RPF-III

PERFORMA FOR SUBMISSION OF FINAL REPORT OF RESEARCH PROJECTS

Part- I : General Information

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

8001 Institute Project Code No. : Path. XV (813)

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 **: Investigations on diseases of vanilla**

803 Priority Area : Crop Protection

8031 Research Approach :

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

804 Specific Area : Identification, development of diagnostics and management of pathogens

805 Duration of Project : 5 years

8051 Date of start : 1st April, 2003

8052 Date of Completion : 31st March, 2008

806 Total cost /Expenditure Incurred : Rs 11,00,000
(Give reasons for variation, if any from original estimated cost)

807 Executive Summary

Survey of vanilla plantations of Karnataka and Kerala, revealed the occurrence of viral disease with incidence ranging from 0-10% in different gardens. Various kinds of mosaic and associated symptoms such as mild mottle, mild mosaic, and mild chlorotic mottle streak were observed. Electron microscopy of leaf dip preparations of the diseased plants showed the presence of at least four kinds of particles resembling. *Potyxvirus, Carlavirus*, *Closterovirus* and *Cucmovirus*. *Cucumber mosaic virus*

(CMV) causing mosaic, leaf distortion and stunting of vanilla, *Cymbidium mosaic virus* (CymMV) causing mild mottle on leaves, *Bean common mosaic virus* (BCMV) causing necrosis of stem and *Bean yellow mosaic virus* (BYMV) causing severe mosaic and leaf deformation were identified and characterized on the basis of biological and coat protein (CP) nucleotide sequence properties.

Virus particles were purified and polyclonal antibodies against viruses produced in rabbit. IgG isolated from antiserum was used to prepare IgG-alkaline phosphatase conjugate. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) method was standardized for the detection of viruses in plants. Coat protein gene of the all the four viruses were amplified through RT-PCR and cloned in plasmid vectors. The nucleotide sequence of the cloned fragment was determined by sequencing the clones by Sanger's chain termination method using automated DNA sequencing facility. The sequences were subjected to BLAST analysis to find out the related sequences in the GenBank and compared to find out the per cent identity of isolate in question with the known isolates. Phylogenetic trees were drawn for each of the four viruses to know their phylogenetic relationship with other known virus isolates.

As vanilla is propagated by stem cutting, spread of viruses occur mainly through use of infected stem cuttings for planting. As symptoms can not be the sole criteria to decide virus-free nature of the plants, sensitive RT-PCR methods were developed for all the four viruses so as to detect the presence of viruses even in symptomless plants. This method can be used in nursery certification programme to certify virus-free vanilla plants.

Survey for fungal diseases revealed that yellowing and premature bean shedding was major problem during April-May, while *Phytophthora* incited stem and bean infection was observed during July, and root rot by *Fusarium sp.* during November. A disease characterized by the appearance of small water-soaked spots which later develop into characteristic brownish sunken lesions on vanilla beans was noticed for the first time during September 2004 in Kozhikode, Kerala where vanilla is intercropped with coconut, arecanut and clove. The disease was named as brown rotand studies on the causal organism and pathogenicity tests showed that the disease is caused by the fungus *Cylindrocladium quinqueseptatum.*

The average crop loss estimated due to yellowing and premature bean dropping ranged from 23-34%. Studies of the affected beans revealed the association of *Colletotrichum vanillae* with the disease. *In vitro* studies using different fungicides and rhizobacteria showed that Thiophanate methyl at 100 ppm was inhibitory to the fungus followed by carbendazim (250 ppm) and among the rhizobacteria, isolates IISR 152 and IISR 909 were inhibitory. The result of the field trial with different irrigation systems showed that bean shedding was comparatively less in sprinkler irrigated compared to mist irrigated and un-irrigated plots, and the fungicides tested were on par except Bordeaux mixture where bean dropping was found comparatively high.

Root rot and stem rot disease caused by *Fusarium oxysporum f. sp. vanillae* (*Fov*) (and in certain cases association of *P. meadii* also observed) is an important

production constraint. Twenty rhizobacterial isolates were screened for growth promotion and against rot pathogens of vanilla such as *P. meadii, F. oxysporum* and *C. vanillae*. Under *in vitro* conditions, all the rhizobacterial isolates tested except *Bacillus polymixa (*isolate IISR 909) and one *Bacillus* sp. (isolate IISR 915) were inhibitory to *P. meadii* up to 74% while *F. oxysporum* was highly inhibited (91.0%) by *Bacillus polymixa* (isolate IISR 909). The maximum growth promotion in terms of shoot length (27cm) in vanilla was observed in plants treated with *Pseudomonas fluorescens,* (isolate IISR 13). The isolates found promising as growth promoters include: *P. .fluorescens* isolates (IISR6, IISR 853) *B. lentus* (IISR 906), *B. polymixa* (IISR 909), *Enterobacter agglomerans* (IISR 912), *Bacillus* spp. (isolates IISR 910, IISR 913, IISR 914, IISR 915 and IISR 149). Of the four different consortia of rhizobacterial isolates, the consortia containing *P. fluorescens* (isolates IISR 13, IISR 51, IISR 6), *Bacillus* spp. (isolate IISR 152, IISR 147, IISR 151 and IISR 153) and *B. polymixa* (isolate IISR 909 showed the maximum disease reduction of 92.9%.

In another study, seventy biocontrol agents including fungi and bacteria were isolated from 65 locations distributed in six districts of Kerala. They included *Fusarium* sp.(24), *Colletotrichum* spp. (20), *Rhizoctonia sp (2), Trichoderma* spp. (7), *Paecilomyces sp*. (1), Mucor spp. (3), non-sporulating fungi (10) and *Pseudomonas fluorescens* (3). When tested under *in vitro* conditions, six isolates showed more than 50% inhibition. None of the non-pathogenic *Fusarium* sp. tested showed promising inhibitory effect. The five promising isolates when tested under in *planta* conditions by challenge inoculation, *Paecilomyces* sp. provided 100% protection against root rot caused by *F. oxysporum* f.sp.*vanillae* while *Trichoderma harzianum and P. fluorescens gave only 40*% protection.

808 Key words **:** vanilla, disease, survey, incidence, virus, fungi, symptoms, identification, diagnostics, RT-PCR, cloning, management, biocontrol agents, root rot, brown rot, tip rot, premature yellowing, bean shedding, mosaic, necrosis.

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

812 Co- Investigator:

8121 Name : Dr M.N. Venugopal

8122 Designation : Principal Scientist

8123 Division/ Section : Crop Protection

8124 Location : Indian Institute of Spices Research

8125 Institute Address : Indian Institute of Spices Research
Cardamom Research Centre, Appangala,
Madikeri, Karnataka.

813 Co- Investigator:

8131 Name : Dr C. N. Biju

8132 Designation : Scientist

8133 Division/ Section : Crop Protection

8134 Location : Indian Institute of Spices Research

8135 Institute Address : Indian Institute of Spices Research
Cardamom Research Centre, Appangala,
Madikeri, Karnataka.

Par-III: Technical Details

820 Introduction and objectives

8201 Project Objectives : To find out etiology of viral and fungal diseaes.
To develop sensitive diagnostics against viral dieseas

To evolve suitable integrated disease management strategy for fungal diseases

8202 Background information and importance of the projects

Commercial scale cultivation of vanilla has begun in India only about ten years back. Occurrence of a mosaic disease reportedly caused by a potyvirus in India was reported (Thomas, 2002). Besides, random survey of vanilla gardens of Kerala and Karnataka revealed the presence mosaic, necrosis and other related symptoms possibly caused by viruses. In all these cases, causal viruses have not been identified yet. As all the viral diseases are known to spread through the planting material, it is essential that these viruses are identified and suitable sensitive detection method developed for quick identification in the planting material. Similarly although the occurrence of root rot, stem rot and bean rot are known, the pathogens associated with them and no package available for the management of these diseases. The present project envisages an effective disease management strategy for controlling fungal diseases of vanilla.

821 Project Technical Profile

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

Viral diseases

1. Random survey of vanilla growing regions of Karnataka and Kerala taken up.
2. Symptomatology and incidence of the viral diseases recorded.
3. Virus isolates showing typical viral like symptoms collected.
4. All these isolates were maintained in the green house under insect proof condition through vegetative propagation.
5. Isolates wee subjected to leaf dip electron microscopy (done at IARI, New Delhi) to know the particle morphology of the viruses involved.
6. Pure culture of the virus isolate established through local lesions obtained by mechanical inoculation of the virus isolates onto *Chenopodium amaranticolor* and *Nicotiana benthamiana*.
7. Each of the viruses was propagated on suitable plant species under insect proof conditions.
8. Purification of viruses (*Cucumber mosaic virus* and *Bean common mosaic virus*) were done using standard protocols.
9. Polyclonal antiserum against viruses were produced in rabbits by injecting purified virus preparations. About 4-5 injections were given at weekly

intervals and serum containing antibodies collected by bleeding the animal 15 days after last injection.

10. Immunoglobulin G (IgG) was purified from crude antiserum by affinity chromatography using standard protocol.
11. IgG and alkaline phosphatase conjugation done by using glutaraldehyde by one step glutarladehyde method described by Avrameas (1969)
12. Double antibody sandwich (DAS) ELISA was standardized using different concentrations of IgG coating, conjugate dilution and antigen dilution.
13. The DAS-ELISA method standardized above was used to detect the presence of virus in field vanilla samples from different regions.
14. Total RNA isolation from diseased vanilla plants done by Acid-guanidium thiocynate phenol chloroform method or by using RNeasy kit from Qiagen.
15. Reverse transcription (RT) PCR was done using all the necessary components and primer pairs designated specifically for each of the virus in an thermal cycler.
16. After RT-PCR, contents were run on the gel and DNA band of interest was eluted and cloned into a TA vector.
17. Positive recombinants were identified by PCR and restriction analysis.
18. Selected clones were sequenced using automated sequencing facility by chain termination method.
19. Sequence data were compiled using Seqaid and multiple sequence alignment was created using CLUSTAL W program. Phylograms were viewed using Treeview software. Related sequences of viruses were downloaded from GenBank using BLAST search.

Fungal diseases

1. Random survey of vanilla growing regions of Karnataka and Kerala was taken up.
2. Symptomatology and incidence of the viral diseases recorded.
3. During survey samples of disease affected plant parts including stem, leaf, beans and roots were collected.
4. The samples collected were subjected to pure culture isolation of pathogens.

5. The fungal isolates obtained were identified based on their cultural and morphological characters. Al the isolates were numbered and maintained in the laboratory.
6. Rooted cuttings of vanilla were used for pathogenicity tests.
7. Seven different fungicides *viz*. carbendazim (bavistin), mancozeb (Indofil M45), zineb (Indofil Z 78), thiophanate methyl (Roko), Bordeaux mixture and carbendazim-mancozeb (saff) were evaluated under *in vitro* conditions. Fungicides were prepared and incorporated into the medium and the pathogen was inoculated in the centre of petridish. The linear growth of the pathogen was measured after 72 h.
8. The rhizobacterial isolates maintained in the IISR biocontrol repository were used to evaluate against pathogens such as *Phytopthora meadi*, *F oxysporum* f. sp. *vanillae* and *Colletotrichum vanillae* by dual culture method.
9. To study induced systemic resistance due to rhizobacteria, vanilla shoots of 10 cm size were cut off from the rhizobacteria treated plants and were inoculated with pathogen. Observations on leaf area infected and length of internode infection recorded.
10. Pot culture experiment was conducted to know the effect of fungicides on disease management. Vanilla plants raised in pots were sprayed or drenched with different fungicides. The experiment had nine treatments with plants each and observations on leaf rot, basal root rot tip rot were taken.
11. A pot culture experiment to know the effect of rhizobacteria consortia was carried out. The consortia was applied at the time of planting and repeated thrice at two month interval. Six month after establishment of the plants, pathogen, *F. oxysporium* f. sp. *vanillae* was applied and plants were observed for four months for disease incidence.
12. Field trail was laid out in three vanilla gardens in Calicut district to study the effect of fungicides (carbendazim, mancozeb, zineb, thiophanate methyl, Borrdeaux misture and carbendazim mancozeb) in controlling immature bean shedding under two different irrigation conditions (mist and sprinkler). The plot under mist irrigation received irrigation for 4 h daily at 10 to 12 h and 15 to 18h. The plot with sprinkler irrigation received irrigation from 11 to 15 h daily.

13. Another field trial was initiated during 2006 with the onset of flowering in vanilla in a farmer's plot to study the effect of growth harmones, fertilizers and fungicides on immature bean shedding in RBD with three replications having 10 vines per replication. The treatments were given at monthly interval starting from February and continued till May.
14. Field trial for the management of root rot was laid out in RBD with three replications having 10 vines per replication at IISR farm, Peruvannamuzhi. The experiment consisted of two promising fungicides, bioconsortia and a fungal biocontrol agent, *Paecilomyces* sp. The treatments were imposed during onset of monsoon and a second application was done after one month.

8212 Total man months involvement of component project workers

a) Scientific 35
b) Technical 10
c) Supporting 15

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

Viral diseases

Survey

A total of 65 vanilla plantations at Idukki, Kozhikode and Wyanad districts of Kerala, and Dakshina Kannada, Udupi and Uttara Kannada districts of Karnataka were surveyed for the incidence and distribution of viral diseases. Mosaic and necrosis were the two kinds of viral diseases found in many of the plantations. The incidence of mosaic disease varied from 0-5% while that of necrosis varied from 0-10% (Table 1). Maximum incidence of mosaic disease was recorded at Adimali under Idukki District of Kerala while many locations surveyed both in Karnataka and Kerala were free from mosaic disease. Similarly, maximum incidence of necrosis disease was observed at Nellipoyil village under Kozhikode taluk of Kerala and many plantations surveyed did not show the presence of necrosis disease. Both the diseases were noticed in all ages of the crop. Arecanut, cashew, gliricidia, erythrina and forest trees

were the live standards found in areas surveyed and vanilla was grown under natural shade in these plantations. Both mosaic and necrosis diseases were found in vanilla crop irrespective of the type of standards used. In general both incidence and severity of the disease were higher at Kerala compared to Karnataka.

Table 1: Distribution and incidence of viral diseases on vanilla in Karnataka and Kerala

Location		**No. of gardens surveyed**	**Disease noticed**	Mean incidence (%)
State/District	**Village**			
Karnataka State Dakshina Kannada District	Adyanadka	Three	Mosaic Necrosis	0.50 0.75
	Peeramogaru	Two	Mosaic Necrosis	0.15 0.25
	Sediyapu	Two	Mosaic Necrosis	0.40 0
	Shibara	One	Mosaic Necrosis	0 1.0
Hassan District	Ankehalli	One	Mosaic Necrosis	0 0
Udupi District	Idu	Three	Mosaic Necrosis	3.0 2.0
Uttara Kannada	Gadikai	Two	Mosaic Necrosis	0 0
	Koppa	One	Mosaic Necrosis	0 2.0
	Kumbarakottige	One	Mosaic Necrosis	0 1.0
	Malenahalli	Two	Mosaic Necrosis	0 1.0
	Neernahalli	Two	Mosaic Necrosis	1.0 0
Kerala State Idukki District	Adimali	One	Mosaic Necrosis	5.0 1.0
	Arakkulam	Two	Mosaic Necrosis	0 0
	Ayyappancoil	Six	Mosaic Necrosis	1.2 1.4
	Kudavathoor	Two	Mosaic Necrosis	2.0 0
	Muttom	One	Mosaic Necrosis	0 4
	Myladumpara	One	Mosaic Necrosis	0 0
	Santhapara	One	Mosaic Necrosis	0 4.0
	Vandiperiyar	One	Mosaic Necrosis	3.0 4.0
Kozhikoe District	Kodenchery	Three	Mosaic Necrosis	0.4 2.7
	Koovapoyil	One	Mosaic Necrosis	2.0 0
	Nellipoyil	Eleven	Mosaic Necrosis	0.6 10.0
	Thiruvambadi	Six	Mosaic Necrosis	2.2 1.0
Wyanad District	Madakkimala	Two	Mosaic Necrosis	0 3.0
	Muttil	One	Mosaic Necrosis	0 0
	Panyaram	Three	Mosaic Necrosis	0 2.0
	Raroth	One	Mosaic Necrosis	0 1.0
	Vythiri	Two	Mosaic Necrosis	0 1.0

Symptomatology

Disease symptoms were more prominent and visible on the younger leaves. Various kinds of mosaic and associated symptoms such as mild mottle, mild mosaic, and mild chlorotic mottle streak were observed (Fig. 1). In a few cases such mosaic is also associated with leaf distortion giving wavy margin to the leaves. The size of the leaves also gets reduced and in advanced stages leaves become brittle and show severe crinkling with torn areas on the leaf lamina. The necrotic lesion on stem and leaves was the other kind of symptoms found in many of the plantations (Fig. 1). The distinct necrotic lesions of varying length (few mm to several cm) were seen on the stem region of the plant. In a few cases necrosis were also seen in the older leaves at the lower surface.

Electron microscopy

Electron microscopy of leaf dip preparations of the diseased plants showed the presence of at least four kinds of particles. Three kinds of flexuous particles of varying length resembling viruses in the genera such *Potyxvirus* or *Carlavirus*, *Potyvirus* and *Closterovirus* were observed (Fig. 2). These particles were seen independently and also in combination in the diseased samples, thus making it difficult to assign a particular virus for a particular kind of symptoms. In addition, isometric particles were also seen in a few samples in addition to flexuous particles.

Identification and characterization of *Cucumber mosaic virus* causing mosaic disease

Cucumber mosaic virus (CMV) causing mosaic, leaf distortion and stunting of vanilla was characterized on the basis of biological and coat protein (CP) nucleotide sequence properties. The characteristic disease symptoms associated with CMV infection in vanilla included mosaic and leaf distortion. The leaves become small and leathery with deformation. In some cases, the internodal length was reduced leading to stunting of the plant. The virus was efficiently sap transmitted to *C. amaranticolor.*

Figure 1: Symptoms of viral diseases on vanilla. (a) Mosaic affected leaf showing chlorotic mottling and streaks (b) Necrosis affected plant showing necrosis.

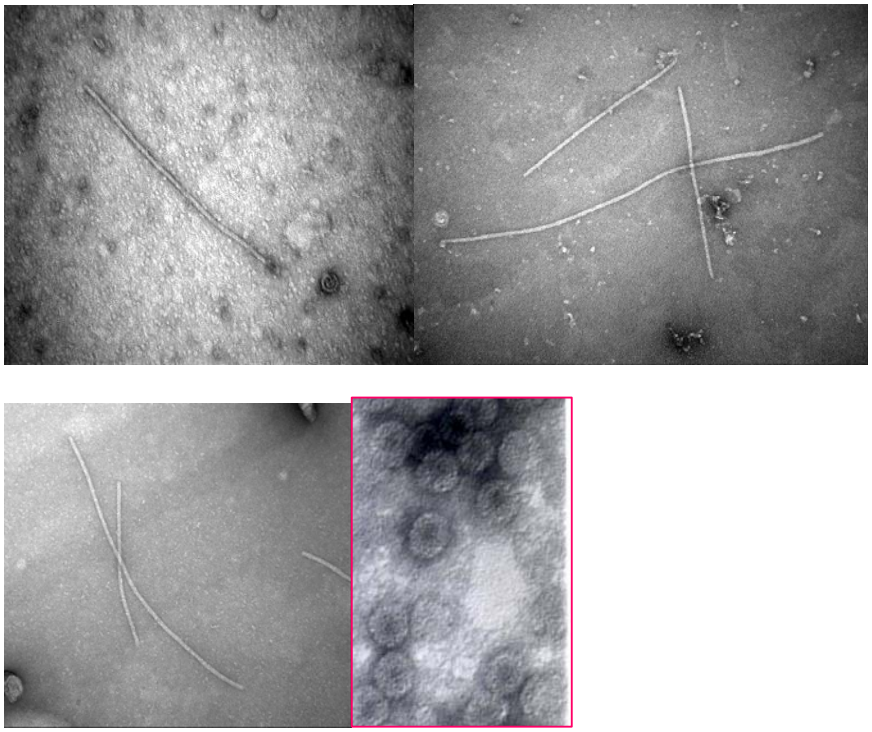

Figure 2: Leaf dip electron microscopy of virus infected vanilla leaves showing different particle morphology

The inoculated leaves produced chlorotic local lesions within 3-5 days of inoculation, which later turned to necrotic lesions. Back inoculated *V. planifolia* produced original symptoms. The virus was also transmitted to plant members belonging to Cucurbitaceae, Fabaceae and Solanaceae (Table 2). Back inoculation to *C. amaranticolor* from test plants showed that only susceptible hosts reproduced the symptoms on *C. amaranticolor.*

Virus Purification

The virus was purified from *N. benthamiana* as it was a good propagating host. In sucrose gradient centrifugation, the gradient fraction from 3.25 to 3.50 cm from the top of the centrifuge tube was ascertained to have the virus particles. Yield of virus obtained per 100g of tissue varied from 1-3 mg depending on the season and harvest time after inoculation. Electron microscopy of negatively stained purified preparations revealed the presence of typical isometric particles of about 28 nm diameter. The purified preparation when run on SDS-PAGE produced a major band corresponding to 25 kDa. SDS-PAGE of purified preparation also revealed some break down of coat protein. The SDS-PAGE separated viral preparations reacted with its homologous antiserum in EBIA and reacted positively with the expected band. EBIA tests clearly confirmed the degradation of coat protein subunits in the purified preparations.

Serological detection

Polyclonal antiserum against the virus was produced in a New Zealand white rabbit by injecting purified virus preparation (0.5 mg of virus with Freund's incomplete adjuvant, (1:1) intramuscularly six times at 10 days interval. The animal was bled 15 days after the last injection and the antiserum was collected. The virus was immunogenic and produced antiserum with an titre of 1:8000 in ELISA. Immunoglobulin G (IgG) was purified from the crude polyclonal antiserum by affinity column chromatography using IgG purification kit (Genei, Bangalore) and quantified by taking O.D values at 280 nm (1.4 O.D = 1mg/ml of IgG). One mg of this IgG was used for enzyme conjugate preparation. One step glutaraldehyde method described by Avrameas (1969) was followed for the preparation of IgG-alkaline

phosphatase conjugate. In order to standardize DAS ELISA for detection of CMV, various concentrations of coating antibody (IgG) and enzyme conjugate were tried

Table 2. Reaction of different plant species to *Cucumber mosaic virus* infecting vanilla

Family /Plant species	Symptoms	Days required for symptom expression
Chenopodiaceae *Chenopodium amaranticolor* L.	CLL, NLL	3-7
Cucurbitaceae *Benincasa hispada* (Thunb.) cogn. ***Cucumis sativus*** L. *Cucurbita pepo* L. *Lagenaria siceraria* (Mol.) *Trichosanthes anguina* L.	Mo VC SL Mo CS	7-10 7-10 - 15-20 3-7
Fabaceae *Cajanus cajan* (L.) Millsp. *Cicer arietinum* Linn. *Glycine max* (L.) Merr. *Vigna mungo* (L.) Hepper *Vigna radiata* (L.) Wilczek *Vigna unguiculata* (L.) Walp.	SL SL SL VN SL SL	- - - 5-10 - -
Malvaceae	SL	-
Poaceae	SL	-
Solanaceae *Capsicum annuum* L. *Lycopersicon esculentum* L. *Nicotiana benthamiana* W. *Nicotiana glutinosa* L. *Nicotiana tabacum* L.	M, LC D, St B, M, Mo, LC B, M, Mo, LC B, M, Mo, LC	5-7 5-7 7-10 7-10 7-10

B-blisters on leaves; CLL-chlorotic local lesion; CS-chlorotic spot; D-deformation of top leaves; LC-curling of leaves; M-mosaic; Mo-mottling; NLL-necrotic local lesion; SL-symptomless; St-streaks on stem; VC- vein clearing; VN-veinal necrosis.

and, IgG at 1µg/ml of coating buffer and CMV IgG-alkaline phosphatase conjugate at 1:2000 dilutions successfully detected the presence of CMV in the infected samples. DAS ELISA method thus standardized was able to detect CMV infection in the vanilla plant samples. Out of 66 plants representing different locations tested, 19 showed positive reaction to CMV antiserum (Table 3). Plants showing symptoms

Table 3. Detection of *Cucumber mosaic virus* (CMV) by double antibody sandwich (DAS) ELISA in vanilla plants representing different locations of Karnataka and Kerala states

Isolates	Visual symptoms	A_{405} value[$]
Kerala state		
Calicut		
Sample 1	LD	0.36
Sample 2	NS, LD, M	0.44
Sample 3	M	0.40
Sample 4	NS	0.36
Sample 5	SL	0.35
Vandiperiyar		
Sample 1	NS, LD, S	0.40
Sample 2	M, CS, LD, NS	0.46
Sample 3	M	0.57
Sample 4	NS	0.37
Sample 5	M, NS, S	1.00
Kannur		
Sample 1	NS, CS, LD, S	0.33
Sample 2	S	0.47
Sample 3	S	0.37
Karnataka state		
Dakshina Kannada		
Sample 1	NS	0.48
Sample 2	NS	0.30
Sample 3	CS	1.00
Sample 4	NS, CS	1.23
Saklespur		
Sample 1	SL	0.91
Sample 2	SL	0.38
Healthy vanilla	SL	0.05

CS-chlorotic streaks; LD- leaf deformation; M-mosaic; NS-necrotic spot; S-stunting of plant; SL-symptomless

[$] Average of three replications taken 1h after addition of substrate.

such as mosaic, mottling, leaf deformation as well as symptomless plants reacted positive to CMV antiserum.

RNA isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For RT-PCR, RNA was extracted using Nucleospin RNA Plant kit (Macherey-Nagel, Duren, Germany). The primers designed for the CP gene sequences of CMV (based on multiple sequence alignments of CP sequences available in GenBank) were used to prime the amplification. Genome sense primer 5' ATGGACAAATCTGAATCAAC 3' was derived from the beginning of the first 20 bases of the coding region while antisense primer, 5' TCAAACTGGGAGCACCC 3' represented last 17 bases of the coding region of the CP gene. The PCR reaction (100 µl) contained 200 ng each of the primers, 20 U Ribonuclease inhibitor (Genei, Bangalore), 10 U AMV reverse transcriptase (Finnzymes OY, Finland), 2.5 U *Taq* Polymerase (Genei, Bangalore), 1x PCR buffer (Genei, Bangalore), 10 mM Dithiothreitol, (Genei, Bangalore) and 10 µM each of the dNTPs (Finnzymes OY, Finland). PCR mix (27 µl) containing the above components was added to the tubes containing the template RNA (73 µl) resulting in a final reaction volume of 100 µl. Amplification was performed in an automated thermal cycler and the programme consisted of one cycle at 42° C for 45 min for cDNA synthesis followed by 40 cycle reaction profile involving 30 s of denaturation at 94° C, 1 min of annealing at 50° C, and 1 min of extension at 72° C and a single cycle of final extension at 72° C for 10 min. The reaction products (20 µl) were analyzed on 1% agarose gel along with 500 bp DNA ladder.

Cloning, sequencing and sequence analyses of coat protein gene

RT-PCR was successful in amplifying the CMV CP gene and a product of expected size (ca. 650 bp) was observed in infected sample. The resultant product was cloned and sequenced. The sequenced region contained a single open reading frame, which comprised 657 bases of nucleotides potentially coding for 218 amino acids (Fig. 3). It was compared with CP gene sequences of all available CMV isolates from India as well as a few representative isolates from other parts of the world belonging to both the subgroups (I and II). Nucleotide and deduced amino acid sequence of CP

gene of CMV infecting vanilla (VP) showed greatest identity with CMV isolate from black pepper (99%) (IN-Pn). Both at nucleotide and amino acid level, VP showed an identity of 89–92% and 92–95% with Subgroup IA isolates of CMV while it was 91-99% and 94-99% with Subgroup IB isolates respectively. An identity of 75-76% and 78-82% were observed with Subgroup II isolates both at nucleotide and amino acid level respectively.

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ATGGACAAATCTGAATCAACCAGTGCTGGTCGTAACCGTCGACGTCGTCCGCGTCGCGGTTCCCGCTCCG      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

AGCTGGTCGTCCTACTATTAACCACCCAACCTTTGTGGGTAGTGAGCGTTGTAAACCTGGATACACGTTC     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

GTATTCAAAAGACGATGCGCTCGAGACGGATGAACTAGTACTTCATGTCGACATTGAGCACCAAGGGATT     630
 Y  S  K  D  D  A  L  E  T  D  E  L  V  L  H  V  D  I  E  H  Q  G  I      210

CCCACTTCTGGGGTGCTCCCAGTTTGA     657
P  T  S  G  V  L  P  V  <       218
```

Figure 3: Nucleotide and deduced amino acid sequence of coat protein gene of *Cucumber mosaic virus* infecting vanilla

Multiple sequence alignment based on deduced CP amino acid sequences of subgroup I isolates showed that VP and IN-Pn differed only at one position (Fig. 4). Analysis of CP identified one amino acid position (G209) as unique to VP. Within subgroup IB isolates, Indian isolates were more close to each other than to subgroup IB isolates from other regions (Fig. 4). One amino acid position (T31) was found to be unique to all Indian isolates. Similarly one amino acid position (Y99) was unique among subgroup IA isolates.

```
VP          MDKSESTSAGRNRRRRPRRGSRSASSSADATFRVLSQQLSRLNKTLAAGRPTINHPTFVG   60
IN-Pn       ************************************************************
IN-PhyM     ****************************L*******************************
IN-Di       ****************************L*******************************
IN-Am       **************HS****************************************
IN-Hb       **************L*****************************************
IN-Pb       ************************************************************
IN-Pl       ************************************************************
IN-Ba       ************************************************************
M48         ****D*A*********************VN*******************************
ChCu        ****************A*CGC******GN******************S********
ABI         ****************************N*******************************
SD          ****************************N*******************************
C7-2        ***********************P*****N*******************************
NT9         ***********************P*****N*******************************
Ban         ***********************P*****N*******************************
D8          ****************A********S**N******************L*******
N           *************************S**N*******************************
P1          **********************V***S**N*******************************
Leg         **********************P**S**N*******************************
Pepo        *******G**************P*****N**************S*************
Kor         ***************L*******P**S**N**************S*************

VP          SERCKPGYTFTSITLKPPKIDKGSYYGKRLLLPDSVTEFDKKLVSRIQIRVNPLPKFDST   120
IN-Pn       ************************************************************
IN-PhyM     ********************************************V***********
IN-Di       ****************************W*****************V***********
IN-Am       ****N**********R*****************************************S
IN-Hb       ************************************************************
IN-Pb       ********************R***************************************
IN-Pl       *********************R**************************************
IN-Ba       ****R***************R**************************************
M48         ***************R*****R********F**E*************************
ChCu        *********************P**************************************
ABI         **********S**********R**************************************
SD          **********S**********R**************************************
C7-2        **********S**********R**************************************
NT9         *********************R**************************************
Ban         ****R***************R***************Y**********************
D8          ********************R***************Y**********************
N           ********************R***************Y**********************
P1          ********************R***************Y**********************
Leg         ********************R*************M*Y**********************
Pepo        ********************R***************Y**********************
Kor         T*************S******R***************Y**********************

VP          VWVTVRKVPASSDLSVSAISAMFADGASPVLVYQYAASGVQANNKLLYDLSVMRADIGDM   180
IN-Pn       ************************************************************
IN-PhyM     ************************************************************
IN-Di       ************************************************************
IN-Am       *******I****************************************************
IN-Hb       ************************************************************
IN-Pb       ***************A**********************************A********
IN-Pl       ***************A**********************************A********
IN-Ba       ***************A*********************T************A********
M48         ***************A***T*******************************P********
ChCu        ***************T******S**************************A********
ABI         ***************A**********************************A********
SD          ***************A**********************************A********
C7-2        ***************A**************N*****************A********
NT9         ********S*******A*************************************A********
Ban         ***************A*****************************F*****A********
D8          ***************A**********************************A********
N           ***************A**************H*****************A********
P1          ***************A**********************************A********
Leg         ***************A**********************************A********
Pepo        ***************A**********************************A********
Kor         ***************P******P**P**********************A********
```

Figure 4: Multiple sequence alignment of coat protein amino acid sequences of CMV infecting vanilla (VP) with other CMV isolates of Subgroup I using Clustal X. Asterisk indicates identity at a given position. Sequences for comparisons were obtained from GenBank

Phylogram illustrating phylogenetic relationship among CMV isolates generated based on CP amino acid sequences showed that VP was most closely related to the members of Subgroup I. In contrast, CMV isolates belonging to Subgroup II formed a different cluster, well separated from Subgroup I isolates. Within subgroup I, VP showed close phylogenetic relationship with subgroup IB isolates than IA isolates as the latter formed a separate cluster. However, within subgroup IB, two major clusters were obtained. One cluster consisted of Indian isolates that aligned together as one group and the other consisted isolates from other Asian countries. The only exception was three Indian isolates (IN-Pb, IN-Pl & IN-Ba) and two other isolates (ChCu & M48) which formed two separate subclusters. Among all the isolates, VP showed maximum evolutionary relationship with IN-Pn.

Detection of CMV through RT-PCR

The RT-PCR method described above was successful in the detection of CMV in field samples (Fig. 5). A modified acid guanidium thiocynate phenol chloroform method was employed after isolation of total RNA from the vanilla samples. RT-PCR was carried out using primers designed for the amplification of coat protein gene of the virus. The method developed could be used in the routine detection of virus in the planting material certification programme.

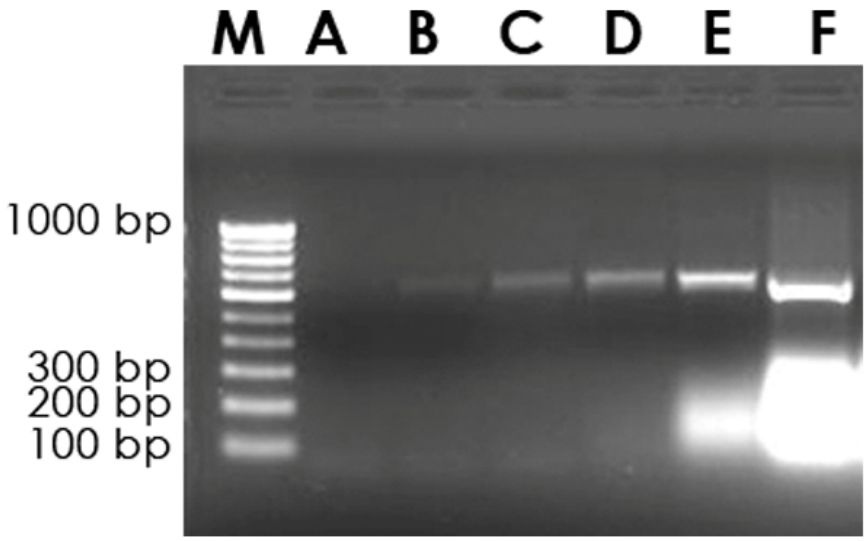

Figure 5: Detection of CMV in field plants through RT-PCR. Lane M: 100bp ladder; Lane A: Healthy (negative control); Lanes B-F: test plants

Identification, characterization and development of diagnostics for *Cymbidum mosaic virus* (CymMV) infecting vanilla

The virus causing mild chlorotic mottle and streaks on leaves of vanilla was identified as a strain of *Cymbidium mosaic virus* based coat protein gene sequence comparison and phylogenetic studies. In RT-PCR, only plants showing mild chlorotic mottle and chlorotic streaks resulted in successful amplification giving a band of expected size. No such band was seen in healthy plants. The PCR amplified DNA fragment was cloned, sequenced and sequence data deposited at GenBank with accession number DQ 208422. The sequenced region contained a single open reading frame, which comprised of 672 nucleotides potentially coding for 223 amino acids (Fig. 6). It was compared with CP gene sequences of all available CymMV isolates from India as well as a few representative isolates from other parts of the world. Similarly partial CP gene sequences of CymMV isolates infecting vanilla from French Polynesia and ReUnion Island were also used for comparison. Nucleotide and deduced amino acid sequence of CP gene of CymMV infecting vanilla in India (IND-

```
TGGGAGAGCCCACTCCAGCTCCGGCTGCCACTTACTCCGCTGCCGACCCCACTTCTGCACCAAGATTGG    70
M  G  E  P  T  P  A  P  A  A  T  Y  S  A  A  D  P  T  S  A  P  R  L  A   24

CCGACCTGGCTGCCATTAAGTACTCACCTGTCACCTCCTCCATCGCCACCCCCGAAGAAATCAAGGCCAT  140
  D  L  A  A  I  K  Y  S  P  V  T  S  S  I  A  T  P  E  E  I  K  A  I    47

AACCCAATTGTGGGTTAACAACCTTGGCCTCCCCACTGACACCGTAGGTACCGCGGCCATTGACCTGGCC  210
 T  Q  L  W  V  N  N  L  G  L  P  T  D  T  V  G  T  A  A  I  D  L  A    70

CGTGCCTACGCTGATGTCGGGGCGTCCAAGAATGCTACTCTGCTCGGTTTCTGCCCTACGAAACCTGATG  280
R  A  Y  A  D  V  G  A  S  K  N  A  T  L  L  G  F  C  P  T  K  P  D  V   94

TCCGTCGCGCCGCTCTTGCCGCGCAGATCTTCGTAGCCAACGTCACCCCCCGCCAGTTTTGCGCTTACTA  350
  R  R  A  A  L  A  A  Q  I  F  V  A  N  V  T  P  R  Q  F  C  A  Y  Y   117

CGCAAAAGTGGTGTGGAATCTGATGCTGGCCACTAACGATCCGCCCGCCAACTGGGCTAAGGCTGGTTTC  420
 A  K  V  V  W  N  L  M  L  A  T  N  D  P  P  A  N  W  A  K  A  G  F    140

CAGGAGGATACCCGGTTTGCCGCCTTTGACTTCTTCGATGCCGTCGATTCCACTGCCGCACTGGAGCCTG  490
Q  E  D  T  R  F  A  A  F  D  F  F  D  A  V  D  S  T  A  A  L  E  P  A  164

CTGAATGGCAGCGCCGCCCTACTGACCGTGAACGTGCTGCGCACTCGATCGGGAAGTACGGCGCCCTTGC  560
  E  W  Q  R  R  P  T  D  R  E  R  A  A  H  S  I  G  K  Y  G  A  L  A   187

CCGTCAGCGCATCCAAAACGGCAACCTTATCACCAACATTGCAGAGGTCACCAAGGGCCATCTTGGCTCC  630
 R  Q  R  I  Q  N  G  N  L  I  T  N  I  A  E  V  T  K  G  H  L  G  S    210

ACCAACACCCTCTATGCTCTGCCTGCACCCCCTACTGAATAA     672
T  N  T  L  Y  A  L  P  A  P  P  T  E  <      223
```

Figure 6: Nucleotide and deduced amino acid sequence of coat protein gene of *Cymbidium mosaic virus* (CymMV) infecting vanilla

Vp) showed highest identity (98.8 and 99.1%) with a CymMV isolate from Singapore (SGR). Identity with other CymMV isolates ranged from 88.3 to 97.7% at nucleotide level. While at amino acid level, except for the two Korean isolates (KRA-Dd and KRA-orc), identity ranged from 92.3 to 98.2%. Korean isolates showed an identity of 69.4% (KRA-Dd) and 75.6% (KRA-orc) with the present isolate. When all CymMV isolates available from India were compared, identity ranged from 94.3 to 96.4 % and 92.3 to 97.3% at nucleotide and amino acid levels respectively. Similarly IND-Vp showed high levels of identities (95.5 to 97.0% and 98.2 to 99.4%) with partial CP sequence of CymMV isolates infecting vanilla from French Polynesia and ReUnion Island at nucleotide and amino acid levels.

Multiple sequence alignment based on deduced CP amino acid sequences of CymMV isolates showed that IND-Vp and SGR differed only at two positions at the N-terminal region (Fig. 7). Analysis of CP identified one amino acid position (R22) as unique to IND-Vp. Similarly one amino acid position (N81) was found to be unique among IND-Vp and SGR isolates. When available partial CP sequence of vanilla isolates of CymMV were considered, IND-Vp and FP-Vt-29a differed only at

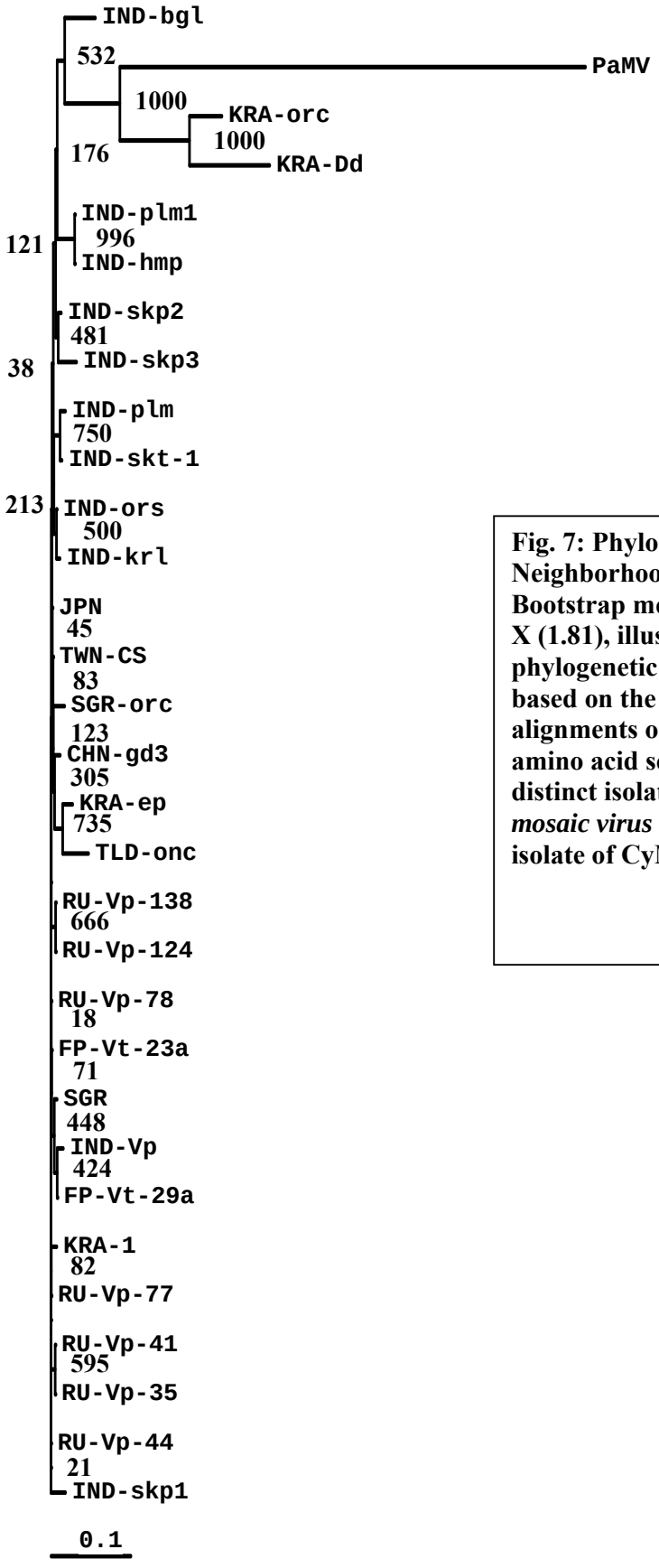

Fig. 7: Phylogram, drawn by Neighborhood Joining Bootstrap method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of the coat protein amino acid sequences of 29 distinct isolates of *Cymbidium mosaic virus* and vanilla isolate of CyMV (IND-Vp).

one

position. Among Indian isolates, CymMV isolate from Bangalore (IND-bgl) showed maximum variation. Multiple alignment showed that core region of CP were more conserved than N- and C-terminal regions. C-terminal region accounted for maximum variation especially in two isolates from Korea (KRA-orc and KRA-Dd).

Phylogram illustrating phylogenetic relationship among CymMV isolates generated based on CP amino acid sequences showed that IND-Vp was most closely related to a vanilla isolate of CymMV from French Polynesia (FP-Vt-29a) followed by a CymMV isolate from Singapore (SGR). Except for two isolates from Korea (KRA-orc and KRA-Dd), results showed that sequences were highly conserved among different isolates of CymMV infecting different hosts and regions. The relatively low sequence identities observed with all Indian isolates of CymMV suggest that vanilla isolate of CymMV did not originate locally.

Detection of CymMV by RT-PCR:

Infection of vanilla by CymMV is often symptomless or cause only mild symptoms. This coupled with easy mechanical transmission of the virus from plant to plant (through injuries caused during cultural operations such as looping of vines and manual pollination) could lead to widespread dissemination of the virus. Hence it is essential to index planting material for the presence of virus before they are being used. In this direction, RT-PCR method was developed in the present studies can be efficiently used to identify CymMV free plants. Of the 65 samples tested, 27 gave positive results for CymMV infection (Fig. .8). This would help in identifying and certifying planting material to check spread of the virus.

Identification, characterization and development of diagnostics for *Bean common mosaic virus* (BCMV) infecting vanilla

The diseased plants showed distinct necrotic lesions of varying length (few mm to several cm). The disease initially starts as a necrotic spot on the stem and slowly gets enlarged and encircles the stem. In an affected plant, necrosis may be seen only at one or few regions on the stem. Rest of the stem region looks apparently healthy without any visual symptoms. A few of the necrosis affected plants also show

mosaic symptoms on leaves. The virus was efficiently sap transmitted to *C. amaranticolor.* The inoculated leaves produced chlorotic local lesions within 3-5 days of inoculation. In host range studies through mechanical inoculation the virus was also transmitted to plant members belonging to Cucurbitaceae, Fabaceae and Solanaceae (Table 4). *N. benthamiana* was found to be a good host for propagation of the virus.

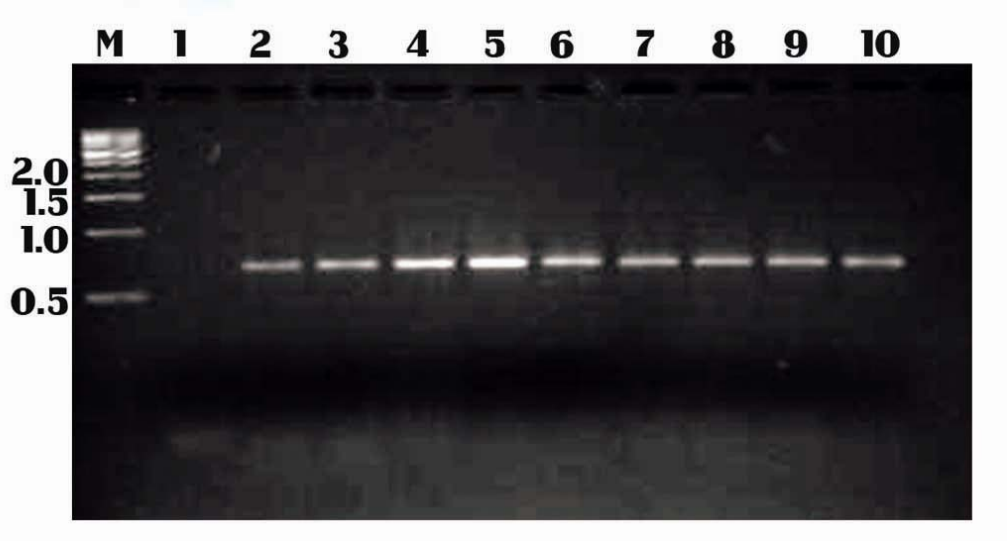

Fig.8. Agarose gel showing RT- PCR amplification of CP gene of *Cymbidium mosaic virus*. Lane 1: 500 bp DNA size ladder and numbers on the left indicate their size in kb; Lane 2: healthy vanilla (negative control); Lane 3: a known CyMV infected vanilla (positive control); Lanes 4-7: vanilla isolates showing varying kinds of symptoms

Cloning, sequencing and phylogenetic analysis of virus associated with stem necrosis

The diseased vanilla plants showing typical symptoms were were subjected to reverse transcription (RT) PCR using primers designed for the conserved region of NIb and coat protein (CP) region of five different potyviruses . A ~1000 bp RT-PCR amplified fragment was consistently seen in only infected plants that used primers specific for *Bean common mosaic virus* (BCMV). The amplicon obtained from one infected vanilla plant was cloned and sequenced. Sequenced region contained 1033 nuceotides

Table 4. Reaction of different plant species to virus causing necrosis in vanilla

Family /Plant species	Symptoms	Days required for symptom expression
Chenopodiaceae *Chenopodium amaranticolor* L.	CLL,	3-7
Cucurbitaceae *Benincasa hispada* (Thunb.) cogn. ***Cucumis sativus*** L.	SL SL M GM M&CS	- - 12-15 10-12 4-6
Fabaceae *Cajanus cajan* (L.) Millsp. *Cicer arietinum* Linn. *Glycine max* (L.) Merr. *Vigna mungo* (L.) Hepper *Vigna radiata* (L.) Wilczek	SL SL SL SL SL C &VN	- - - 5-10 - 12-15
Malvaceae *Abelmoschus esculentus* L.	SL SL	- -
Poaceae	SL	-
Solanaceae *Capsicum annuum* L. *Lycopersicon esculentum* L. *Nicotiana benthamiana* W. *Nicotiana glutinosa* L.	SL SL M &LC SL SL	- - 7-10 - -

CLL - chlorotic local lesion; CS - chlorotic spot; GM - green mosaic; LC - curling of leaves; M - mosaic; SL - symptomless; VN - veinal necrosis.

comprising part of NIb and on coat protein gene of the virus. The coat protein gene consisted of 809 nucleotides potentially coding for 269 amino acids (Fig. 9). Sequence analyses based on coat protein per cent sequence identity at the nucleotide and deduced amino acid indicated that the causal virus is a strain of BCMV. The

phylogram depicting phylogenetic relationship among different potyvirus isolates also clearly indicated that virus is a strain of BCMV (Fig. 10).

```
TCAGGAACTGGACAACCGCAGCCACCAATAGTGGATGCTGGCGTGGATGCTGGGAAAGATAAGAGAGAGA      70
S  G  T  G  Q  P  Q  P  P  I  V  D  A  G  V  D  A  G  K  D  K  R  E  R      24

GAAGCAATAGAGGACAAGACCCTGAATGCAGGGAGGGGTCAGGAAACGACAACCGTGGTGCGGGAGATTC     140
   S  N  R  G  Q  D  P  E  C  R  E  G  S  G  N  D  N  R  G  A  G  D  S      47

AACAGTGAGAGACAAGGATGTGAACGCAGGCTCCAAAGGGAAAGTTGTCCCACGGCTTCAAAAGATCACA     210
 T  V  R  D  K  D  V  N  A  G  S  K  G  K  V  V  P  R  L  Q  K  I  T       70

AAAAGGATGAACTTGCCCATGGTGAAGGGGAATGTTATTTTAAATCTAGACCATCTGTTGGATTACAAGC     280
K  R  M  N  L  P  M  V  K  G  N  V  I  L  N  L  D  H  L  L  D  Y  K  P      94

CAGAACAAACTGATCTTTTTAACACAAGAGCAACAAAGATGCAGTTTGAAATGTGGTACAATGCTGTGAA     350
   E  Q  T  D  L  F  N  T  R  A  T  K  M  Q  F  E  M  W  Y  N  A  V  K     117

GGGCGAGTATGAGATAGATGACGCACAGATGTCAATTGTAATGAATGGCTTCATGGTGTGGTGTATTGAC     420
 G  E  Y  E  I  D  D  A  Q  M  S  I  V  M  N  G  F  M  V  W  C  I  D      140

AATGGCACTTCACCGGATGTTAATGGTACATGGGTGATGATGGATGGAGATGAGCAAGTTGAATACCCAC     490
N  G  T  S  P  D  V  N  G  T  W  V  M  M  D  G  D  E  Q  V  E  Y  P  L     164

TCAAACCAATGGTTGAGAATGCAAAACCAACACTCCGTCAAATCATGCACCATTTCTCAGATGCAGCTGA     560
   K  P  M  V  E  N  A  K  P  T  L  R  Q  I  M  H  H  F  S  D  A  A  E     187

AGCATACATTGAGATGAGAAATTCCGAGAAACCGTACATGCCTAGGTACGGATTACTTCGGAATTTGAGG     630
 A  Y  I  E  M  R  N  S  E  K  P  Y  M  P  R  Y  G  L  L  R  N  L  R      210

GATAAAAATCTAGCTCGCTACGCTTTTGATTTCTATGAGGTGACATCCAAAACATCGGATCGAGCCAGAG     700
D  K  N  L  A  R  Y  A  F  D  F  Y  E  V  T  S  K  T  S  D  R  A  R  E     234

AAGCAGTAGCACAGATGAAGGCAGCAGCCCTCAGCAACGTTAGCAGCAAGTTGTTTGGACTTGACGGTAA     770
   A  V  A  Q  M  K  A  A  A  L  S  N  V  S  S  K  L  F  G  L  D  G  N     257

TGTTGCAACAACTAGCGAGAATAGTGAGAGGCACACTGC     809
 V  A  T  T  S  E  N  S  E  R  H  T  ?      269
```

Figure 9: Nucleotide and deduced amino acid sequence of the coat protein gene of *Bean common mosaic virus* associated with vanilla

Figure10: Phylogram, drawn by Neighborhood Joining Bootstrap method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of the coat protein amino acid sequences of distinct isolates of bean common mosaic virus (BCMV), other potyviruses and vanilla isolate of BCMV (Vanilla-Ind).

Purification and Production of antiserum against BCMV

BCMV infecting vanilla was isolated and pure culture established through local lesion in *Chenopodium amaranticolor*. Cowpea was found to be better propagation host for the virus. The virus was propagated under insect proof

conditions. The leaves harvested three weeks after inoculation from these plants were used in the purification. Purification procedure involved homogenizing each 100 g tissue in a mixture of 50 ml chloroform, 50 ml carbon tetrachloride and 200 ml cold 0.5 M potassium phosphate buffer, pH 7.5, containing 0.02 M sodium sulphite. Centrifuged the homogenized mixture for 5 min at 4000 ***g***, discarded the pellet and filtered the supernatant fluid through filter paper. Added polyethylene glycol (PEG, M. Wt 6000) to 6% (w/v), stirred for 1 h at 4°C, and recovered the precipitated virus particles by centrifugation at 12,000 ***g*** for 10 min. Allowed the pellet to resuspend undisturbed for at least 6 h, then clarified by centrifugation for 10 min at 12,000 ***g***. Added a 20% PEG solution in 0.02 M Tris buffer, pH 8.2 (2 ml per 5 ml virus preparation), kept the mixture at 4°C for 1 h, then centrifuged at 17,000 ***g*** for 10 min. Resuspended the precipitate in 0.25 M potassium phosphate buffer, pH 7.5, and maintained overnight at 4°C before centrifuging at 12,000 ***g*** for 10 min. Further purification was done through sucrose density gradient centrifugation (10-40%) at 25000 rpm (SW 41Ti rotor, Beckman) for 3h. Virus containing zone was recovered and concentrated by centrifugation at 84,500 ***g*** for 90 min.

Purified virus preparation when checked under electron microscopy showed the presence of typical flexuous particles resembling potyviruses (Fig. 11). Purified preparation was injected into new Zealnad white rabbit for polyclonal antiserum production. A total of five intramuscular injections at 10 days interval were given and serum containing antibodies were collected after 15 days from the last injection. After storing for 1 h at room temperature, the serum was incubated for over night at 4°C. On the next day, contents were centrifuged for 5 min at 8000 rpm and supernatant was collected and used in DAC ELISA to test the titre of antiserum. From the crude polyclonal antiserum, immunoglobulin G (IgG) was purified through affinity chromatograohy coupled with protein A. IgG-alkaline phosphatase conjugate was prepared by one step glutaraldehyde method. Double antibody sandwich (DAS) ELISA was standardized for the reliable detection of the virus in diseased vanilla samples collected from different regions using this conjugate.

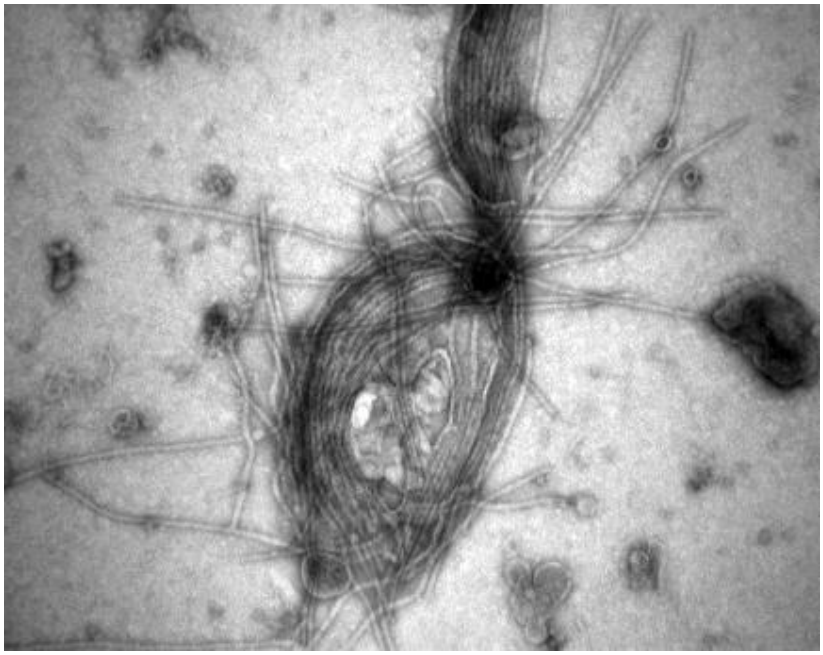

Figure 11: Electron micrograph of purified preparations of *Bean common mosaic virus.*

Detection of BCMV through RT-PCR

Reverse transcription PCR based method was developed for the quick and sensitive detection of the virus in vanilla planting material. The method involved isolation of total RNA from plants by acid guanidium thiocyanate phenol chloroform method and subjecting them to RT-PCR using coat protein gene specific primers. After RT-PCR, contents were run on the gel and observed for the presence of expected band (800 bp) (Fig. 12).

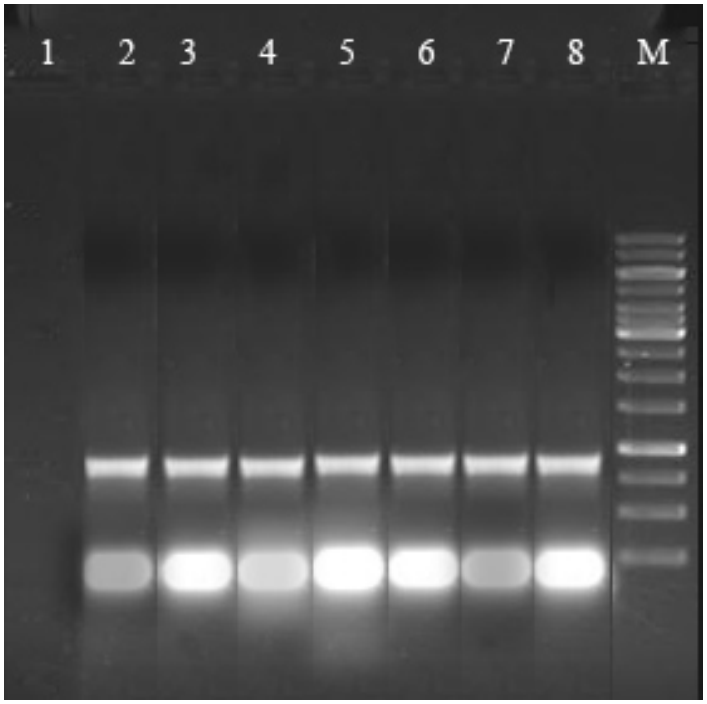

Figure 12: Detection of bean common mosaic virus (BCMV) infecting vanilla through RT-PCR. Lane M: 1 kb DNA Marker; Lanes 1-8: Different test samples.

Occurrence of *Bean yellow mosaic virus* (BYMV) on vanilla

Vanilla plants showing typical symptoms of chlorosis and leaf deformation collected from Madikeri district of Karnataka when subjected to RT-PCR using primers specific for five different potyviruses, gave specific amplification with primers specific to *Bean yellow mosaic virus* (BYMV). The PCR product was eluted from the gel, cloned in a TA vector and sequenced by automated sequencing facility at Bangalore Genei. The cloned region contained portion of NIb and coat protein gene. The sequenced region of coat protein contained 778 based potentially coding for 259 amino acids (Fig. 13). Sequence analysis based on per cent nucleotide and deduced amino acid identity and phylogenetic analysis clearly showed that vanilla isolate is a strain of BYMV (Fig 14).

```
S  E  Q  E  K  L  N  A  G  E  N  K  K  D  K  G  R  R  I  D  G  N  P  S      24

CAGAGAAATCCGAGGAACAAAGCAGTAGGCAGATATTACCTGACAGGGATGTCAACACAGGAACTGTTGG     140
  E  K  S  E  E  Q  S  S  R  Q  I  L  P  D  R  D  V  N  T  G  T  V  G      47

AACATTTTCAATTCCGAGACTTAAAAAGATAGCGGGGAAGCTTAACATTCCAAAAATTGGTGGAAAAGTA     210
 T  F  S  I  P  R  L  K  K  I  A  G  K  L  N  I  P  K  I  G  G  K  V      70

GTTCTTAATTTAGATCATTTATTGGATTACAATCCGCCACAGGATGACATCTCAAACACCATAGCGACAC     280
V  L  N  L  D  H  L  L  D  Y  N  P  P  Q  D  D  I  S  N  T  I  A  T  Q     94

AAGCACAATTTGAAGCGTGGTATGATGGTGTTAAGCAAGCATATGAAGTAGATGATTCACAGATGGGCAT     350
  A  Q  F  E  A  W  Y  D  G  V  K  Q  A  Y  E  V  D  D  S  Q  M  G  I     117

TATACTAAATGGACTGATGGTATGGTGCATAGAGAATGGCACATCAGGAGACTTACAAGGTGAATGGACA     420
 I  L  N  G  L  M  V  W  C  I  E  N  G  T  S  G  D  L  Q  G  E  W  T      140

ATGATGGATGGCGAGGAGCAAGTGACATACCCGCTCAAACCAATCTGGGACAATGCGAAGCCAACATTCC     490
M  M  D  G  E  E  Q  V  T  Y  P  L  K  P  I  W  D  N  A  K  P  T  F  R     164

GCCAAATAATGTCTCATTTCTCACAGGTTGCAGAAGCTTACATAGAGAAAAGAAATGCGACAGAGAGGTA     560
  Q  I  M  S  H  F  S  Q  V  A  E  A  Y  I  E  K  R  N  A  T  E  R  Y     187

TATGCCACGGTATGGCCTCCAGAGAAACTTGACTGACTACGGTTTGGCTAGATATGCCTTTGATTTCTAT     630
 M  P  R  Y  G  L  Q  R  N  L  T  D  Y  G  L  A  R  Y  A  F  D  F  Y      210

CGACTGACTTCTAAGACTCCTGTGCGTGCTAGGGAGGCACATATGCAAATGAAAGCAGCAGCAGTTAGAG     700
R  L  T  S  K  T  P  V  R  A  R  E  A  H  M  Q  M  K  A  A  A  V  R  G     234

GTAAGTCAAACCGGTTATTTGGACTTGATGGCAATGTTGGAACAGACGAGGAGAACACAGAGAGACACAC     770
  K  S  N  R  L  F  G  L  D  G  N  V  G  T  D  E  E  N  T  E  R  H  T      257


AGCAGGTG     778
 A  G  ?     259
```

Figure 13: Nucleic acid sequence (shown as DNA) and deduced protein sequence of *Bean yellow mosaic virus* (BYMV) infecting vanilla.

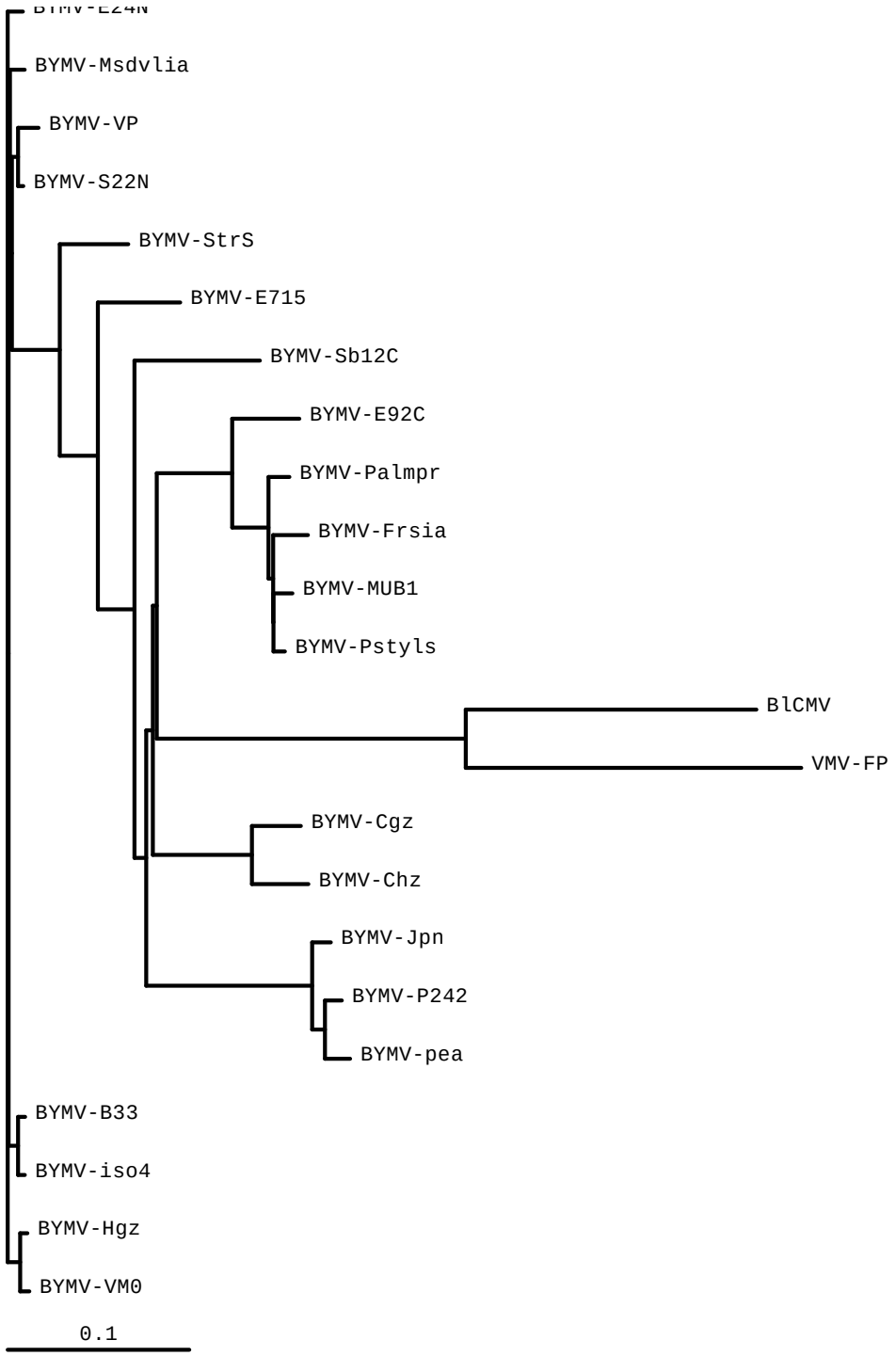

Figure 14: Phylogram, drawn by Neighborhood Joining Bootstrap method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of the coat protein amino acid sequences of distinct isolates of *Bean yellow mosaic virus* (BYMV) infecting vanilla (BYMV-VP) with strains of BYMV and other distinct potyviruses.

Detection of BYMV through RT-PCR

Reverse transcription PCR based method was developed for the quick and sensitive detection of the virus in vanilla planting material. The method involved isolation of total RNA from plants by acid guanidium thiocyanate phenol chloroform method and subjecting them to RT-PCR using coat protein specific primers. After RT-PCR, contents were run on the gel and observed for the presence of expected band (850 bp) (Fig. 15)

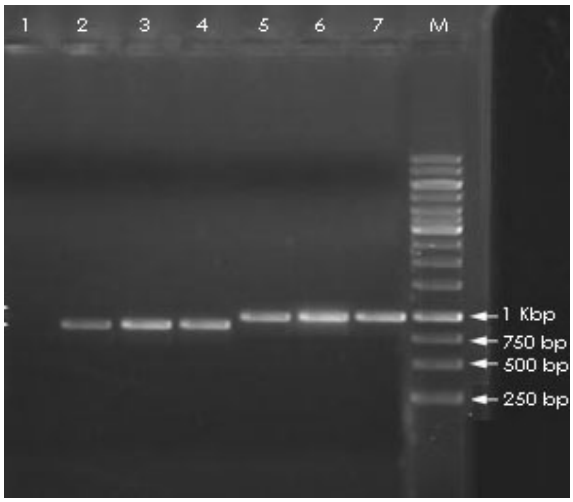

Figure 15: Detection of Bean yellow mosaic virus through RT-PCR. Lane M: 1 kb DNA ladder; Lane 1: Healthy (negative) control; Lanes 2-7: Test samples.

Fungal diseases

Survey

The survey was undertaken in three years *viz.* 2003, 2004 and 2005 at different periods (Table 4). Seventy two locations in five districts of Kerala (Kozhikode, Ernakulam, Malappuram, Idukki and Wyanad) were covered in the survey. The age of the surveyed vanilla vines ranged from 2-5 years. Both organic and inorganic system of cultivation was followed by farmers. During March - May 2003, high intensity of yellowing and premature bean shedding was observed as the major problem on bearing vines in all the areas irrespective of the management practices adopted by the farmer Fig. 16). The disease was characterized by dropping off remnants of flowers from the tip of immature beans which otherwise will remain attached to the beans till half way through maturity. This was followed by yellowing

Table 4: Survey of vanilla gardens for fungal diseases

District	Location	Disease
April 2003 Calicut	Thiruvampady	Premature Yellowing and bean shedding
	Manjuvayal	Premature Yellowing and bean shedding
	Pullurampara	Premature Yellowing and bean shedding
	Nellipoyil	Premature Yellowing and bean shedding
	Kulathoor	Premature Yellowing and bean shedding
	Karinattu	Stem rot
	Irattyl	Inflorescence rot
	Pekkuzhiyil	Stem yellowing (root rot)
	Alakkal	Premature Yellowing and bean shedding
	Meenmutti	Premature Yellowing and bean shedding
	Kurottupara (1)	No disease
	Kurottupara (2)	No disease
	Choorappara	Premature Yellowing and bean shedding
	Koodathai	Premature Yellowing and bean shedding
	Kodencherry(1)	Premature Yellowing and bean shedding
	Kodencherry(2)	Premature Yellowing and bean shedding
May 2003 Wyanad	Vythiri	Premature Yellowing and bean shedding
	Muttil(1)	No disease
	Muttil(2)	Negligible yellowing
	Pariyaram (1)	Premature Yellowing and bean shedding
	Pariyaram (2)	No disease
	Madakkimala	Tip rot
August 2003Calicut	Muthappanpuzha	Root rot
	Thiruvampady	No fungal infection
	Anakkampoyil	No fungal infection
	Mannukuzhy	No fungal infection
	Neervelil	No fungal infection
	Neervelil(2)	No fungal infection
	Koorottupara	Stem rot
	Mundoor	No fungal disease
	Mundoor	No fungal disease
Idukki	Thodupuzha	No fungal disease
	Kudavathoor	No fungal disease
	Varingal	No fungal disease
	Arakkulam	No fungal disease
	Ayyappankovil	No fungal disease
	Kalthotty	Stem & bean rot
	Mamoottyl	Root rot
	Myladumpara	Stem leaf and bean rot
	Vandiperiyar	Leaf spots
	Chottupara	Bean rot

	Santhanpara	No fungal disease
	Adimali	Bean rot
Karnataka	Sakleshpur	Flower dropping
	Mudigere (1)	Bean rot
	Mudigere (2)	No fungal disease
	Mudigere (3)	No fungal disease
March 2004	Vellaram kunnel	Flower shedding
Calicut	Chempukadavu (1)	Flower shedding
	Chempukadavu (2)	Flower shedding
	Chempukadavu (3)	Root rot
	Chempukadavu (4)	Root rot
	Pulloorampara	Premature Yellowing and bean shedding
	Thasmarasserry	Premature Yellowing and bean shedding
	Thamarasserry	Premature Yellowing and bean shedding
Novemeber 2005 Wyanad	Vengapally	Root rot
	Vythiri	Stem rot
	Nadavayal	Stem rot , yellowing and drying, internode rotting
	Batheri	Aerial root drying & Stem rot
	Nadavayal	No fungal infection
	Poothady	No fungal infection
	Nadavayal	No fungal infection
	Cheppila	< 1% infection
	Pulpally	No fungal infection
	Pulpally	No fungal infection
	Padichira	Leaf axil rot
	Batheri	Leaf axil rot
	Padichira	Wilt & Root rot
	Batheri	stem rot, & basal rot
	Kanakkancherry	No infection
	Padichira	Root rot
	Meppady	Root rot

and premature bean shedding. Crop loss assessment from the infected areas showed that the percent crop loss due to this disease ranged from 23-34 % and in severe cases it extended up to 57%. Incidence of the disease was reported by farmers even from February till the onset of south west monsoon showers in May. The other major disease noticed was root rot caused by *Fusarium oxysporum* f sp. *Vanillae*. The symptoms were yellowing and drying of the stem and leaves thus leading to total death of plants. The disease became severe during 2005 onwards wiping out vanilla plantations in several locations. Besides, sporadic incidence of stem rot and bean rot caused by *Phytopthora meadii* and brown rot caused by *Colletotrichum* sp was also observed in some of the plantations surveyed.

Isolation, pathogenicity and identification of pathogens

Representative disease samples including root, stem and leaves were collected from each plantation and subjected to pathogen isolation. Fungal isolates obtained from each diseased sample were sub-cultured onto PDA slants and maintained in the repository after giving the IISR number prefixed by the letter 'V'. The details of isolation made are given Table 5. Fungus, *Colletotrichum vanillae* was found associated with all the infected beans affected by premature yellowing and bean shedding samples. Occasionally *Phytophthora meadii* was isolated from root, stem and bean rot diseases. Among 64 isolates obtained from Wayanad area, 24 isolates were of *Fusarium* species and 20 isolates were of *Colletotrichum* sp. The rest of the isolates belonged to *Rhizoctonia, Mucor* and *Trichoderma* and some non sporulating fungi which were non-pathogenic.

During September 2004, a new disease of vanilla beans characterized by the formation of brown spots was noticed in Chembukadavu area of Kozhikode District (Kerala) where vanilla is grown intercropped with coconut (*Cocos nucifera* L.), arecanut (*Areca catechu* L.) and clove (*Syzygium aromaticum*). The intensity of infection ranged from 10 - 90% on the beans. The infection initiated as small water soaked spots on the beans which later developed into characteristic brownish sunken lesions. The lesions were either elongated, round or oval with sunken centre and the size varied from 1-10 mm. Such lesions coalesced and formed larger lesions in severe cases. The lesions showed characteristic brown to black periphery and reddish brown depressed or sunken centre resembling anthracnose symptoms. Leaves of the affected plants also showed similar spots. An affected bean had more than 5-10 lesions and almost all the beans in a bunch were infected.

All the fungal isolates obtained were tested for pathogenicity by inoculating on to excised vanilla cuttings. Infection was observed within seven days. The *Colletotrichum* sp. showed pathogenicity was identified as *C. vanillae* Massae

Fig 16. Symptoms of various fungal diseases

because of their host specificity. Other isolates of *Colletotrichum* belonged to *C. gloeosporioides* which has sparse or no setae formation.

Table 5: Disease symptoms and associated organisms

Disease Symptoms	**Associated Organism (s)**
Aerial root drying	*F. oxysporum f sp.vanillae Rhizoctonia solani.*
Root rot	*F.oxysporum f sp.vanillae*
Stem rot & drying	*F. oxysporum f sp.vanillae*

Typ rot	*Fusarium sp.*
Stem rot & yellowing	*Fusarium sp, Rhizoctonia sp.*
Yellowing & fruit rot	*F. oxysporum f sp.vanillae*
Yellowing	*F. oxysporum f sp.vanillae*
Flower drop	*F. oxysporum f sp.vanillae*
Leaf spots	*Colletotrichum vanillae.*
Leaf axil rot	*C. vanillae.*
Leaf rot	*C. vanillae*
Flower shedding	*C. vanillae*
Premature yellowing & bean shedding	*C. vanillae*
Bean shedding	*C. vanillae*
Stem rot	*C. vanillae.*
Bean rot	*Phytophthora meadii*
Root rot	*P. meadii*
Stem rot	*P. meadii*
Brown spot ,	*Cylindrocladium quinquiseptatum*
Leaf rot, Bean rot	*Mucor racemosus*

The *Mucor* sp. was also isolated from rot affected leaf, stem and beans from three plantations and proved pathogenicity as per Koch's postulates. The species was identified as *Mucor racemosus* which is the first report of its kind on vanilla. Mycelium of the fungus was yellowish, very extensive and septate in older aerial hyphae. Budding cells are formed in media. Mycelium breaks up into oidia is characteristic of *Mucor recemosus.* Stem rot was characterised by the rotting of the internodal area, which initiated on the nodal regions and extended to both ways. This was the severe disease problem noticed in many of the vanilla gardens. Root rot is the major devastating problem characterized by the drying and rotting of the roots touching the soil. Even aerial roots also showed drying. *Fusarium* was isolated from these roots in addition to its isolation from stem and leaves. The *Fusarium* species isolated from root and stem rot infection was identified as *F. oxysporum f.sp vanillae.*

Disease management

In vitro evaluation of fungicides against Colletotrichum vanillae

Seven different fungicides *viz.* carbendazim (Bavistin), mancozeb (Indofil M45), zineb (Indofil Z-78), thiophanate methyl (Roko), Bordeaux mixture and carbendazim-mancozeb (saff) were evaluated under *in vitro* conditions to study the efficacy of fungicides in inhibiting *Colletotrichum* of Vanilla (Table 6). The results of fungicide sensitivity studies indicated that thiophanate methyl (100ppm) and carbendazim were highly inhibitory to the fungus even at 250ppm. The ED_{90} value was found to be below 50ppm for these fungicidies, while other fungicides are effective only at higher concentrations.

In vitro evaluation of Rhizobacteria against Colletotrichum vanillae

Twenty isolates of Rhizobacteria maintained in the repository of IISR were tested under *in vitro* conditions to evaluate their efficacy in controlling *C. vanillae*. Among them, IISR 152 and 909 were inhibitory to the pathogen showing an inhibition rate of 87.88 and 81.11% respectively (Table 7). Isolates IISR 6, IISR 853, IISR 912 and IISR 915 were also found promising which gave an inhibition up to 62-66%.

Table 6: *In vitro* effect of fungicides on *Colletotrichum vanillae*

Fungicides	**% inhibition**							ED 90 Value
Concentration (ppm)	**50**	**100**	**250**	**500**	**1000**	**1500**	**2000**	**ppm**
Bordeaux mixture	10.78	17.5	28.28	50.78	100	100	100	900
Carbendazim	92.63	94.86	100	100	100	100	100	47.96
Carbendazim+ Mancozeb	81.79	86.9	88.0	89.7	91.84	95.39	100	493.64
Copper oxy chloride	0.00	8.94	51.30	93.0	100	100	100	450
Metalaxyl-mancozeb	21.7	52.36	77.8	82.89	86.97	91.45	100	1004.46
Mancozeb	7.89	11.18	13.42	15.78	23.94	55.0	59.2	2368
Thiophanate Methyl	96.84	98.8	100	100	100	100	100	46.46

Table 7: Effect of rhizobacterial isolates on *Colletotrichum vanillae*

Rhizobacterial isolates	Inhibition (%)	Rhizobacterial isolates	Inhibition (%)
IISR 6	63.7	IISR 913	59.7
IISR 13	31.5	IISR 914	39.5
IISR 51	**69.8**	IISR 915	63.7
IISR 853	49.6	IISR 147	49.6
IISR 859	39.5	IISR 148	54.4
IISR 906	39.5	IISR 149	39.7
IISR 907	64.5	IISR 150	46.4
IISR 909	**73.8**	IISR 151	49.6
IISR 910	51.6	IISR 152	**87.9**
IISR 912	65.7	IISR 153	36.3

Field trial for the management of immature bean shedding

A field trial was laid out in three vanilla plantations in Calicut district to study the effect of fungicides in controlling immature bean shedding. The experiment consisted of two main plot treatments (mist and sprinkler irrigation) and six subplot treatments with fungicides *viz.* carbendazim (Bavistin-0.2%), mancozeb (Indofil M45 0.2%), zineb (Indofil Z-78 0.2%), thiophanate methyl (Roko 0.2%), Bordeaux mixture 1% and carbendazim-mancozeb (saff 0.2%). Observation on flower dropping and bean shedding was taken at monthly intervals. The plot under mist irrigation received irrigation for 4 h daily between 10 to 12 h and 15 to 17h. The plot with sprinkler irrigation received irrigation from 11 to 15 h daily.

The results showed that bean shedding was comparatively less in sprinkler irrigation system when compared to mist irrigation and all the treatments were on par except Bordeaux mixture where the bean dropping was found comparatively higher. Mancozeb 0.20%was found effective in managing the flower dropping.

Table 8a Table Incidence of flower dropping

	% disease (flower dropping)			
Treatments	Mist	No irrigation	Sprinkler	Mean
Carbendazim 0.2%	21.10	19.15	19.03	19.76(11.43) B
Bordeaux mixture 1%	19.67	25.15	15.88	20.23(11.96)B
Thiphanate methyl 0.2%	38.80	17.01	46.05	33.95(31.89)A
Carbendazim+Mancozeb0.2%	22.17	17.10	18.32	19.20(10.82)B
Mancozeb 0.2%	18.58	18.50	4.60	13.89(5.76)B
Zineb 0.2%	26.15	21.54	23.95	23.88(16.39)B
Control	18.16	24.24	14.14	18.85(10.44)B
LSD at alpha 0.05	ns	ns	ns	9.84

Table 8b: Incidence of yellowing and fruit dropping

	% disease (yellowing and fruit dropping			
Treatments	Mist	No irrigation	Springler	Mean
Carbendazim 0.2%	30.52(25.79)ABC	28.45(22.69)ABCD	21.15(13.02)DEF	26.71(20.20)ABC
Bordeaux mixture 1%	32.30(28.55)AB	30.17(25.26)ABC	24.62(17.36)BCDE	29.03(23.55)AB
Thiphanate methyl 0.2%	29.52(24.28)ABC	28.68 (23.03)ABCD	22.83(15.05)CDEF	27.01(20.62)ABC
Carbendazim+Mancozeb0.2%	26.56(19.99)ABCD	31.38 (27.11)AB	18.98(10.58)EF	25.64(18.72)BC
Mancozeb 0.2%	28.28(22.45)ABCD	31.00(26.53) AB	16.27(7.85)FG	25.18()18.10)C
Zineb 0.2%	29.55(24.32)ABC	33.01 (29.68)A	28.95(23.43) ABC	30.50(25.76)A
Control	27.27(20.99)ABCD	32.82 (29.38)A	10.89 (3.57)G	23.66(16.10)C
LSD at alpha 0.05	6.6	6.6	6.6	3.811
Grand Mean	29.14(23.71)A	30.79 (26.20)A	20.53 (12.30)B	

Another field trial was initiated during 2006 with the onset of flowering in vanilla in a farmers plot at Kodencherry to study the effect of different treatments such as growth hormones, fertilizers and fungicides on immature bean shedding. The trial was laid out in RBD with three replications having 10 vines /replication. The treatments were given at monthly intervals starting from February with the onset of flowering and continued till May 2006. Observations were recorded at monthly intervals on bean shedding. Only negligible incidence of immature bean shedding was observed during the period due to frequent summer showers (nine from February to May) received. From this it was inferred that immature bean shedding is closely

associated with high temperature besides *Colletotrichum* infection which is predisposed by high temperature and low relative humidity.

Effect of fungicides on root rot management

This pot culture experiment consisted of nine treatments with 10 plants per treatment. Observations were recorded on root rot and inter node rot. Among the different fungicides tested, except carbendazim 0.1%, all the other fungicides were significantly superior to control (Table-9).

Table-9 Effect of fungicides on root rot management

Treatment	% disease incidence
Carbendazim + Mancozeb 0.2%	5.945
Carbendazim 0.2%	4.636
Carbendazim 0.1%	23.25
Thiophanate methyl 0.2%	10.69
Carbendazim + Mancozeb +Neem oil 0.2+0.5%	6.367
-Mancozeb 0.25%	9.254
Phytomin0.3%	7.778
Phytoguard0.5%	5.714
Control	43.0
LSD	20.12

Effect of rhizobacteria on disease management

In vitro studies on suppression of fungal pathogens by rhizobacteria

The rhizobacterial isolates were tested against two pathogens, *P. meadii* and *F. oxysporumn* f. sp. *vanillae* for in *vitro* inhibition using dual culture plating method. The dual cultured plates were incubated at 28±2°C. The radial growth of the pathogen towards the bacterial growth was measured after 72h and the percent inhibition was calculated by comparing with control (pathogen alone). Most of the isolates were inhibitory to *P. meadii* causing up to 74 % inhibition (Table 10). In the case of *F. oxysporum* f. sp. *Vanillae*, IISR 909 was found highly inhibitory (91.0%). Isolates, IISR 6, 51, 853, 147, 148 and 152 showed 52-58% inhibition. Other isolates showed

below 50% inhibition. Interestingly four isolates (IISR 6, 13, 153, and 912 showed multiple antagonism against both the pathogens. These isolates were used in the bioconsortium for the management of root rot of vanilla caused by *F. oxysporum* f. sp. *vanillae.*

Table 10: *In vitro* effect of individual rhizobacteria on pathogen inhibition

Rhizobacterial isolate	**Species**	Inhibition (%)		
		P. meadii	*F. oxysporum f.sp. vanillae*	*C. vanillae*
IISR 6	*Pseudomonas fluorescens*	59.6 ab	58.3 ab	44.4 ab
IISR 13	*P. fluorescens*	64.9 ab	44.4 ab	58.3 ab
IISR 51	*P. fluorescens*	59.6 ab	55.5 ab	27.8 ab
IISR 853	*Bacillus* sp.	60.5 ab	55.5 ab	16.7 b
IISR 859	*Bacillus* sp.	73.8 a	0.0 b	16.7 b
IISR 906	*Bacillus* sp.	71.1 ab	44.4 ab	13.9 b
IISR 907	*Bacillus* sp.	63.1 ab	30.6 ab	11.1 b
IISR 909	*Bacillus* sp.	21.1 bc	91.7 a	38.9 ab
IISR 910	*Bacillus* sp.	63.2 ab	23.7 b	22.2 b
IISR 912	*Bacillus* sp.	64.0 ab	44.4 ab	50.0 ab
IISR 913	*P. fluorescens*	59.6 ab	2.8 b	30.7 ab
IISR 914	*P. fluorescens*	49.1 ab	18.1 b	16.7 b
IISR 915	*Bacillus lentus*	0.0 c	16.7 b	50.0 ab
IISR 147	*Bacillus* sp.	56.1 ab	51.4 ab	16.7 b
IISR 148	*B. polymixa*	56.1 ab	52.8 ab	27.8 ab
IISR 149	*Bacillus* sp.	65.9 ab	38.9 ab	16.7 b
IISR 150	*Enterobacter agglomerans*	64.1 ab	44.4 ab	33.3 ab
IISR 151	*Bacillus* sp.	65.9 ab	30.6 ab	16.7 b
IISR 152	*Bacillus* sp.	67.2 ab	58.3 ab	27.8 ab
IISR 153	*Bacillus* sp.	64.9 ab	47.2 ab	77.8 a

Greenhouse studies

In vivo evaluatin of rhizobacteria against P. meadii

To study the induction of systemic resistance (if any) due to these rhizobacterial isolates, vanilla shoots of 10cm size were cut off from the individual rhizobacteria treated plants and were inoculated with the pathogen under green house conditions. Internodal region and leaves of these detached cuttings were inoculated with 5mm dia culture discs of *P. meadii* and incubated for 72-120 h under humid conditions. There were five replications /treatment. Observations on leaf area infected as well as lesion length of the internode infection was recorded after 72 h of inoculation and calculated the percent area. The lesions produced within 72h of inoculation showed the susceptibility of plants where as no infection indicated the resistance acquired due to rhizobacterial inoculation. Plants treated with rhizobacterial isolates *viz.* IISR 51, IISR 907, IISR 147 and IISR 148 resisted infection whereas plants treated with other isolates showed varying levels of infection both on the leaves and stem.

Effect of mixture of rhizobacteria on Fusarium infection

The three month old plants grown in polybags were transplanted into earthen pots containing 10kg potting mixture. The consortium was applied at the time of transplanting and repeated thrice at two months interval. Six months after establishment, the plants were inoculated with 4 x1 0^8 spores/g *of F. oxysporum* f. sp. *vanillae*. The plants were observed for four months for disease incidence. The data were analyzed statistically using MSTATC package.

The mixture of rhizobacteria was promising in controlling the infection caused by *F. oxysporum* f. sp. *vanillae*. Though plants initially took up infection, there was no further spread of the disease in any of the treated plants, whereas, in the control plants, the infection extended upwards and the whole plant collapsed within a period of one month. This clearly showed the protection was offered by the mixture of rhizobacteria in managing *Fusarium* infection. All the four mixtures showed minimum disease incidence in all the treatments and were significantly superior to control giving more than 88-92% disease reduction (Table 18). The results very

clearly revealed the potential of the rhizobacterial mixture in the suppression of *Fusarium* infection in vanilla, though the effect varied with the isolates used. Difference in degrees of protection against *P. meadii*, was observed among the rhizobacteria. It may be due to the fact that rhizobacterium activates different defense

Table 11. Effect of mixture of rhizobacteria on *Fusarium* infection

Treatment	*Fusarium* infection (%)
IISR 13, 51, 152 and 909	8.6
IISR 13, 51, 152 and 909 + IISR 148, 149, 906 and 907	8.1
IISR 13, 51, 152 and 909 + IISR 6, 147, 151 and 153	5.2
IISR 6, 51, 147, 148, 149, 906 and 907	7.9
Control – (only pathogen)	73.3
LSD	24.3

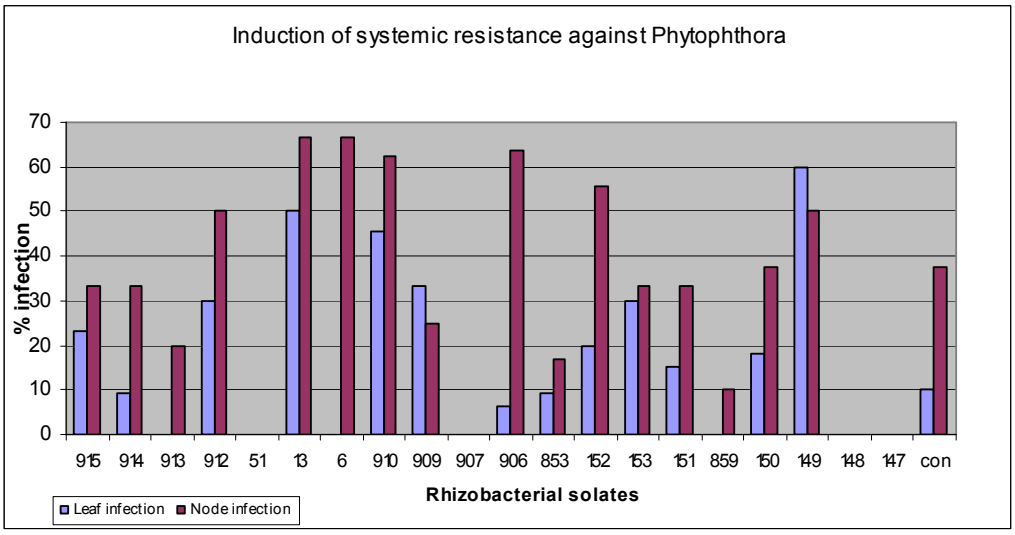

Fig 17: In vivo effect of rhizobacterial isolates on *P. meadi*

mechanisms within the induced resistance path way resulting in differential induction in symptoms.

Field evaluation of bio-consortium for the management of root disease caused by *F. oxysporum* f.sp *vanillae*

The field trial for the evaluation of bioconsortium for root rot (*F. oxysporum* f.sp *vanillae*) disease of vanilla was done for three years. The trial consisted of nine treatments including Copper oxychloride and bioagent *Trichoderma.* Treatments T2 (IISR 6, 8, 13, 51, 151 P1AR6), T3 (IISR 6, 8, 13, 51, 151 , 853) , T4 (IISR 6, 8, 13, 51, 151, 859) and T6 (*T. harzianum*) were found at par in reducing the disease incidence (Table 12) when compared to absolute control where no treatments were given. Among the treatments T3 was found superior to all other treatments through it was at par with T2, T4 and T6.

Table 12: Evaluation of bio-consortium on root rot disease

Sl.No	Treatments	Disease incidence (%)
T1	IISR 6, 8, 13, 51, 151, PB21C	44.44
T2	IISR 6, 8, 13, 51, 151, P1AR6	22.46
T3	IISR 6, 8, 13, 51, 151, 853	16.67
T4	IISR 6, 8, 13, 51, 151, 859	50.00
T5	IISR 6, 8, 13, 51, 151, PB21C, P1AR6, 853, 859	38.89
T6	*Trichoderma(p-26*)	28.01
T7	COC 0.3%	55.57
T8	Control -Nutrient broth	27.77
T9	Absolute control	38.89
	LSD	19.42

A field trial was laid out in RBD at IISR farm at Peruvannamuzhi to study the effect of bioconsortium on root diseases of vanilla. The trial consisted nine treatments including control. There were three replications /treatment and six plants/ replication. Two rounds of treatments were given at one month interval.

Effect of fungicides and biocontrol agents on disease management:

A field experiment to evaluate the effect of fungicides and bioagents on root rot of vanilla caused by *F.oxysporum* f. sp *vanillae* was laid out at IISR farm Peruvannamuzhi. The experiment was in RBD with three replications having 10 plants /treatment under shade net conditions. The experiment consisted of two promising fungicides along with bacterial bioconsortium and a fungal biocontrol isolate *Paecilomyces* sp which was found promising under *in vivo* conditions. The treatments were imposed during the onset of monsoon in the month of June. The bioconsortium was prepared in Nutrient broth and adjusted to get a CFU of 10^{10} cell/ml and applied @ 2 lit/vine. *Paecilomyces* was multiplied in chopped and sterilized sorghum grains and suspended in water and applied @ of 2 l/vine having a CFU of $4x10^8$ spores /ml. Fungicides were applied @ 2 lit/vine. The second application was given in July. Observations on disease incidence (basal rot /root rot) were recorded at monthly intervals and final observation was statistically analyzed. The experiment was conducted for two years. The result showed that all the treatments were at par, but significantly superior over control. Among the treatments fungicide carbendazim (0.2%) is found better treatment in reducing the disease incidence and also establishment of the plants followed by carbendazim+ mancozeb (0.2%) and *Paecilomyces* sp (100g/vine in sorghum based formulation) (Table 13).

Table 13: Effect of fungicides and bioconsortia on management of root rot

Treatments	Concentration	% Disease incidence by *Fusarium* sp
Bio Consortium IISR 13, 51, 152 and 909	2 lit/vine (48hr culture)	37.960
Carbendazim	0.2%	25.892
Carbendazim+ Mancozeb	0.2%	33.035
Paecilomyces sp.	2 lit/vine (spore load $4x10^8$ spores/ml)	34.375
Control (water drenching)		59.375
LSD		18. 355

8222 Questions- Answered

What are the different viruses that infect vanilla and methods to identify virus-free plants?

Different viruses infecting vanilla *vix.*, *Cucumber mosaic virus* (CMV), *Cymbidium mosaic virus* (CymMV), *Bean common mosaic virus* and *Bean yellow mosaic virus* were identified and characterized based on cloning and sequencing of coat protein gene. Polyclonal antibodies were raised against viruses and DAS-ELISA method developed for detection of the viruses in planting material. Oligonucleotide primer pairs specific for each of the viruses were synthesized and RT-PCR method developed for the reliable detection of viruses in planting material. As viruses are systemic in nature and can not be killed once they infect a plant, use of virus free material for planting is important. As symptoms can not be used as a reliable criteria, use of serological or nucleic acid based diagnostics are necessary to identify virus free plants. Thus the ELISA or PCR based diagnostics developed in the project can be used identify virus –free plants.

What are the cause and strategies for management of fungal diseases infecting vanilla?

The major fungal diseases of vanilla noticed were: yellowing and premature bean shedding associated with *Colletotrichum vanillae* during April –May, stem and bean rot caused by *Phytophthora meadii* during July-August and root rot and drying of stem by *Fusarium* sp. *In vitro* studies using different fungicides and rhizobacteria against *C. vanillae* showed that thiophanatemethyl at 100 ppm and carbendazim (250 ppm) were highly inhibitory to the fungus. Among the rhizobacteria, (isolates IISR 152 and IISR 909 were highly inhibitory to the fungus. Root rot of vanilla caused by *Fusarium oxysporum* f.sp. *vanillae* could be controlled using the rhizobacterial consortia containing *Pseudomonas fluorescens* (isolate IISR 13, IISR 51, IISR 6), Bacillus species (isolate IISR 152, IISR 147, IISR 151 and IISR 153 and *B. polymixa* (isolate IISR 909). *Paecilomyces* sp. was identified as a potential biocontrol agent against root rot of vanilla caused by *F. oxysporum* f.sp. *vanillae*.

8223 Process/ Product/ Technology/ Developed

ELISA and RT-PCR based detection for different viruses infecting vanilla to identify virus-free plants

Developed disease management strategy for Premature yellowing and bean shedding.

Developed biocontrol and chemical control strategy for management of root rot of vanilla.

Developed bioconsortium for the management of root rot of vanilla

8224 Practical Utility (not more than 150 words)

Viruses are systemic in nature and can not be killed once they infect a plant. Hence for management of viral diseases in vanilla, use of virus-free planting material is

important. As symptoms can not be used as a reliable criteria, use of serological or nucleic acid based diagnostics are necessary to identify virus-free plants. Thus the ELISA or PCR based diagnostics developed in the project can be used identify virus – free plants. Planting materials should be taken only from such virus-free plants in order to check further spread of the viruses.

Yellowing and premature bean shedding associated with *Colletotrichum vanillae* during April –May, stem and bean rot caused by *Phytophthora meadii* during July-August and root rot by *Fusarium* sp are the major fungal diseases causing economic losses to vanilla. Thiophanate methyl at 100 ppm or carbendazim at 250 ppm could be used to manage *C. vanilla* while among rhizobacteria, isolates IISR 152 and IISR 909 were found highly inhibitory to the fungus. Root rot of vanilla caused by *Fusarium oxysporum* f.sp. *vanillae* could be controlled using the rhizobacterial consortia containing *Pseudomonas fluorescens* (isolate IISR 13, IISR 51, IISR 6), *Bacillus* spp (isolate IISR 152, IISR 147, IISR 151 and IISR 153 and *B. polymixa* (isolate IISR 909). *Paecilomyces* sp. was identified as a potential biocontrol agent against root rot of vanilla caused by *F. oxysporum* f.sp. *vanillae*.

Constraints, if any: Nil

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

8231 Research papers

Bhat, A.I., Bhadramurthy, V., Siju, S. and Hareesh, P.S. 2006. Detection and identification of *Cymbidium mosaic virus* infecting vanilla (*Vanilla planifolia* Andrews) in India based on coat protein gene sequence relationships. **J. Plant Biochemistry & Biotechnology**, 15: 33-37.

Bhat, A.I., Venugopal, M.N., R.P.Pant and Bhai, R.S. 2004. Occurrence and distribution of viral diseases on vanilla (*Vanilla planifolia* Andrews) in India. **J. Spices & Aromatic Crops**, 13: 143-148.

Madhubala, R., Bhadramurthy, V., Bhat, A.I., Hareesh, P.S., Retheesh, S.T. and Bhai**,** R.S. 2005**.** Occurrence of *Cucumber mosaic virus* on Vanilla (*Vanilla planifolia* Andrews) in India. **J Biosci**. 30: 339-350.

Suseela Bhai, R. and Anandaraj, M. 2006 Brown rot- A new Disease of vanilla (*Vanilla planifolia* Andrews**): J. Spices and Aromatic Crops** 15:139-140.

Suseela Bhai R, Remya and Eapen, S.J. 2008. *In vitro* and *in planta* assays for biological control *o*f *Fusarium* root rot disease of vanilla (*Vanilla planifolia* Andrews) (**Communicated to J.Biocontrol**)

Suseela Bhai, R..and Kumar, A. 2008. Effect of rhizobacteria on *Phytophthora meadii*, *Fusarium oxysporum* f.sp. *vanillae* and *Colletotrichum vanillae* infecting vanilla (*Vanilla planifolia* Andrews.). **J. Biocontrol. 22 (in press**).

Suseela Bhai, R., Bhat, A.I and Anandaraj, M. 2006. Yellowing and Premature bean dropping in Vanilla (*Vanilla planifolia* Andrews). **J. Plantation Crops** 34. 115-117

Popular articles

Bhat , A.I., Sarma, Y.R., Anandaraj, M and Suseela Bhai, R. 2003. Viral diseases can be a future threat to vanilla in India. **Indian J. Arecanut, spices and Medicinal plants.** 5(3): 107-110.

Bhat , A.I., Venugopal, M.N. Suseela Bhai, R and Anandaraj, M., 2004. Stem necrosis - a new virus disease affecting vanilla in India. **Spice India**. 17 (7): 5-7.

Bhat, A.I. 2003. Diagnostics for virus detection-its importance in spices. **Spice India**. 16 (9): 30-33.

Papers presented in Seminars/conferences

Bhadramurthy, V and Bhat, A.I. 2007. Occurrence and characterization of *Bean common mosaic virus* on vanilla (*Vanilla plan folia* Andrews) in India. Poster presented at the 10th International Plant virus epidemiology symposium, 15-20 October, 2007, ICRSAT, Hyderabad.

Bhat, A.I., Bhadramurthy, V., Madhubala, R., Hareesh, P.S., Retheesh, S.T., Siju, S. and Venugopal, M.N. 2006. Detection and characterization of viruses infecting vanilla (*Vanilla planifolia* Andrews) in India (abstract). International Symposium on Management of vector-borne viruses, 7-10 February, 2006, ICRISAT, Hyderabad, p80.

Suseela-Bhai, R., Bhat, A.I. and Anandaraj, M. 2003. Premature yellowing and bean shedding in vanilla (*Vanilla planifolia* Andrews) (abstr). Paper presented at the southern chapter meeting of the IPS held at U.A.S., Dharwad, December 18-20, 2003. p88.

Suseela Bhai R 2007. Occurrence of fungal diseases in vanilla (*Vanilla planifolia* Andrews)in Kerala. Paper presented at SYMSAC IV on 'Threats and solutions to spices and aromatic Industry'. OUAT, Bhuvaneswar, Orissa pp 385

8233 Reports

MSc Dessertations

Investigations for biocontrol organisms against pathogens of vanilla (*vanilla planifolia* Andrews)", Ms. Remya B : Bharathidasan University, Trichy, December 2005 – March 2006

Investigations on the occurrence of fungal diseases on vanilla (*vanilla planifolia* Andrews) in Wyanad region**"** Ms. Jithya Danesh: Bharathiyar University, Coimbatore, December 2005 – March 2006

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

Symposium on "Recent developments in the diagnosis and management of Plant diseases for meeting global Challenges" at UAS Dharwad from December 18-20, 2003.

Vanilla in India-aspects and prospects" 29-30th April 2004 Sirsi (organized by ISS)

Vanilla –Sustainable solutions. 22nd December 2004. CII, Kerala at Cochin

International Symposium on Management of vector-borne viruses, 7-10 February, 2006, ICRISAT, Hyderabad

SYMSAC IV- Threats and solutions to spices and aromatic plant industry. 25-26th Nov. 2007 Orissa University of Agriculture and Technology , Bhubaneswar, Orissa

10th International Plant virus epidemiology symposium, 15-20 October, 2007, ICRSAT, Hyderabad

824. Infrastructural facilities developed

8242. Details of field, laboratory, note books and final material and their location) Field note book and experimental data book are available in the Crop Protection Division.

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

The present study identified, characterized and developed RT-PCR based diagnostics for four different viruses infecting vanilla in India. This methodology can be used in the vanilla nursery certification programme to identify virus-free plants. Similarly, fungal pathogens causing diseases and their management strategies have been worked out. In future more research emphasis may be given on non-chemical means of management. Identification of resistant sources against all pathogens and their use in breeding to get resistant lines should be carried out.

Part-IV : Project Expenditure

(Summary)

1992 - 2005

830 Total Recurring Expenditure

8301 Salaries: (Designation with pay scale)	**Estimated**	**Actual**
i) Scientific	-	7,50,000
ii) Technical	-	1,20,000
iii) Supporting	-	30,000
Sub-Total	-	9,00,000
Consumables		
i) Chemicals	-	80,000
ii) Glasswares	-	20,000
iii) Others	-	50,000
Sub-Total	-	1,50,000
8303 Travel	-	20,000
8304 Miscellaneous (other costs)	-	30,000
8305 Sub-Total (Recurring)	-	11,00,000

831 Total Non – Recurring Expenditure
(Equipments and works)

i) Construction of animal house	-	-

823 Total (830 and 831)	-	11,00,000

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-Investigators:

1. Dr. R. Suseela Bhai
(From April 2003 to March 2007)

2. Dr. M.N.Venugopal
(From April 2003 to March 2007)

3. Dr C.N.Biju
(From April 2007 to March 2008)

Signature & Comments of the Head
of the Division/ Section

Signature & Comments of the
Director