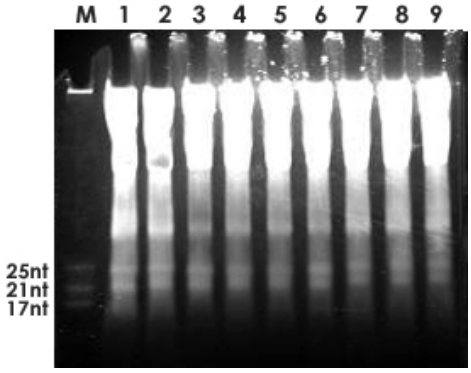

Fig. 15 Total RNA isolated from a transgenic line of sense construct challenge inoculated with PYMoV, run in 15% denaturing PAGE. Lane M:miRNA marker, Lane 1-10: total RNA from the same plant. Two bands in the 21-25 nt region indicate the presence of small RNA.

5. 0 Testing transgenic plants of CMV construct

5.1. PCR

Out of 74 transgenic plants carrying the CMV coat protein gene in the sense orientation when subjected to PCR (AIB 1, 2; AIB 119, 120; Table 5), 12 plants showed the presence of transgene (Fig. 16).

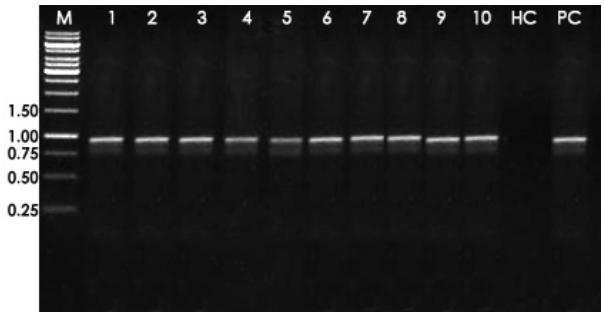

Fig. 16a PCR amplification of transgenic plants of CMV-CP sense construct using primers specific for kanamycin region. Lane M: 1Kb ladder, Lane 1-10: transgenic plants, Lane HC: Healthy (untransformed) control, Lane PC: Positive control

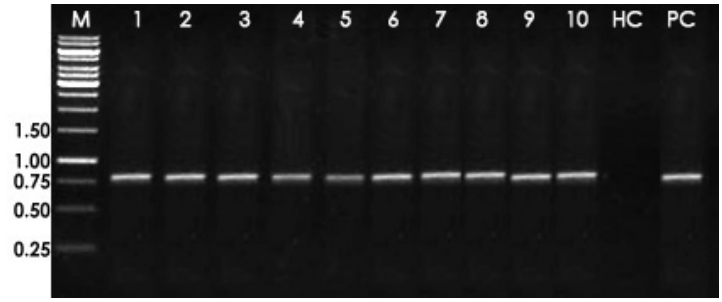

Fig. 16b PCR amplification of transgenic plants of CMV-CP sense construct using primers specific for transgene (CMV-CP). Lane M: 1Kb ladder, **Lane 1-10: Transgenic plants, Lane HC: Healthy (untransformed) control**, **Lane PC: Positive control**

5.2 Southern analysis

About 20 µg of genomic DNA from transgenic plants was digested with restriction enzyme *Eco*RI (which has a unique site in the construct), separated by electrophoresis in a 0.8% agarose gel and subsequently transferred to a nylon membrane (Porablot NY plus, *Macherey Nagel*, Germany) by capillary method. A 650 bp DNA fragment corresponding to the transgene (CMV coat protein) was labeled with digoxigenin and used as a probe for hybridization according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). The minimum size of hybridized fragment expected when *Eco*RI was used was 5.8 kb while size of the linearized recombinant plasmid used as positive control would give a 13 kb band. The hybridized membrane was washed, subjected to chemilluminescent development using CSPD substrate, and then exposed to X-ray film. Linearized recombinant plasmid pBI121with the same restriction enzyme was used as a positive control while genomic DNA from a non-transformed plant served as a negative control. The results of Southern analysis showed presence of the transgene in nine plants with two insertions (Fig 17).

5.3 Northern Hybridization

In order to confirm production of the transcript in the transgenic plants they were subjected to northern hybridization. Total RNA was extracted from 100 mg leaf tissue using Trizol extraction method according to the manufacturer's instructions (Sigma). About 20 µg of total RNA was separated by electrophoresis in a 1% agarose gel at 80 V for 1 h and subsequently transferred to a nylon membrane (Porablot NY plus, *Macherey Nagel*, Germany) by capillary method. A 650 bp DNA fragment corresponding to the transgene was labeled with digoxigenin and used as a probe for hybridization. The hybridized membrane

was washed, subjected to chemilluminescent development using CSPD substrate, and then exposed to X-ray film. Total RNA from a non-transformed plant served as a negative control and total RNA from a plant infected with CMV served as a positive control. Among the nine plants tested eight showed strong signal while one plant showed weak signal in northern hybridization indicating the production of transcript (Fig. 18).

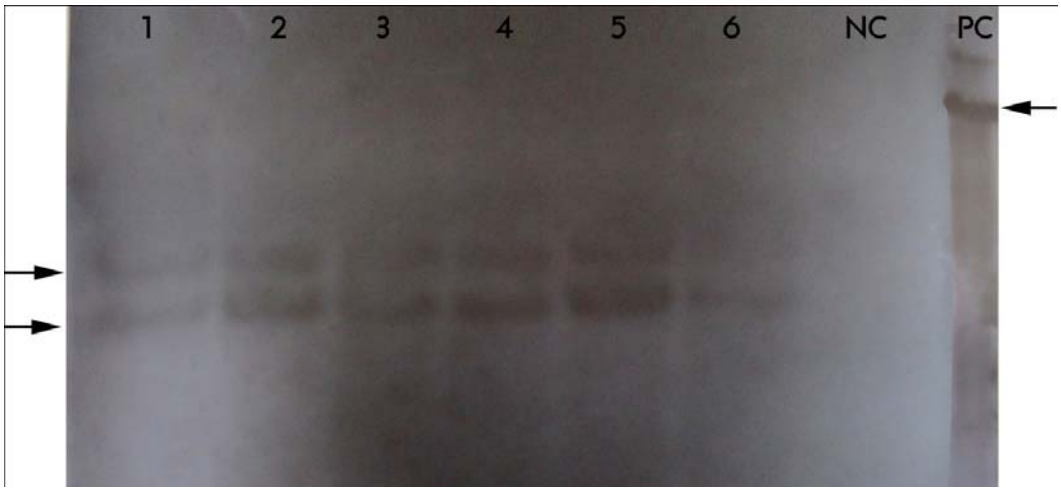

Fig. 17 Testing transgenic plants carrying CMVCP gene by Southern hybridization. Lane 1-6: transgenic plants, NC: non-transformed control and PC: positive control

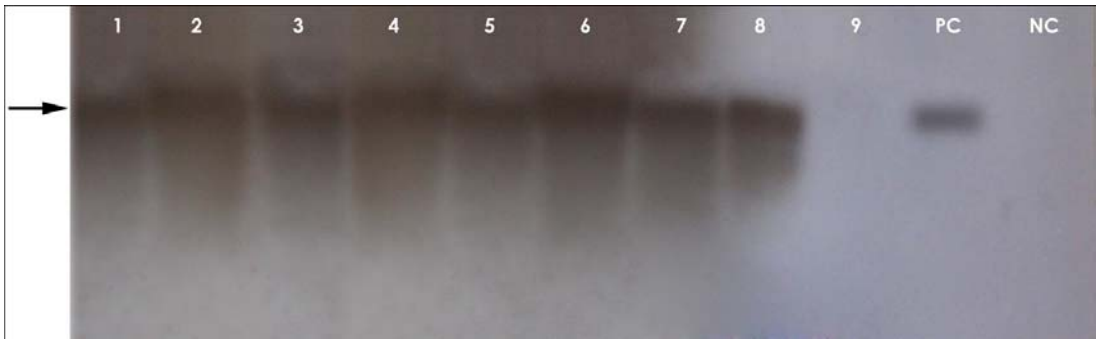

Fig. 18 Northern hybridization analysis of transgenic lines carrying *Cucumber mosaic virus* coat protein construct using transgene specific probe. Lanes 1-9: Transgenic plants; Lane PC: Positive control and Lane NC: Negative control

5.4 Western blotting

The nine plants that showed positive reaction in northern hybridization was subjected to western blotting to check for production of the transgene protein. Leaf samples were ground in lysis buffer followed by incubation in ice for 15 min. The samples were then centrifuged at 12000g for 20 min at 4 °C. Supernatant was added with 1.5 volumes of ice cold ammonium acetate in 80% methanol and incubated at -20 °C for 2 h. This was followed

by centrifugation at 4000g for 20 min at 4 °C. Pellet washed twice with cold 0.1M ammonium acetate in 100% methanol, twice with cold 80% acetone and finally once with 70% ethanol. Pellet was completely re-suspended each time with vortexing and incubated at -20 °C for at least 15 min between each wash. Protein pellets were solubilized in water and loaded in 15% SDS-PAGE gel according to Laemmli's protocol. After the run, gel was blotted onto PVDF membrane at 20 V for 4 h through electro-blotting. The membrane was placed in the blocking solution overnight at 4 °C. On the next day it was incubated in cross-absorbed primary antibody specific to CMV coat protein at 1:1000 in TBS for 1 h followed by three washes. The membrane was then incubated in anti- rabbit IgG labeled with alkaline phosphatase in TBS in the ratio 1:7500 for 1 h. After three washes, the membrane was developed by placing it in substrate solution containing NBT and BCIP. A positive (known infected plant) and a negative control (known healthy plant) were used in the method. All transgenic plants including the positive control showed positive reaction while negative control did not show any results (Fig. 19).

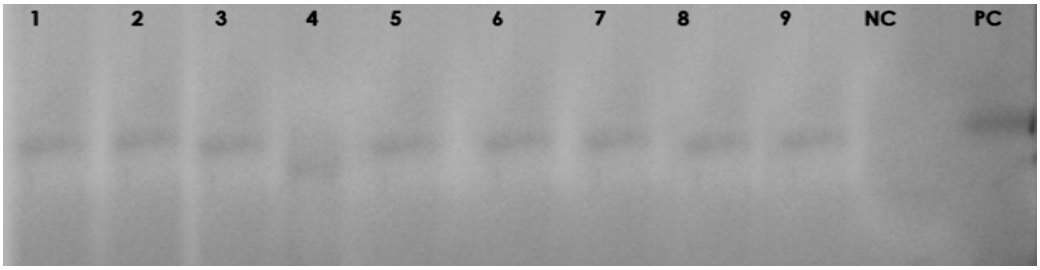

Fig 19. Western blot analysis of transgenic plants carrying CMV coat protein construct. Lanes 1-9: transgenic plants, Lane NC: non-transformed control and lane PC: positive control.

6.0 Testing transgenic black pepper lines for *Cucumber mosaic virus* (CMV) resistance in the contained green house

Challenge inoculation of short listed CMV transgenic lines was done through grafting (Fig. 20). CMV infected black pepper plants were taken as root stocks. A somatic embryo derived non transformed plant grafted on CMV infected black pepper served as positive control. Among these, five plants were tested 90 days after grafting for presence of the virus through RT-PCR (using DNase treated total RNA as template) using CMV 2b gene specific primers (AIB 183, 184; Table 5). Results showed presence of the virus two plants while other plants did not show presence of the virus indicting their resistance to CMV (Fig. 21a). The results were confirmed through real-time RT-PCR using CMV 2b specific primers (AIB 239, 240; Table 5) which also showed presence of the virus only in two plants with a Ct value of

22 and 23 respectively while non-transformed grafted positive control plants showed a Ct value of 23 (Fig. 21c). All plants were also subjected RT-PCR (AIB 1, 2; Table 5) and real-time RT-PCR (AIB 139, 140; Table 5) using transgene (CMV coat protein) specific primers to know transcript production. Result of RT-PCR showed presence of coat protein transcript in all plants (Fig. 21b). Among these, intensity of the band was high in two plants (lanes 3

Fig. 20 Screening transgenic black pepper plants for resistance to *Cucumber mosaic virus* through graft inoculation under contained green house conditions

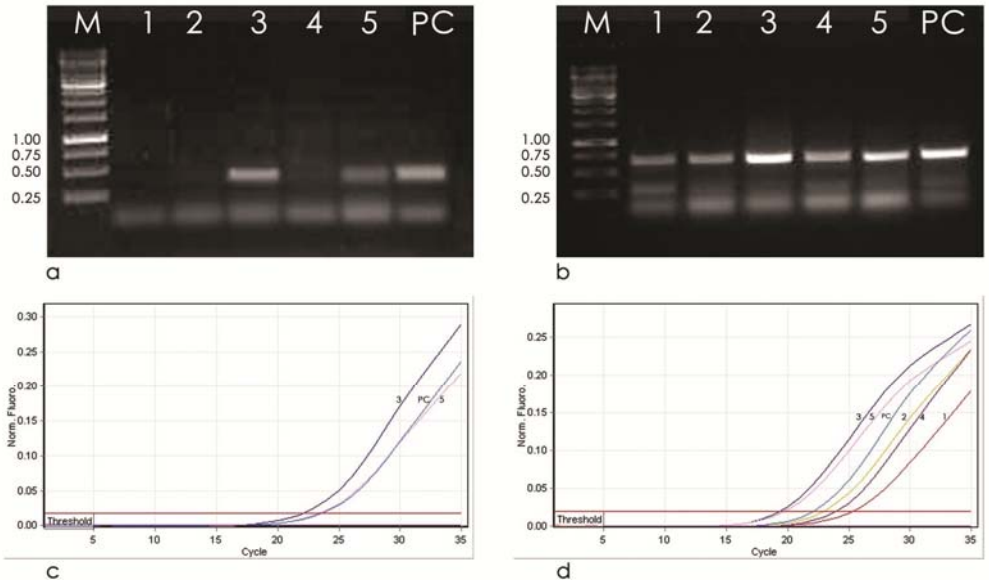

Fig. 21. RT-PCR and real-time RT-PCR of challenged transgenic plants carrying CMV coat protein gene. (a) RT-PCR with CMV 2b specific primers (b) RT-PCR with CMV coat protein specific primers (c) real-time RT-PCR with CMV 2b specific primers (d) real-time RT-PCR with CMV coat protein specific primers. Lane M: DNA size markers; Lanes 1-5: challenged transgenic plants, lane PC: positive control (non-transformed somatic embryo derived black pepper plant grafted on CMV infected black pepper root stock)

and 5 in Fig. 21 b) that tested positive for CMV when tested using CMV 2b specific primers indicating that increased transcript is contributed from the virus. Real-time RT-PCR using coat protein specific primers (AIB 139, 140; Table 5) also confirmed the above results with two plants showing increased transcript production compared to other plants including the positive control which is due to the presence of the virus (Fig. 21d).

(c) Objective-wise Achievements :

(i) *Production of transgenic black pepper using coat protein gene of Cucumber mosaic virus and portion of ORF III of Piper yellow mottle virus as transgene.*

- Somatic embryos were obtained from the micropylar region of germinating seeds of different black pepper varieties in growth regulator free SH medium

- The cyclic secondary somatic embryos (pro-embryogenic mass) obtained from different varieties of black pepper was multiplied and maintained in SH medium containing sucrose under dark. The concentration of sucrose played an important role in the prolifieration of embryogenic mass. The optimum sucrose concentration was found to be 1% in the case of IISR Shakthi, Subhakra and Panniyur 1 whereas it was 1.5% in the case of IISR Malabar Excel and Karimunda.

- In order to obtain plantlets, pro embryogenic mass was transferred to SH medium with 3% to 3.5% sucrose under dark with shaking at 110 rpm for 30 days. Optimum sucrose concentration for conversion of somatic embryo to plantlets was found to be 3.5% for most of varieties. Well differentiated plantlets were allowed to grow under 12 h light until they produced 2-3 leaves. The well developed plants were then transferred to WPM and rooted plantlet was then hardened under green house conditions.
- Genetic fidelity testing of somatic embryo derived plants with their mother plants was done using SSR markers. Total DNA isolated from plants was subjected to PCR to amplify SSR regions using six primer pairs of SSR primers. The PCR products when analyzed on gel showed an amplicon ranging from 164 to 298 bp in different primer combination. The banding pattern among mother and somatic embryo derived black pepper plants were same across different varieties indicating genetic uniformity of somatic embryo derived plants with their mother plants.

- Three constructs (*Cucumber mosaic virus* coat protein gene in sense orientation, portion of open reading frame of III of *Piper yellow mottle virus* in sense and antisense orientation) prepared in binary vector pBI121 were used for transforming four varieties of black pepper (Panniyur 1, IISR Malabar Excel, IISR Shakthi and Subhakara) using *Agrobacterium* mediated transformation.

- *Agrobacterium* mediated transformation included infecting pro-embryogenic mass with overnight grown culture of *A. tumefaciens* EHA 105 containing the construct. After agro-infection, explants were co-cultured in dark on basal SH medium with 1.5% sucrose for 48h. The co-cultured embryogenic mass was transferred to selection medium (SH basal + 1.5% sucrose + 100 µg/ml cefotaxime + 25 µg/ml kanamycon), and were cultured for 3-5 weeks under dark.

- The vigorous growing points observed in the first round of selection 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 3.5% sucrose for further development of embryos into 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 WPM with 3.5% sucrose, 0.8% agar, 100 µg/ml kanamycin and 0.2% charcoal. The non-bleached plants (upto 30 days) were hardened in sterile potting mixture in the green house.

- An alternate method to PCR, called loop-mediated isothermal amplification (LAMP) and real-time LAMP based assays were developed for quick and sensitive detection of transgenic black pepper plants. Primers (six each) were designed based on the nucleotide sequence of two target regions [kanamycin and Cauliflower mosaic virus (CaMV) 35S promoter] integrated into the genome of transgenic black pepper. Both assays successfully detected transgenic plants and no cross-reaction was recorded with non-transgenic plants. The sensitivity of LAMP was up to 10^4 times that of conventional PCR while real-time LAMP was up to 10^3 times that of LAMP and 10^7 times to that of PCR. The addition of 6 mM magnesium sulphate and 0.4 M betaine with 1 h reaction time proved optimal for amplification through LAMP assay. The assays were validated by testing putative transformants of black pepper.

- Of the 67 putative transgenic plants of PYMoV sense and antisense contructs subjected to PCR and LAMP, 26 plants showed positive reaction for the transgene (PYMoV) and kanamycin indicating presence of inserted DNA sequences in the transgenic plants. Of the 42 transgenic plants subjected to Southern hybridization using digoxiginin labeled transgene specific probe five plants showed positive reaction with single band in each plant indicating singe copy of the transgene.

- Out of 74 transgenic plants carrying CMV coat protein gene when subjected to PCR, 12 plants showed presence of the transgene. The results of Southern analysis showed presence of the transgene in nine plants with two insertions. Of these, eight plants showed strong signal while one plant showed weak signal in northern hybridization indicating the production of transcript. All nine plants showed reaction in western blotting for the presence of transgenic coat protein.

(ii) Testing of transgenic black pepper lines for viral resistance in the green house

- Short listed transgenic lines based on PCR test of both sense (11 plants) and antisense constructs (15 plants) were subjected to challenge inoculation with PYMoV through mealybug and graft inoculation Three plants of sense construct and one plant of antisesnse construct showed mild symptoms till 300 days after challenge inoculation. All challenged plants irrespective of the kind of symptoms showed presence of the virus when tested through PCR. When a result of southern hybridization was compared, three of the symptoms remission plants were positive in Southern as well.

- Nine transgenic plants of CMV were challenged with virus through graft inoculation. When these plants were analysed after 90 days of inoculation through RT-PCR and

real-time RT-PCR, two plants showed presence of the virus indicating the susceptibility while three plants did not show presence of the virus indicating their resistance to the virus.

(d) Conclusions

- The cyclic secondary somatic embryos (pro-embryogenic mass) were produced in different varieties of black pepper and used as explants for transformation.

- Method for regeneration of somatic embryos into plantlets was developed and their genetic fidelity was confirmed through SSR in all varieties.

- Transformation of embryogenic mass of four varieties was done using three different viral constructs. The putative transformants that survived selection using kanamycin were hardened and presence of transgene in them was confirmed.

- A method based on loop-mediated isothermal amplification (LAMP) was developed as an alternative to PCR for quick screening of transgenic plants.

- Challenge inoculation of transgenic plants carrying PYMoV sense and antisense constructs under green house conditions showed mild to severe symptoms though all plants showed presence of the virus. Four plants with symptom remission showed presence of siRNA.

- Challenge inoculation of transgenic plants carrying CMV coat protein region under green house showed presence of the virus in two plants while three plants were free from virus indicating their resistance to the virus.

11. Financial Implications (Lakh Rs.) :

11.1 Expenditure on

(a) Manpower : Rs 10.37161lakhs
(b) Research/Recurring Contingencies : Rs 6.06840 lakhs
(c) Non-Recurring Cost (Including Cost of Equipment) : Nil
(d) Any Other Expenditure incurred: 1.88079 lakhs

11.2 Total Expenditure: Rs 18.32080 lakhs

12. Cumulative Output :

(a) Special Attainments/Innovations :

- The method of cyclic secondary somatic embryos (pro-embryogenic mass) production in different varieties of black pepper was standardized.
- *Agrobacterium* mediated transformation of different varieties of black pepper using pro-embryogenic mass as explant was developed.
- Transgenic black pepper plants harbouring CMV and PYMoV sequences produced and their transgenicity confirmed through PCR, LAMP and Southern hybridization tests.

- A new method of screening and rapid identification of transgenic black pepper plants using LAMP and real-time LAMP assays were developed.
- Challenge inoculation of transgenic plants carrying PYMoV sense and antisense constructs under green house conditions showed mild to severe symptoms though all plants showed presence of the virus. Four plants with symptom remission showed presence of siRNA.
- Challenge inoculation of transgenic plants carrying CMV coat protein region under green house showed resistance against the virus in three plants.

(b) List of publications (1 copy each to be submitted) :

i. Research Papers

Sasi, S., Revathy, K.A. and Bhat, A. I. 2015. Rapid identification of transgenic black pepper using loop-mediated isothermal amplification (LAMP) and real-time LAMP assays. Journal of plant Biochemistry and Biotechnology DOI 10.1007/s13562-015-0302-1(in press).

ii. Reports/Manuals

iii. Working and Concept Papers

iv. Popular Articles

v. Books/Book Chapters

vi. Extension Bulletins

Bhat, A.I., Devasahayam, S. and Anandaraj, M. 2013. Viral disease and its management in black pepper. Technical Bulletin, Indian Institute of Spices Research, Kozhikode, Kerala, India, 14 pp

(c). Intellectual Property Generation (Patents: filed/obtained; Copyrights: filed/obtained; Designs: filed/obtained; Registration details of variety/germplasm/accession) :

(d) Presentation in Workshop/Seminars/Symposia/Conferences (relevant to the project)

Bhat, A. I., Revathy, K.A., Sasi, S and Jiby, M.V. 2014. Production and evaluation of transgenic black pepper expressing *Cucumber mosaic virus* and *Piper yellow mottle virus* sequences (Abstr). Paper presented at the VIROCON 2014 held at TNAU, Coimbatore during 18-20 December, 2014, p167.

Sasi, S., Revathy, K.A. and Bhat, A.I. 2014. Rapid identification of transgenic black pepper using loop-mediated isothermal amplification (LAMP) and real-time LAMP assays (Abstr.) In *International Symposium on Plantation Crops, Hotel Gateway, Kozhikode, 10-12 December 2014. Dinesh R, Santhosh J Eapen, Senthilkumar C M, Ramakrishnan Nair R, Devasahayam S, John Zachariah T & Anandaraj M (Eds.) Abstracts- PLACROSYM XXI,* p55-56.

Revathy K A, Shina Sasi and Bhat A I, 2014. Characterization of *Cucumber mosaic virus* infecting black pepper based on RNA2 and RNA3 sequences (Abs.). In *International Symposium on Plantation Crops, Hotel Gateway, Kozhikode, 10-12 December 2014.*

Dinesh R, Santhosh J Eapen, Senthilkumar C M, Ramakrishnan Nair R, Devasahayam S, John Zachariah T & Anandaraj M (Eds.) Abstracts- PLACROSYM XXI, p.158.

Sasi, S., Bhat, AI, and Nair, RRN. 2015. Somatic embryogenesis and testing somatic embryo derived plants of black pepper for *Piper yellow mottle virus* (Abstr). Paper presented at the National symposium on Understanding host-pathogen interaction through science of omics, held during March 16-17, 2015, IISR, Kozhikode. p 7.

Revathy, KA, Bhat, AI. 2015. RNAi vector construction using 2b gene from black pepper isolate of *Cucumber mosaic virus.* Paper presented at the National symposium on Understanding host-pathogen interaction through science of omics, held during March 16-17, 2015, IISR, Kozhikode.p 143.

(e) Details of technology developed (Crop-based; Animal-based, including vaccines; Biological- biofertilizer, biopesticide, etc; IT based-database, software; Any other-please specify):

- Standardized method of somatic embryogenesis in different varieties of black pepper
- A method for regeneration of plantlets from pro-embryogenic mass of different varieties of black pepper developed.
- A SSR based method was developed for genetic fidelity testing of somatic embryo derived plants.
- A method for production of transgenic plants of black pepper through *Agrobacterium* mediated transformation
- A LAMP based method for quick screening of transgenic black pepper plants.
- A method for screening transgenic plants for resistance to viruses.
- Two CMV resistant transgenic plants and four plants with PYMoV sequences showed symptom remission.

(f) Trainings/demonstrations organized :

- Two SRFs working in the project were trained in the development and testing of transgenic black pepper plants for resistance to viruses.
- Two SRFs from ICRI Myladampura were trained in the detection of viruses in black pepper.

(g) Trainings received :

(h) Any other relevant information :

13. (a) Extent of achievement of objectives and outputs earmarked as per RPP-I :

Objective wise	Activity	Envisaged output of monitorable target(s)	Output achieved	Extent of achievement (%)
1. Production of transgenic black pepper using coat protein gene of Cucumber mosaic virus and portion of ORF III of *Piper yellow mottle virus* as transgene	1.Production of somatic embryos	Pro-embryogenic mass of different varieties of black pepper from seeds	Produced, multiplied and maintained pro-embryogenic mass of eight varieties of black pepper	100
	2. Regeneration of somatic embryo into plantlets	Regenerated plantlets from somatic embryos	Protocol for regeneration of plantlets from pro-embryogenic mass standardized	100
	3. Genetic fidelity testing of regenerated plantlets	Quality testing of regenerated plantlets	Genetic fidelity of regenerated plantlets of all varieties to their corresponding mother plants confirmed through SSR primers	100
	4 *Agrobacterium* mediated transformation of black pepper using CMV coat protein construct	Transgenic plants harbouring CMV coat protein gene	Seventy four transgenic black pepper plants carrying CMV coat protein gene produced and hardened in the green house	100
	5. *Agrobacterium* mediated transformation of black pepper using portion of ORF III of PYMoV sense construct	Transgenic plants harbouring PYMoV ORF III sense sequence	Thirty four transgenic black pepper plants carrying PYMoV sense construct produced and hardened in the green house	100

6. Agrobacterium mediated transformation of black pepper using portion of ORF III of PYMoV antisense construct	Transgenic plants harbouring PYMoV ORF III antisense sequence	Thirty threet transgenic black pepper plants carrying PYMoV antisense produced and hardened in the green house	100
7. Development of LAMP based assay for screening transgenic plants	Assay for quick screening of transgenic plants	Developed and validated LAMP and real-time LAMP based assays for the quick identification of transgenic plants based on CaMV 35 S promoter and kanamycin gene specific primers.	100
8. Screening transgenic plants of CMV through PCR, Southern and northern hybridization and western blotting	Confirmation of transgenicity of plants produced	The 74 transgenic plants when subjected to PCR, 12 plants showed presence of the transgene; Southern analysis showed presence of the transgene in nine plants with two insertions. Of these, eight plants showed strong signal while one plant showed weak signal in northern hybridization and all nine plants showed positive reaction in western blotting	100
9. Screening transgenic plants of sense construct of PYMoV through PCR and Southern hybridization	Confirmation of transgenicity of plants produced	Of the 33 plants tested via PCR, 11 plants showed positive reaction and two plants showed positive signal in Southern hybridization with single insertion	100

	10. Screening transgenic plants of antisense construct of PYMoV through PCR and Southern hybridization	Confirmation of transgenicity of plants produced	Of the 38 plants tested via PCR, 15 plants showed positive reaction and three plants showed positive signal in Southern hybridization with single band indicating single copy of transgene.	100
2 Testing of transgenic black pepper lines for viral resistance in the green house	1.Challenge inoculation of transgenic plants of PYMoV with the virus	Determination of resistance or susceptibility of transgenic plants to PYMoV	11 plants with sense and 15 plants with antisense constructs were subjected to challenge inoculation with PYMoV through mealybug and graft inoculation Three plants of sense construct and one plant of antisesnse construct showed mild symptoms till 300 days after challenge inoculation.	100
	2.Testing for presence of PYMoV in challenged plants	Determination of presence or absence of virus	All challenged plants irrespective of the kind of symptoms showed presence of the virus when tested through PCR.	100
	3. Testing for presence of siRNA in plants showing symptom remission	Testing for presence of siRNA in plants	All four plants that showed symptom remission when checked showed presence of siRNA .	100
	4 Challenge inoculation of transgenic plants of CMV with the virus	Determination of resistance or susceptibility of transgenic plants to CMV	Nine transgenic plants of CMV that were positive in PCR were challenged with virus through graft inoculation.	100
	5. Testing for presence of CMV in challenged plants	Testing for presence of CMV	When challenged plants were analysed after 90 days of inoculation through RT-PCR and real-time RT-PCR, three plants showed presence of the virus indicating susceptibility while two plants did not show presence of the virus indicating resistance to CMV	100

(b) Reasons of shortfall, if any : NA

14. Efforts made for commercialization/technology transfer : NA

15. (a) How the output is proposed to be utilized?

- The *Agrobacterium* mediated transformation protocol developed can be used to transform black pepper with any useful construct for pest and disease resistance and to enhance quality and productivity of the crop.

- Since CMV is known to infect many crops, the CMV construct prepared in this study can be used to transform other crops to get CMV resistant varieties.

- As somatic embryogenesis is known to eliminate viruses in some crops, somatic embryogenesis standardized for different varieties of black pepper in this study can be attempted to get virus-free black pepper plants.

- The LAMP assay developed in this study to differentiate transgenic and non-transgenic black pepper plants can also be used to screen transgenic plants of any crops that harbor CaMV 35S promoter and or kanamycin sequences.

(b) How it will help in knowledge creation?

- Protocol for somatic embryo production, their regeneration and genetic fidelity testing of somatic embryo derived plants of seven varieties of black pepper reported.

- Production of transgenic black pepper plants harboring PYMoV and CMV sequences and confirmation of transgenicity reported for the first time.

- For the first time developed a quick and sensitive screening method of transgenic plants based on LAMP and real-time LAMP.

- Screening method to test copy number and transcript production in transgenic black pepper based on Southern and northern hybridization reported.

16. Expected benefits and economic impact (if any) :

- Developed a method for production of transgenic plants of black pepper through *Agrobacterium* mediated transformation

- A LAMP based method for quick screening of transgenic black pepper plants

- Identified two CMV resistant and four transgenic plants with PYMoV sequences showing symptom resmission.

17. Future line of research work/other identifiable problems :

- Testing promising transgenic plants under different environmental conditions using

different isolates of PYMoV and CMV.

- Preparation of more gene constructs especially hairpin constructs based on CMV and PYMoV sequences and their testing in black pepper for resistance against viruses.

18. Details of research data (registers and records) of the project deposited with the institute : Six number of registers pertaining to the project is with PI of the project.

Signature of PI
(A. Ishwara Bhat)

Co-PI
(D. Prasath)

19. Signature of Head of Division :

20. Observations of PME Cell based on Evaluation of Research Project after completion :

21. Signature (with comments if any along with rating of the project in the scale of 1 to 10 on the overall quality of the work) of Director :