VIRAL DISEASES OF VANILLA (*Vanilla planifolia* Andrews) IN KERALA

By

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DECLARATION

I hereby declare that this thesis entitled " **Viral diseases of vanilla (*Vanilla planifolia* Andrews) in Kerala"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled " **Viral diseases of vanilla (*Vanilla planifolia* Andrews) in Kerala"** is a record of research work done independently by Mr. Abhilash Dinakar (2003-11-21) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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Abhilash Dinakar.

LIST OF ABBREVIATIONS

APS-	Ammonium persulphate
BSA-	Bovine serum albumin
cm-	Centimeter
CMV-	Cucumber mosaic virus
CymMV-	Cymbidium mosaic virus
DAC-ELISA	Direct antigen coating-ELISA
DI-	Disease Incidence
EDTA-	Ethylene diamine tetra acetic acid
ELISA-	Enzyme linked immuno sorbant assay
g-	Gram
M-	Molar
mg-	Milligram
mm-	Millimeter
Mr-	Molecular mass
ORSV-	Odontoglossum ring spot virus
PAL-	Phenylalanine ammonia lyase
PBST-	Phosphate buffer saline tween
PBST-PO -	Phosphate buffer saline tween -polyvinyl pyrrolidone ovalbumin
PDI-	Plant disease index
PO-	Peroxidase
PPO-	Polyphenol oxidase
PTA-ELISA-	Plate trapped antigen-ELISA
PVP -	Polyvinyl pyrrolidone
rpm-	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
TEMED	Tetramethyl ethylene diamaine
TGA	Thioglycolic acid
V-	Volume
VAMD-	Vanilla mosaic virus disease
VanMV-	Vanilla mosaic virus
VNV-	Vanilla necrosis virus
W-	Weight
µg.-	Microgram
µl -	Micro liter
µM-	Micro molar

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INTRODUCTION

1. INTRODUCTION

Vanilla (*Vanilla planifolia.* Andrews) is the second most expensive spice crop in the world. It is the only spice orchid grown for its fruit. The natural essence vanillin obtained from the dried beans of vanilla is widely sought after as flavouring agent in ice creams, confectionaries and pharmaceuticals apart from its medicinal value. The crop is cultivated in many tropical countries and the largest producer of vanilla is Madagascar followed by Indonesia, Comoros and Reunion. It is also cultivated in Kerala, Karnataka and Tamil Nadu states of India producing approximately 90 tonnes of dried beans per annum. The very high price the crop fetched, the highly suitable weather and wide gap between demand and production prompted the promising farmers of Kerala to expand the area under vanilla cultivation. Thus more and more farmers ventured into vanilla cultivation during the past three years by procuring planting materials frantically from what so ever source they could obtain. This might be the reason for the reported out breaks of a few virus diseases of vanilla in Kerala.

Fungal and viral diseases are major constraints to vanilla production now. Among these, viral diseases are very devastating and considered as potential threat to vanilla cultivation. Eight different viral diseases have been reported to occur in vanilla. Incidence of cymbidium mosaic virus (CymMV) (*Potexvirus*) and odontoglossum ring spot virus (ORSV) (*Tobamovirus*) were the early viruses detected in vanilla (Wisler *et al.*, 1987; Pearson and Pone, 1988) occurring in the South Pacific and French Polynesian Islands.

Vanilla mosaic virus (VanMV) incidence was reported form French Polynesia, Cook Islands, Fiji and Vanuatu resulting in very severe leaf distortion and mosaic by Wisler *et al.* (1987) and Zettler and Wisler (1990). Another very destructive viral disease of vanilla is vanilla necrosis virus (VNV) reported from Fiji, Tonga and Vanuatu causing distorted leaves, chlorotic patches and necrotic lesions on older leaves and stem resulting in defoliation and death of vine (Pearson *et al.*, 1990,1993). Incidence of uncharacterized Rhabdo virus like particles in *V.tahitensis and V. planifolia* was reported by Pearson *et al.* (1993).

Wang *et al.* (1993) reported that VNV is a strain of watermelon mosaic virus. Severe stunting of *V. tahitensis* in French Polynesia and *V. planifolia* in Reunion Islands was reported to be due to the incidence of a strain of Cucumber mosaic virus (CMV) (Farreyrol *et al.*, 2001). Occurrence of two viruses serologically related to vanilla mosaic virus (VanMV) and dasheen mosaic virus (DsMV) was reported by Grisoni *et al.* (2004).

In India the first report of a mosaic disease of vanilla was by Thomas (2002) in Myladumpara area of Kerala state. Later several reports came indicating the incidence of other viruses such as VanMV, VNV, CymMV, ORSV and CMV on *V. planifolia*. (Bhai *et al.*, 2003; Bhat *et al.*, 2003, 2004a ; Sudharshan *et al.*, 2003; Madhubala *et al.*, 2005) from Karnataka and Kerala.

Information on the nature, type and the extent of incidence of viral diseases on vanilla are sparse. Vanilla being a vegetatively propagated crop it is of utmost importance to investigate the occurrence and severity of viral diseases, which will help in identifying the problems and management practices to be followed in avoiding incidence of such diseases in future. The present study was taken up with the following objectives.

1. Surveying major vanilla growing tracts of Kerala to study the prevalence of viral diseases
2. Identify the most serious viral disease
3. To study the symptomatology and transmission to understand the nature and spread of the virus in field
4. Physical properties and host range study.
5. Pathophysiological studies to understand the mechanism of damage.
6. Purification and characterization to study the details of the virus.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Vanilla is cultivated in several countries such as Madagascar, Indonesia, Comoros Islands, Reunion Islands, Tonga, Vanuatu, Cook Islands French Polynesia and India. Among these, Madagascar is the largest producer of natural vanilla beans. Several species of vanilla are under cultivation *viz. Vanilla planifolia* Andrews Syn. *V. fragrans* (Salisb.) Ames (Bourbon type), *V. tahitensis* J.W. Moore *and V. pompona* .A limiting factor in the successful cultivation of vanilla is the incidence of fungal and viral diseases.

2.1 OCCURRENCE

In vanilla cultivation a major constraint for its production world wide is the wide spread incidence of devastating virus diseases. The first report of incidence of a virus disease in vanilla was by Wisler *et al.* (1987) who reported the occurrence of cymbidium mosaic virus (CymMV) and odontoglossum ring spot virus (ORSV) in *V. tahitensis* from French Polynesia. The occurrence of CymMV was also reported on *V.planifolia* from the Pacific countries by Pearson *et al.* (1993) and from Indian Ocean countries by Grisoni *et al.* (2004)

A *potyvirus* causing mosaic and severe malformation of leaves was described in French Polynesia on *V. tahitensis* and *V. pompona* by Wisler *et al.* (1987) which was serologically related to dasheen mosaic virus (DsMV) and named as vanilla mosaic virus (VanMV).

In Tonga a *potyvirus* was found associated with leaf distortion, chlorotic and necrotic spotting of leaves, stem necrosis and vine death in 1986 (Pearson and Pone 1988), which was subsequently named as vanilla necrosis virus (VNV) (Pearson and Pone 1989) and further characterized by Pearson *et al.* (1990). This was later demonstrated to be a strain of watermelon mosaic virus (WMV) by Wang *et al.* (1993)

Another vanilla necrosis *potyvirus* from Tonga was reported by Liefting *et al.* (1992) which was different from VanMV and WMV.

Pearson *et al.* (1993) summarized the occurrence of these viruses on vanilla in several South Pacific countries. They further reported the occurrence of

Rhabdo virus like particles in vanilla samples from Fiji and Vanuatu. Recently a strain of cucumber mosaic virus (CMV) was also reported to cause infection and disease in *V. tahitensis* and *V. fragrans* from French Polynesia. (Farreyrol *et al.*, 2001). In a more recent survey carried out in French Polynesia using ELISA technique for identification, three distinct *potyviruses* were identified and found to occur in vanilla *viz.* VanMV, WMV and a virus related to bean common mosaic virus (Grisoni *et al.*, 2004).

In India the occurrence of virus disease in vanilla was first reported by Thomas (2002). He described the occurrence of a mosaic disease possibly caused by a *potyvirus* in Idukki district of Kerala. A recent survey conducted in vanilla gardens of Kerala revealed the occurrence of more than one kind of viral disease producing necrotic lesions on stem and leaves and mosaic symptoms with chlorotic yellow patches on leaves. (Bhai *et al.*, 2003; Bhat *et al.*, 2003)

Sudharshan *et al.* (2003) reported the occurrence of viral diseases of vanilla in several districts of Karnataka, which produced distinct symptoms such as mosaic, leaf curl and leaf distortion and was inferred to be caused by CymMV.

Bhat *et al.* (2004a) reported another important disease, the stem necrosis of *V. planifolia*, which is fast spreading and might pose a serious threat to vanilla cultivation in India. A leaf curl symptom by CymMV has also been reported from India by Sudharshan *et al.* (2003). The infection by CMV has also been reported from India by Madhubala *et al.* (2005)

2.2 SURVEY

Surveys were carried out by several scientists on the occurrence of vanilla viruses in vanilla cultivating belts.

In 1986 surveys were conducted in the islands of Moore, Tahiti in French Polynesia for the presence of CymMV and ORSV in vanilla (Wisler *et al.*, 1987). and reported its wide spread occurrence in those areas.

Pearson and Pone (1988) conducted survey on incidence of vanilla virus diseases in Tonga as part of vanilla development project and reported the wide spread occurrence of virus diseases. A detailed survey conducted by Pone (1989)

indicated that vanilla *potyvirus* was spreading very fast in Tonga and the level of infection was as high as 50% in some fields. Similar field surveys were also conducted by Person *et al.* (1993) for vanilla *potyvirus* and Farreyrol *et al.* (2001) for CMV in vanilla.

Survey conducted in French Polynesia by Grisoni *et al.* (2004) revealed the presence of two new viruses infecting *V. tahitensis*, which were serologically related to VanMV and DsMV

In India, vanilla virus surveys conducted in Karnataka and Kerala revealed the occurrence of the disease in both the states with a mean disease incidence ranging from 0-10%. High incidence of the disease up to 60% were also seen in a few gardens (Bhat *et al.*, 2004b). Another survey conducted by Bhat *et al.* (2004a) in India reported the occurrence of vanilla mosaic disease ranging from 0-5%.

2.3 SYMPTOMATOLOGY

Wisler *et al.* (1987) described some of the characteristic symptoms of *potyvirus* infecting *V. tahitensis* in French Polynesia, which included leaf distortion and mosaic patterns.

Pearson and Pone (1988) reported that the *potyvirus* infecting *V. tahitensis* showed symptoms such as irregular chlorotic yellow patches on leaves and the leaves developed uneven surfaces and irregular wavy edges. In older leaves the distortion was very severe and brown scab like spots appeared together with necrotic brown lesions on vines. Eventually the vine started to die, either from tip or base of leaves leaving a bare vine. New shoots may then develop but ultimately they also got affected.

Vanilla mosaic virus was responsible for leaf distortion and mosaic in *V. tahitensis* whereas in *V. fragrans* vanilla necrosis virus caused more severe symptoms which included distortion of leaf margin of young leaves, sunken chlorotic patches on leaves and on older leaves necrotic raised scab like lesions were seen. As the disease progressed necrotic lesions were found on the stem and the vines defoliated. (Pearson *et al.*, 1990)

Severely stunted *V. tahitensis* plants with conspicuous stem and leaf deformation, leaf mosaic and distortion caused by CMV was reported by Farreyrol *et al.* (2001) She also reported mosaic, leaf deformation, leaf deformation and mosaic, stunting and necrosis, chlorotic or greasy spots on CMV infected *V. fragrans*

Grisoni *et al.* (2004) reported two new viruses in *V. tahitensis*, which produced pronounced mosaic in islets on young leaves of vanilla. It is characterized by thinning of the leaf lamina delimiting large green patches that appear in relief and was associated with severe deformation of the leaf edge.

In India a virus disease of vanilla was first reported by Thomas (2002). The symptoms of the disease included chlorotic spots or streaks parallel to venation. In few occasions leaves of the infected plants showed wavy margin and reduced leaf size. The affected leaves become hard and leathery.

Sudharshan *et al.* (2003) reported a mosaic symptom on the foliage of *V. planifolia.* The symptoms appeared as mild mosaic or mottling with chlorotic specks or streaks mainly on leaves and rarely on stem. Such mosaic was also associated with leaf blistering and mild distortion. He further reported leaf curl symptom on *V. planifolia* from Karnataka. The symptoms included diffuse chlorotic patches on young leaves followed by leaf distortion. Necrotic lesions were also seen on older leaves.

Another important viral disease that was fast spreading and posing a serious threat to vanilla cultivation in India was reported by Bhat *et.al* (2004b). It was characterized by the appearance of brown necrotic patches on stem region with shriveled appearance. The affected stem showed distinct necrotic lesions of varying length (few mm to several cm). In a few cases the necrosis was also seen on older leaves at the lower surface in the form of scab. This often gave the appearance of sun scorch. When the affected stem region was cut open, array of dead tissues of varying depth were seen. The disease initially started as necrotic spots on stem and slowly got enlarged and encircled the stem. It led to the death of tissues at the affected region. In the affected plant, necrosis was seen only at one or few regions of the stem. Rest of the stem region looked apparently healthy

without any visual symptoms. A few of the necrosis affected plants also showed mosaic symptoms on leaves.

2.4 TRANSMISSION

Transmission studies of vanilla viruses were conducted by several scientists, which included mechanical transmission (sap), insect transmission and graft and bud transmissions.

2.4.1 Mechanical transmission

Sap transmission tests of vanilla *potyvirus* infecting *V. fragrans* were carried out by Pearson *et al.* (1990). They showed that this virus was sap transmissible to *Nicotiana benthamiana* in which it induced systemic vein chlorosis. Destruction of young leaves was also noticed after 10-15 days of inoculation. They further observed that the virus infecting *V. pompona* in French Polynesia was not mechanically transmissible to *N. benthamiana*. Their studies also revealed that the *potyvirus* infecting *V. fragrans* was not mechanically transmissible to different hosts *viz. Cassia occidentalis, Chenopodium amaranticolor, C.murale, C.quinoa, Datura stramonium, Gomphrena globosa, N. clevelandii, N.tabaccum* etc.

Wang and Pearson (1992) reported that the *potyvirus* infecting *V.tahitensis* from French Polynesia and Cook Islands was not mechanically transmitted to *Capsicum annuum, C. amaranticolor, C.murale, C.quinoa, Coriandrum sativum, Cymbidium swartz, Cyphomandra betacea, D. stramonium, G.globosa, N. clevelandii, N.tabaccum, N glutinosa, Petunia hybrida, Phaseolus vulgaris, Solanum melongina, Tetragonia expansa, Zea mays, Zinnia elegans* etc.

Farreyrol *et al.* (2001) observed that none of the plant species mechanically inoculated with vanilla mosaic virus developed visible symptoms and no virus particle was observed in electron microscopy of negatively stained sap extract. She further reported the transmission of CMV (vanilla strain) to *N. benthamiana* in French Polynesia in which the plant exhibited necrosis, vein discoloration and mottling.

Their findings were later reaffirmed by Madhubala *et al.* (2005). They reported that CMV (vanilla strain) could be successfully transmitted to several plant species upon mechanical inoculation with the infected sap under green house conditions.

2.4.2 Insect transmission

Transmission of vanilla *potyvirus* from *V. tahitensis* to *Vanilla pompona* by the aphid *Myzus persicae* was reported by Wisler *et al.* (1987). Pearson *et al.* (1990) reported that vanilla *potyvirus* was readily transmitted from infected *N. clevelandii* seedlings to healthy *N. clevelandii* by *M. persicae* and *Aphis gossypii* but failed to transmit the virus from infected to healthy *N. benthamiana* and from infected *V. fragrans* to healthy ones and also to *N. clevelandii*. Similarly *A. gossypii* also failed to transmit the virus from infected to healthy *N. benthamiana* and from *V. fragrans* to *N. clevelandii*. *M. persicae* also failed to transmit the vanilla *potyvirus* from *V. pompona* to *N. clevelandii*.

Wang and Pearson (1992) reported that *M. persicae* did not transmit the *potyvirus* affecting vanilla in French Polynesia from *V. tahitensis* to *N. clevelandii*.

2.4.3 Graft and bud transmission

Bhat *et al.* (2003) highlighted the vegetative mode of spread of viruses infecting vanilla crop. They reported that some of the viruses belonging to the genera *Tobamovirus* and *Potexvirus* were transmitted through contact between plants, cutting knives and other implements used in agricultural operations.

2.5 PHYSICAL PROPERTIES

Perusal of literature showed that no such studies were conducted so far on viral diseases affecting vanilla. However study on the physical properties forms a part of basic study on plant viruses. Johnson and Grant (1932) studied the physical properties of *Cucumis* virus 1 infecting different host plants and reported that the virus had a thermal inactivation point (TIP) of 60-65°C, dilution end point (DEP) of 1:10000 and longevity *in vitro* (LIV) at room temperature of 24- 28 hours.

Shawkat and Felga (1979) isolated watermelon mosaic virus (WMV) from *Cucurbita pepo* and CMV from brinjal and found that the viruses were inactivated at 65 °C and have DEP and LIV of 10^{-3}- 10^{-4} and 4-8 days respectively.

Gupta *et al.* (1992) reported that tobacco mosaic virus from brinjal had a TIP of 90 °C, DEP of 10^{-6} and LIV for a long period at room temperature.

2.6 PATHOPHYSIOLOGY

2.6.1 Chlorophyll

Reduction in total chlorophyll, chlorophyll a and chlorophyll b due to virus infection was reported by many workers (Johri and Padhi, 1985 ; Shukla *et al.*, 1992 ; Sarma *et al.*, 1995 ; Dantre *et al* ., 1996 Thind *et al.*, 1996)

Smitha (2001) estimated total chlorophyll, chlorophyll a and chlorophyll b of banana bract mosaic disease infected banana plants and found that the virus infection lead to reduction of these items in infected plants compared to healthy.

2.6.2 Total phenol

Farkas *et al.* (1960) found that the activation of phenol oxidizing enzymes was less in host due to systemic virus infection so that the phenol level is high during infection.

Total phenol was reported to be high in virus infected leaves of many plants (Chakrabortty *et al.*, 1994; Sarma *et al.*, 1995; Dantre *et al.*, 1996).

Jeeva (2001) reported that in sweet potato feathery mottle virus infection on sweet potato contributed to an increase in the activity of hexose monophosphate (HMP) shunt pathway which produces intermediaries required for synthesis of phenolic compounds.

2.6.3 Carbohydrate

Reduction in carbohydrate content in host plant in response to pathogen attack was reported by Goodman *et al.* (1967). Narayanasamy and Ramakrishnan (1966) reported decreased carbohydrate content in pigeon pea sterility mosaic virus infected pigeon pea.

2.6.4 Protein

According to Uritani (1971) there was enhanced protein content in virus infected plants when compared to healthy plants and the increased protein content in virus affected plants might be due to the production of new PR proteins.

Increased protein content due to virus infection has been reported by Sarma *et al.* (1995). Investigations on enhanced protein content in virus infected plants were reported by Singh and Singh (1984) and Mali *et al.* (2000).

2.6.5 Defense related enzymes

Changes in the defense related enzymes due to infection by different viruses have been reported in many plants (Batra and Kuhn, 1975; Wagih and Coutte, 1982 ; Rathi *et al.*, 1986)

Farkas *et al.* (1960) reported an increased in PPO content in virus infected plants with necrotic lesions. Gomathi *et al.* (1993) reported the increased activity of PO, PPO and PAL enzyme activity in banana infected with banana streak mosaic virus.

2.7 HOST RANGE

Host range studies of vanilla *potyvirus* infecting *V. fragrans* were carried out by Pearson *et al.* (1990). They showed that this virus was transmissible to *N. benthamiana* in which it induced systemic vein chlorosis. Their studies also revealed that the *potyvirus* infecting *V. fragrans* was not transmissible to different hosts *viz. Cassia occidentalis, C. amaranticolor, C. murale, C. quinoa, D. stramonium, G. globosa, N. clevelandii, N. tabacum* etc. They further observed that the virus infecting *V. pompona* in French Polynesia was not transmissible to *N. benthamiana*

Wang and Pearson (1992) reported that the *potyvirus* infected *V.tahitensis* from French Polynesia and Cook Islands was not transmitted to *Capsicum annuum, C. amaranticolor, C. murale, C. quinoa, Coriandrum sativum, Cymbidium swartz, Cyphomandra betacea, D. stramonium, G. globosa,*

N.clevelandii, N. tabacum, N. glutinosa, P. hybrida, P. vulgaris, S. melongina, T. expansa, Z. mays, Z. elegans etc.

Grisoni *et al.* (2004) reported two new viral strains affecting *V. tahitensis*. These viruses could be transmitted to several plant species upon mechanical inoculation including *N. benthamiana*, *P. vulgaris*, Watermelon (cv. Sugar Baby), Melon (cv. Cantaloup) *V. pompona* and *V. tahitensis.*

Madhubala *et al.* (2005) reported that CMV (vanilla strain) could be successfully transmitted to several plant species upon mechanical inoculation with the infected sap under green house conditions such as *C.annuum, Cucumis sativus, N. benthamiana, Trichosanthes angiuna, Lycopersicon esculentum* etc.

2.8 CHARACTERIZATION

2.8.1 Purification studies

Vanilla *potyvirus* was purified from systemically infected *N. benthamiana* and *N. clevelandii* leaves by Pearson *et al.* (1990) to get a final concentration of 2 mg of virus from 25 g of infected leaves. An A_{260}/A_{280} absorbance ratio of 1.28 was obtained for the purified virus.

Wang and Pearson (1992) developed a purification procedure for VanMV. The virus purification yielded approximately 2 mg virus per 100 g of *V. tahitensis* leaf tissue with an absorbance ratio of 1.09 for A_{260}/A_{280}. The virus was also attempted to be characterized by electron microscopic analysis. Pearson *et al.* (1990) through electron microscopic studies found that negatively stained sap from diseased *V. fragrans* contained flexuous filamentous particles with mean length of 765 ±14.6nm.

Wang and Pearson (1992) also observed flexuous filamentous particles in *V. tahitensis* plants through electron microscopy. The mean particle size of 83 negatively stained vanilla mosaic virus particles from a purified preparation, was 763nm x 0.7nm with 92% of the particle falling within a length of 680-900nm.

2.8.2 Electrophoretic analysis of proteins

Pearson *et al.* (1990) determined the relative molecular mass (Mr.) of vanilla poty virus by sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS PAGE) using the procedure by Laemmli (1970). The *potyvirus* produced a single major polypeptide band. 32.7 KDa

The purified virus isolates from *V. tahitensis* were analyzed in SDS PAGE by Wang and Pearson (1992). It was visualized by staining with 0.1% solution of bromophenol blue in a 5:2:5 mixtures of methanol, acetic acid and water. In this procedure a single major polypeptide band of 34 KDa was noted.

Madhubala *et al.* (2005) used the purified preparation of CMV on *V.planifolia* to conduct the SDS PAGE test. They obtained a major band corresponding to 25 KDa protein.

2.8.3 Antiserum production

Antiserum against vanilla *potyvirus* was produced by administering four intramuscular injections of purified virus to a New Zealand white rabbit over a period of eight weeks. Antiserum produced against the purified virus has a homologous titre of 1/1024 (Pearson *et al.*, 1990)

Wang and Pearson (1992) developed polyclonal antisera against *potyvirus* isolates from *V. tahitensis* by administering a series of subcutaneous injections of purified virus particles on New Zealand white rabbit. The purified virus was emulsified with 0.5 ml of Freunds incomplete adjuvant and injected. Repeated injections were made after one week and four weeks.

Vanilla isolate of CMV from Kerala and Karnataka was purified and antiserum was successfully developed in New Zealand white rabbit by Madhubala *et al.* (2005).

2.8.3.1 *Determination of antibody titre*

Grisoni *et al.* (2004) conducted plate trapped antigen enzyme linked immuno sorbant assay (PTA-ELISA) for detection of vanilla viruses in French Polynesia using a dilution of 1:1000 for polyclonal antibodies against *potyviruses* marketed by DSMZ (Braybscgweig, Germany)

Infection of CMV belonging to subgroup IB in *V. planifolia* was detected using Direct Antigen Coating ELISA (DAC- ELISA) by Madhubala *et al.* (2005). They reported that the virus was immunogenic and produced antiserum with a titre of 1:8000

2.8.3.2 *Immunological studies*

Pearson *et al.* (1990) conducted immunological studies of vanilla *potyvirus* using soluble dye immunoblot ELISA technique as described by Gibson (1988). They were able to detect the virus using this technique.

Wang and Pearson (1992) described the serological comparison of *potyvirus* isolates using Soluble Dye Immunoblot ELISA method. They found that VanMV antiserum reacted strongly with purified VanMV from French Polynesia and leaf extracts of *potyvirus* infected *V. tahitensis* from Cook Islands.

Pearson *et al.* (1993) successfully conducted detection tests for CymMV, ORSV, VanMV and VNV by soluble dye immunoblot ELISA using polyclonal antisera. A *potyvirus* group test was also carried out by them using indirect ELISA in infected vanilla using monoclonal antibody.

Farreyrol *et al.* (2001) confirmed the infection of *V. tahitensis* by CMV with double antibody sandwich ELISA in 23.5% of 179 samples from French Polynesia.

Grisoni *et al.* (2004) conducted PTA-ELISA for detection of vanilla viruses in French Polynesia using polyclonal antibodies marketed by DSMZ (Braybscgweig, Germany)

MATERIALS AND METHODS

3. MATERIALS AND METHODS

All the laboratory works and green house studies connected with the project were carried out in the Department of Plant Pathology College of Agriculture Vellayani during 2003-2005. The infected and healthy planting materials of vanilla were collected from the farmer's fields during the preliminary survey. The plants were maintained as per the package of Practice recommendations of Kerala Agricultural University (2002)

3.1 SURVEY

A detailed random survey was conducted in the vanilla growing tracts of Idukki and Kottayam districts of Kerala in two seasons, first during summer (March- April) and then after the rainy season (August- September) of 2003-2004. A total of 30 plots in three taluks of Kottayam district and 30 plots in three taluks of Idukki district having a minimum area of 0.5 acres were selected at random for the survey (Table No.1 and 2)

From each of the selected plots 100 plants were randomly selected and data on incidence of various virus diseases in general and on the disease incidence and disease index of the most commonly occurring virus disease in particular was recorded based on the standard disease assessment key. The data obtained on per cent disease incidence and disease index was analyzed using appropriate statistical technique. Analysis of variance has been conducted on the data to ascertain whether the districts and the taluks within the districts are varying in respect to the prevalence of the disease. As the data pertains to disease intensity it was transformed to their square root. (Panse and Sukhatme, 1954)

3.1.1 Disease Incidence (DI)

The per cent disease incidence was calculated by recording the number of plants infected out of 100 plants in each plot.

$$DI = \frac{\text{Total number of plants infected}}{\text{Total number of plants assessed}} \times 100$$

Table1. List of plots selected for survey in Kottayam district.

Sl.No.	Name of taluks		
	Kanjirapally	Kottayam	Changanacherry
1	M.Kurian Vazhathadathil	Thomas Kutty Pampady	Thomas.V.V Vellukallumpurath
2	T.Mathew Kannettuveetil	V.E Abraham Velliyamkunnathu	Jose.E Kallarakkal
3	Tony Cherian Thadaparampil	K.T Scaria Umberayil	Johnny Michael Kinnathukara
4	Shaju Manuel Nellikunnel	T.C.Mathew Thiruthiparampil	Tony George Kallivayalil
5	Joy Malakkuzhiyil Ancherimuttam	C.J Mani Chakkatapadayi	Baby Zacharia Arackalpadiyil
6	M.M.Thomas Melevettom	Thomachan Chacko Parakkulamthada	Ivan.P.Punnen Parekulam
7	C.V.Antony Chennotu	K.A.Philipose Kannukuzhiyil	Thomas Taharappel Kizhaparayoor
8	K.M.Jose Kadaviladi	A.S.Chacko Kaithamattam	M.P. Thankachan Mylakampuram
9	M.N.Vijayan.Nair Ambady	Santhosh Manayilveedu	Baby Kattadichira
10	Joy Augustine Malayinkizhu	Veeramani Toothilpattu	Abraham Imanuel Kochuparampil.

Table 2. List of plots selected for survey in Idukki district.

Sl.No.	Name of taluks		
	Todupuzha	Peerumedu	Devikulam
1	Issac Mathew Paraserriyil	Santha B Taipura	Immauel Ponnattu
2	Abraham Thomas Puthenchanda	Thankachan Mayalkarapurethu	Sabu Ponnattu
3	Radhakrishnan Totatilveedu	Philipose Koodarathil	Jessy Joy Kulangara
4	Thomas George Vadekedathu	Issac Mannayil Mannayil House	Joseph Kunnethel
5	Pillachan Muthenparayil	Santhi Amma Chekkeravilla	Joy Joseph Pazhampara
6	George Augustine Madathilveedu	Jacob.P.T Dhalia	Jose D Kappan Kappil
7	Shaji.M Puthenparambu	Pappachan Maidanam	Mathew Joseph Valumel
8	Narayanan Nair Kacherimukku	Mauel Jose Vadiyencheri	George Peter Palathinal
9	Sadasivan Tillimangalam	Sabu. Thadathilputhenveedu	Jose Ponnattu
10	Mathew John Vadekketharayil	Jose David Thamaraserry	A.E.Narayanan Ariplakkal

3.1.2 Disease index

The Plant Disease Index (PDI) of each plot was calculated on 0-4 scale as described by Mayee and Datar (1986)

Scale

0- No symptom

1- Light chlorotic mosaic symptom

2- Light mosaic and leaf crinkling

3- Necrotic lesions on leaves, mosaic and crinkling of leaves

4- Severe leaf distortion, necrotic lesions and drying of leaves

$$PDI = \frac{\text{Sum of individual grades}}{\text{No of plants assessed} \times \text{maximum grade assigned}} \times 100$$

3.2 SYMPTOMOTOLOGY

The symptoms produced on naturally infected vanilla plants in the field were recorded. Infected vanilla planting materials were brought from different vanilla growing tracts of Idukki and Kottayam districts and were maintained in green house. These plants were carefully monitored to study the pattern of symptom produced by each specimen. The symptoms produced on other plants on mechanical transmission were also evaluated. The variability of symptoms produced by the most commonly occurring virus disease on vanilla under natural condition were also evaluated

3.3 TRANSMISSION STUDIES

All transmission studies were carried out under insect proof glass house conditions. The culture of the virus was maintained by repeated transmission on *Chenopodium amaranticolor* by mechanical inoculation using 0.1 M phosphate buffer (pH 7.2) at room temperature

3.3.1 Transmission through planting material

Vanilla cuttings of length 1 m were collected from both healthy and infected plants (10 each) from each location and were planted in pots. A potting mixture consisting of 2:1:1 sand, silt and organic manure were used to fill the pot. On top of it coconut husk and coir pith were put and the planting was done. These plants were sprayed with 0.05% monocrotophos and kept in insect proof glass house for 6 months and expression of symptoms were recorded as and when it appeared on new growth.

3.3.2 Mechanical transmission

The culture of vanilla mosaic virus maintained on infected vanilla plant in the screen house was used for the transmission studies. The extract was taken from young infected leaves showing clear chlorotic or mosaic symptoms. The inoculum was prepared by grinding 1 part of infected leaf tissue and 1 part of pre cooled buffer using chilled mortar and pestle. The following buffers were used for sap transmission studies. (Table.3) (Appendix I)

Table 3. Different buffers used for sap transmission

Sl.No.	Buffer	Molar conc.	pH
1	Citrate	0.1M	6.2
2	Tris	0.1M	7.2
3	Phosphate (Sodium phosphate)	0.1M	7.2
4	Borate (Sodium borate)	0.1M	8.0

The homogenate was filtered through a thin layer of absorbent cotton to clarify the inoculum and maintained in chilled condition in an icebox and immediately used for inoculation. Mechanical inoculation was performed by leaf rubbing using the clarified sap. The inoculum was rubbed on young leaves of 10 healthy vanilla plants collected from healthy planting material. The leaves of the test plants were previously dusted with celite powder (600 mesh) and were inoculated by gently rubbing on the upper surface of the leaves using the pestle

moistened with the inoculum. The excess inoculum was removed by washing with distilled water using a wash bottle after 10 minutes and the inoculated plants were kept in insect proof condition. The plants were observed for development of symptoms up to 3 months.

Based on the preliminary studies *C. amaranticolor* was identified as the ideal local lesion host of vanilla mosaic virus and sap transmission studies were conducted using it following the standard procedure as described above. Forth to eighth leaves of the *C.amaranticolor* at 8-15 leaf stage were inoculated. Different buffers as mentioned above were used for the same. The buffer, which gave maximum number of local lesions on the local lesion host, was chosen as the buffer for subsequent studies. Thus 0.1M phosphate buffer pH (7.2) was used for all further studies.

3.3.3 Insect transmission

The insect transmission studies were conducted using the insect vectors *viz., Aphis gossypii, Aphis craccivora* and *Bemisia tabaci.*

Healthy colonies of *A. gossypii* and *A. craccivora* were maintained on brinjal and cowpea respectively and were used for transmission studies.

3.3.3.1 *Transmission using Aphid vectors*

Healthy apterous aphid colonies maintained on the respective host plants were used for transmission studies. Using a camel hairbrush the aphids were collected and transferred to petriplates. The mouth of the plate was covered with a muslin cloth and aphids were starved for a period of 1 hour and thirty minutes (pre acquisition fasting). The starved aphids were allowed to feed on detached infected young vanilla leaves for 30 minutes (acquisition feeding period). Then 20 viruliferous insects each were transferred to healthy vanilla plants maintained on poly bags and allowed to feed for 24 hrs (infection feeding period). Then these insects were killed using monocrotophos (0.05%).The per cent insect transmission was recorded.

3.3.3.2 *Transmission using whiteflies*

Whitefly (*B. tabaci*) colonies were maintained on healthy brinjal plants. Plastic transmission cages designed by Nene (1972) were used for transmission

studies. The top portion of the infected plant showing typical symptom of the disease was introduced into the transmission cage through a rectangular slit at the opening of the cage. White flies were collected with an aspirator and released into the transmission cage. The cage was covered with black cloth except at the region of the wire netting which was kept facing the light source while releasing white flies. The cap of the transmission cage was immediately screwed on and the remaining portion of the rectangular slit of the cage was kept closed by cotton wool. The cages were kept in position using a stick. After 24 hours the cotton was removed and gently tapped with a glass rod to disturb the white flies. This stimulated the white flies to move away from the leaves to the side of the cage facing the light source. It was collected and the viruliferous white flies were released on each test plant (10 Nos.) for inoculation feeding. After inoculation feeding the insects were killed by spraying the plants thoroughly with 0.05% monocrotophos. All the inoculated plants were labeled and kept for observation in insect proof area

3.3.4 Graft and bud transmission

Wedge grafting and approach grafting techniques were employed to test the graft transmission of vanilla mosaic virus. Young infected shoots showing mosaic symptom were used for preparing the scion. The base of the scion was trimmed to wedge shape and inserted into the cleft made on the stem of the healthy vanilla plants kept in insect proof glass house. The scion was inserted into the cleft of the stock and then tied firmly with waterproof parafilm strip. Fifteen plants were maintained. These experimental plants were kept under observation for four months for the development of symptoms

Approach grafting was done using healthy and diseased vanilla plants. Healthy plant was taken and grafted to a diseased plant by making a sidewise slanting incision and removing a shield. Identical cut was also made on diseased plant. Then both of the cut portions were held together and tied firmly. This was then covered using waterproof parafilm strip. Fifteen plants were maintained. The plants were kept under observation for the development of symptoms.

For bud transmission leaf axil was taken from infected plant with a small portion of leaf. Then the bud portion of the healthy plant was scooped out from leaf axil and the diseased leaf axil was placed firmly over the cut so as to make a perfect fit. This was then tied firmly using waterproof parafilm strip. Fifteen plants were maintained and the plants were also kept in insect proof conditions and were observed for the development of symptoms.

3.4. PHYSICAL PROPERTIES

3.4.1 Dilution end point

The sap for determining the physical properties of the virus was prepared by adding one ml of 0.1 M phosphate buffer (pH7.2) for every one gram of infected leaf tissue. It was then crushed into pulp with the help of sterile pestle and mortar and the homogenate was strained through muslin cloth. Serial dilution of the sap *viz.*10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} was made with phosphate buffer. The different dilutions were used for inoculation on separate test plants starting from the highest dilution. The local lesion host *C. amaranticolor* was used as the test plant. Ten replications were maintained for each of the dilutions. The inoculated leaves were labeled and kept under insect proof condition for the observation and development of local lesions

3.4.2 Thermal inactivation point

The sap was extracted as mentioned in the above experiment. Five ml of the sap was pipetted out into thin walled glass test tubes. Care was taken not to smear the upper part of the test tube. It was then placed in a thermostatically controlled water bath. The water bath was filled with water until the level reached 3 cm above the level of sap in the tube. The test tubes were kept for 10 min in the water bath at the required temperatures. The different temperatures tested to estimate the thermal inactivation point were 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75° C. After 10 minutes of treatment the tube was removed and cooled immediately in running water. The control and treated saps were used for inoculation on test plants by smearing it on the leaves of *C. amaranticolor* sprinkled with celite powder. Ten fully opened leaves were inoculated in each

treatment. Observations on the number of local lesions produced on leaves were recorded.

3.4.3 Longevity *in vitro*

The sap for testing the longevity *in vitro* was prepared in the same manner as mentioned in para 3.4.1. Five ml sap each was pipetted into test tubes and closed with aluminium foil. Five test tubes each were kept at room temperature and also in refrigerator. One tube each containing the sap of each treatment was taken at specific periods *viz.* 0, 24, 48, 72, 96 and 120 hours for the sap maintained at room temperature and for the sap kept under refrigerated condition. This was then inoculated on the leaves of *C. amaranticolor*. Ten leaves were inoculated with the respective sap and the inoculated plants were kept under insect proof conditions and observed for the development of local lesions.

3.5 HOST RANGE STUDIES

To determine the host range of the virus under study 12 plants species belonging to Amaranthaceae, Apocyanaceae, Chenopodiaceae, Solanaceae and Orchidaceae as detailed in Table 4 were tested. Ten seedlings each were inoculated with the sap. The plant which did not show visible symptoms of infection after six weeks, were indexed by back inoculation to *C. amaranticolor* to find out whether they are symptomless carriers of the virus.

3.6 PATHOPHYSIOLOGY

Physiological changes in vanilla as a result of viral infection were studied. The leaf samples were collected from the infected as well as healthy vanilla plants and total chlorophyll, chlorophyll a, chlorophyll b, carbohydrates, phenols and protein were estimated The analysis of defense related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase were also conducted.

Table 4.List of host tested to study the host range of vanilla mosaic virus

Sl.No	Family name	Species name
1	Amaranthaceae	*Gomphrena globosa*
2	Apocyanaceae	*Catharanthus roseus*
3	Chenopodiaceae	*Chenopodium amaranticolor* *Chenopodium quinoa*
4	Solanaceae	*Datura stramonium* *Datura metel* *Nicotiana glutinosa* *Nicotiana benthamiana* *Nicotiana tabacum* *Capsicum annum* *Lycopersicon esculentum*
5	Orchidaceae	*Dendrobium spp.*

3.6.1 Total chlorophyll

Chlorophyll was estimated by the method described by Arnon (1949). One gram of leaf sample (W) was ground in a mortar with 20 ml of 80 % acetone. The homogenate was centrifuged at 5000 rpm for 5 min and the supernatant was transferred to a 100 ml volumetric flask. The above procedure was continued until the residue become colourless. The final volume (V) in the volumetric flask was made up to 100 ml. The absorbance of the solution at 645nm and 663nm was determined using a spectrophotometer (Systronics UV-VIS spectrophotometer-118) against the solvent as blank (80%acetone). The chlorophyll content was calculated using the following equation and expressed as milligrams chlorophyll per gram leaf tissue.

Total chlorophyll =20.2 (A645) + 8.02(A663) x V / 1000 x W

Chlorophyll a = 12.7 (A663) - 2.69(A645) x V / 1000 x W

Chlorophyll b = 22.9 (A645) – 4.68(A663) x V / 1000 x W

3.6.2 Total phenols

Phenol content was estimated by the procedure of Bray and Thorpe (1954). One gram of leaf sample was ground in 10 ml of 80 % ethanol using a pestle and mortar. The homogenate was centrifuged at 10000 rpm for 20 minutes (Hettich, EBA 12/12) The supernatant was saved and the residue was extracted with five times the volume of 80 % ethanol and centrifuged. The supernatant was collected and pooled together and then evaporated to dryness. The resultant residue was dissolved in five ml. of distilled water. An aliquot of 0.1 ml. of this sample was pipetted out into a test tube and the volume was made up to three ml with distilled water. Folin- Ciocalteau reagent (0.5 ml) and two ml of 20 % sodium carbonate solution were added to each tube after 3 minutes. The reaction mixture was thoroughly mixed and kept in boiling water for one minute. After cooling the absorbance was measured at 650 nm against the reagent blank using a spectrophotometer (Systronics UV-VIS spectrophotometer-118). Standard curve was prepared and phenol content was expressed as catechol equivalent per gram of leaf on fresh weight basis.

3.6.3 Total carbohydrates

Total carbohydrate content was estimated following the method by Hodge and Hofrieter (1962). Leaf samples of 100 mg each were weighed into test tubes and hydrolyzed with five ml. of 2.5 N HCl by keeping it at 100°C in a water bath and cooled to room temperature. This was then neutralized with solid sodium carbonate until the effervescence ceased. Later the volume was made up to 10 ml. with distilled water and centrifuged at 10000 rpm for 10 minutes. To 0.5 ml of the supernatant, 0.5 ml of distilled water and four ml. of anthrone reagent (200mg anthrone in 100 ml of ice cold 95% sulphuric acid) were added. The reaction mixture was heated for eight minutes at 100° C in a water bath and cooled rapidly. When the colour of the solution became green to dark green the absorbance was determined at 630 nm in a spectrophotometer (Systronics UV-VIS spectrophotometer-118) Amount of carbohydrate present was calculated from standard curve prepared using D-glucose and expressed in terms of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.6.4 Protein

Total soluble protein content was estimated as per the procedure by Bradford (1976). One gram of leaf sample was homogenized in three ml. of 0.1 M sodium phosphate buffer (pH 7.0) using a pre chilled pestle and mortar. The extract was centrifuged at 10000 rpm for 10 minutes at 4° C and the supernatant was used for the estimation. The reaction mixture (0.5ml of distilled water and five ml. of working dye solution and 0.5 ml of leaf extract) was incubated for 5 minutes at room temperature. One ml of distilled water with five ml. of dye solution served as control. Reading was taken at 595 nm in a spectrophotometer (Systronics UV-VIS spectrophotometer-118). Bovine serum albumin (BSA) was used as protein standard. The protein content was expressed as microgram albumin equivalent of soluble protein per gram of leaf on fresh weight basis. (Appendix II)

3.6.5 Defense related enzymes

3.6.5.1 *Peroxidase (PO) activity*

Peroxidase activity was determined according to the procedure described by Srivastava (1989). Leaf sample of 200 mg. was homogenized in one ml of 0.1 M sodium phosphate buffer (pH6.5) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenization was done at 4°C using a sterile pestle and mortar, the homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant was used as the enzyme extract for the assay of peroxidase activity. The reaction mixture consisted of one ml of 0.05M pyrogallol and 50 µl of enzyme extract. It was taken in both reference and sample cuvettes and kept in spectrophotometer (Systronics UV-VIS spectrophotometer-118) and reading was adjusted to zero at 420 nm. The enzyme reaction was started by adding one ml of one per cent hydrogen peroxide into the sample cuvettes and change in absorbance was measured at 30 seconds interval. (Appendix III)

3.6.5.2 *Polyphenol oxidase (PPO) activity*

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.* (1965). The enzyme extract was prepared as per the procedures given for the estimation of peroxidase. The reaction mixture contained one ml of 0.1M sodium phosphate buffer (pH6.5) and 50 µl of enzyme extract. The reaction was initiated after adding one ml of 0.01M catechol. The observations were recorded in a spectrophotometer (Systronics UV-VIS spectrophotometer-118). The change in absorbance was recorded at 495nm. and polyphenol oxidase activity was expressed as change in absorbance of the reaction mixture per minute per gram of leaf on fresh weight basis. (Appendix III)

3.6.5.3 *Phenyalanine ammonia lyase (PAL) activity*

Phenyalanine ammonia lyase activity was analyzed based on the procedure laid out by Dickerson *et al.* (1984). The enzyme extract was prepared by homogenizing one-gram leaf sample in 5 ml of 0.1 M sodium borate buffer (pH 8.8) containing a pinch of polyvinyl pyrrolidone (PVP) using chilled mortar

and pestle. The homogenate was centrifuged at 10000 rpm for 10 minutes at 4° C and the supernatant was used for the assay of PAL activity. The reaction mixture contained 3ml of 0.1M sodium borate buffer (pH8.8), 0.2 ml. of enzyme extract and 0.1 ml of 12 µM L- phenylalanine prepared in the same buffer. The blank contained 3ml of 0.1M sodium borate buffer (pH8.8), 0.2 ml of enzyme extract. The reaction mixture and the blank were incubated at 40°C for 30 minutes and the reaction was stopped by adding 0.2 ml. of 3 M hydrochloric acid. The absorbance was read at 290 nm. in a spectrophotometer (Systronics UV-VIS spectrophotometer-118) PAL activity was expressed in micromoles of cinnamic acid produced per minute per gram fresh weight basis of leaf. (Appendix IV)

3.7 CHARACTERIZATION OF VIRUS

3.7.1 Electrophoretic analysis of protein by SDS-PAGE (Sodium dodecyl sulphate poly acrylamide gel electrophoresis.)

Electrophoretic separation of soluble proteins of the virus under study was carried as per the procedure described by Laemmli (1970) Leaf samples of healthy and virus infected plants were taken for analysis. Five hundred milligrams each of healthy and diseased leaf samples were homogenized separately in 200 µl of cold denaturing solution at 4°C. (Appendix V). The supernatant was mixed with chilled acetone in the ratio 1:4 and the protein was allowed to precipitate by keeping the mixture at 4°C for 30 minutes. The sample was centrifuged at 5000 rpm for 15 minutes at 4°C. The precipitate was resuspended in 20 µl of denaturing solution and vortexed. The homogenate was centrifuged at 5000 rpm for 15 minutes. The supernatant was mixed with equal volume of sample buffer and kept in a boiling water bath for 3 minutes to complete the reaction between protein and SDS. These samples were used for SDS- PAGE. The protein concentration was adjusted in each sample to strength of 100 µg of protein following Bradford method. The following reagents were used for characterization of proteins by SDS- PAGE

a) Acrylamide stock (30 %)

Acrylamide - 29.2g

Bis – Acrylamide – 0.8 g

Double distilled water –100ml.

b) Separating gel buffer stock

1.5M Tris HCl pH 8.8 (Tris base 18.15g was dissolved in 50 ml of double distilled water, the pH was adjusted to 8.8 with 6 N HCl and made up the volume to 100 ml with double distilled water and stored at 4°C)]

c) Stalking gel buffer stock

0.5 M Tris HCl pH 6.8- (Tris base 6.0 g was dissolved in approximately 60 ml of double distilled water and adjusted the pH to 6.8 with 6 N HCl and volume was made up to 100ml with double distilled water and stored at 4°C)

d) Polymerizing agent

Ammonium per sulphate (APS) 10 percent prepared freshly before use

Tetramethyl ethylene diamine (TEMED) fresh from the refrigerator

e) Electrode buffer (pH 8.3)

Tris base -6g

Glycine - 28.8g

SDS -2.0 g

Double distilled water- 2 litre

f) Sample buffer (SDS reducing buffer)

Double distilled water – 2.6 ml

0.5 M Tris HCl pH6.8 – 1.0 ml

2- mercaptoethanol - 0.8 ml

Glycerol - 1.6 ml

SDS 20% - 1.6 ml

0.5% Bromophenol blue – 0.4 ml.

g) Staining solution

Coomassie brilliant blue R- 250 - 0.1g

Methanol - 40 ml

Glacial acetic acid - 10 ml

Double distilled water - 50 ml

h) Destaining solution

Same as staining solution without Coomassie brilliant blue R-250

Procedure

Separating gel was first casted followed by stacking gel by mixing the various solutions as indicated,

a) Preparation of separating gel (12%)

Tris HCl pH 8.8 - 5.0 ml

Double distilled water - 6.7ml

Acrylamide 30 % - 8.0 ml

SDS 10 % - 0.2 ml

The above solution is mixed well and degassed for 3 minutes and then the following items were added immediately

10 % Ammonium per sulphate – 0.1ml

TEMED -0.01ml

The separating gel was mixed well and poured immediately between glass plates and a layer of water was added above the polymerizing solution to quicken the polymerization process. Then stacking gel was casted.

b) Preparation of stacking gel (4%)

Tris HCl pH6.8 - 2.5 ml

Double distilled water - 6.1 ml

SDS 10 % - 0.1 ml

Acrylamide 30 % - 1.3 ml

Then the solution was mixed well degassed for 3 minutes and 10 % APS (0.05ml) and TEMED (0.1ml) were added.

The water layered over the separating gel was removed and washed with a little electrode buffer and then the stacking gel was poured over the polymerized

separating gel, after keeping the comb in position. After polymerization samples were loaded into wells and the electrophoresis was performed at 100 V till the dye front reached the separating gel. Then the voltage was increased to 200 V and continued till the dye reached the bottom of the gel. The gel was removed immediately after electrophoresis between the glass plates and incubated in the staining solution for overnight with uniform shaking. Then the gel was transferred to the destaining solution. The protein appeared as bands and the gel was photographed.

3.7.2 Purification

Purification of vanilla mosaic virus was carried out using the method for the purification of *potyviruses* described by Khurana *et al.* (1987).

Emerging new leaves of vanilla infected with the virus were used for the work. Hundred grams of leaf sample was taken and was homogenized in (1:4) 0.1 M phosphate buffer pH (7.2) containing 0.5M urea, 0.01M Ethylene diamine tetra acetate (EDTA) and 1% thioglycolic acid (TGA). The homogenate was filtered through cheesecloth. The filtrate was clarified by centrifugation at 5000 rpm for 15 minutes in a refrigerated centrifuge (Hettich EBA 12 R). The supernatant was collected and was mixed with equal quantity of chloroform, n- butanol mixture (1:1v/v). It was mixed in a shaker at 4°C for half an hour. Then it was centrifuged at 5000 rpm for 15 min at 4°C. The aqueous phase was collected. To this 4 % polyethylene glycol (PEG 6000) and 0.2 M sodium chloride were added and the mixture was kept at 4°C for one hour with constant stirring. Then it was kept overnight at 4°C. Next day the virus precipitated was pelleted at 10000 rpm in a refrigerated micro centrifuge at 4°C (Hettich MICRO 24-48 R) for 30 min. Supernatant was removed carefully and the pellet was resuspended in 0.01M phosphate buffer (pH7.0) containing 5 mM EDTA and 0.5 M urea. Again it was clarified by centrifugation at 5000 rpm for 5 minutes. The supernatant was taken and stored at 4°C and was used as antigen source for immunizing rabbits. (Appendix VI)

3.7.3 Production of antiserum against virus

Antiserum was produced against the vanilla mosaic virus in New Zealand white rabbit (1.5 years old) by giving 4 intra muscular injections with the partially purified virus at weekly intervals. Before injection, the partially purified virus preparation was emulsified with one ml of Freunds incomplete adjuvant (Difco) (1:1v/v). Ten days after last injection the rabbit was bled through the marginal ear vein. Blood was collected in a sterile test tube and was kept in slanting position for 2 hours at room temperature. The tube was then kept without disturbance in refrigerator at 4°C. Next day the clear serum was pipetted out using a micropipette and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was pipetted out using a micropipette and dispensed into 1.5 ml eppendorf tubes. A pinch of sodium azide was added to the clarified serum to prevent microbial contamination. Then the vials were stored under refrigerated conditions.

3.7.3.1 Determination of antibody titre

The titre of the antiserum was determined by direct antigen coating enzyme linked immunosorbent assay (DAC-ELISA). The procedure described by Huguenot et al (1992) was followed for detection

One gram of infected sample was homogenized in 5 ml of coating buffer (carbonate buffer) containing 2 %w/v polyvinyl pyrrolidone (PVP) in a chilled mortar and pestle. Healthy plant extract was prepared by using leaves of healthy vanilla plants. The homogenate was centrifuged at 5000 rpm for 10 minutes at 4°C. Samples were dispensed at the rate of 100µl into Nunc immunological plates. The treatments were replicated thrice. After incubation for 2 hours at 37°C the wells were washed with Phosphate buffer saline tween (PBS-T) three times each for duration of 3 minutes in the ELISA plate washer. Then the PBS-T was aspirated in the instrument. Then blocking was done with 100 µl of 1% bovine serum albumin (BSA) for 30 minutes at 37°C. After incubation blocking agent was removed, and plate was washed with PBS-T as before. The antibody raised in rabbit was diluted in healthy plant sap for cross absorption and 100 µl of each dilution was added to the well. In addition to cross absorption test the antibody raised in the rabbit was directly added to the well with out cross

absorption. The dilutions of 1:250, 1:500 and 1:1000 were used for the determination of the titre of the developed antiserum. Three replications were maintained for each treatment and incubated overnight at 4°C. On the next day the plates were washed with PBS-T and then treated with 100 micro litre of alkaline phosphatase conjugated goat- antirabbit immunoglobulin diluted in PBST-PO (Polyvinyl pyrrolidone Ovalbumin) 1:10000 v/v. and incubated for two hours at 37 °C. After that wells were washed with PBS-T as before. The substrate *p*-nitopheny phosphate (*p*-NPP) in diethanole amine buffer (1mg/ml) was added to each well (100 µl / well) and incubated for one hour at 37°C. The absorbance was read at 405 nm in ELISA reader. (Appendix VII)

3.7.3.2 Immunological studies

3.7.3.2.1 *Microprecipitin test*

Thirty microlitre of antiserum raised in rabbit and same quantity of vanilla virus infected leaf extract in 0.1M phosphate buffer (pH7.2) were mixed on a microscopic slide. Antibody treated with healthy leaf extract served as control. The mixture was incubated at 25°C under high humidity for 30-45 minutes and examined under microscope.

3.7.3.2.2 *Direct antigen coating enzyme linked immuno sorbent assay: (DAC ELISA)*

One gram of infected sample was homogenized in 5 ml of coating buffer (carbonate buffer) containing 2 %w/v polyvinyl pyrrolidone (PVP) under chilled condition. Healthy plant extract was prepared by using leaves of healthy vanilla plants. The homogenate was centrifuged at 5000 rpm for 10 minutes at 4°C. Samples were dispensed at the rate of 100 µl into Nunc immunological plates. The treatments were replicated thrice. After incubation for two hours at 37°C the wells were washed with phosphate buffer saline tween (PBS-T) three times each for duration of 3 minutes in the ELISA plate washer. Then the PBS-T was aspirated in the instrument. Then blocking was done with 100 µl of 1% bovine serum albumin (BSA) for 30 minutes at 37°C. After incubation blocking agent was removed, plate was washed with PBS-T as before. The antibody raised in rabbit was diluted in healthy plant sap for cross absorption to 1:500 Three

replications were maintained for each treatment and incubated overnight at 4°C. On the next day the plates were washed with PBS-T and then treated with 100 µl of alkaline phosphatase conjugated goat- antirabbit immunoglobulin diluted in PBST-PO 1:10000 v/v. and incubated for two hours at 37 °C. The wells were washed with PBST as before. The substrate p- nitopheny phosphate (p-NPP) in diethanol amine buffer (1mg/ml) was added to each well (100 µl/ well) and incubated for one hour at 37°C. Then the reaction was stopped by adding 50 µl of 4 % sodium hydroxide. The absorbance was read at 405 nm in ELISA reader.

RESULTS

4. RESULTS

Results of the study on viral diseases of vanilla in Kerala are presented below.

4.1 SURVEY

Survey on the incidence of vanilla virus diseases was carried out in the vanilla growing areas of Kottayam and Idukki districts following a stratified two-season survey. The strata were taken as three taluks of respective districts. The type of symptoms and the most common symptom were observed. During the survey, three distinct types of vanilla virus disease symptoms were observed. These distinct symptoms are as detailed below

Symptom Type No.1 (SYM-T-1)

Development of chlorotic areas intermingled with green areas, which gradually develops into mosaic pattern on leaves. As the disease progresses necrotic spots developed on these areas associated with leaf crinkling, distortion and drastic reduction of leaf size (Plate No.1)

Symptom Type No.2 (SYM-T-2)

Light chlorosis on leaves, which are more pronounced when leaf is shown against a source of light with out any distortion or reduction of leaf size. (Plate No.2)

Symptom Type No.3 (SYM-T-3)

Linear necrotic patches develop on leaf lamina, which gradually progress to stem. Later these areas enlarge and the affected plant gives a burnt appearance. (Plate No.3)

Result of the survey on the incidence of different viral diseases on vanilla in Kottayam and Idukki districts is shown in Table 5. The study revealed that the most predominant virus disease occurring in the areas surveyed was the one that produced the Symptom Type No.1, the most predominant symptom of which was mosaic, and hence it is named as vanilla mosaic virus disease (VMVD). The disease was found to be prevalent in 21 plots out of 30 surveyed in Kottayam district and 23 out of 30 plots in Idukki district. The disease which produced Symptom Type No.2 was observed in 9 plots out of 30 in Kottayam and 7 plots out of 30 in Idukki district, while Symptom Type No.3 was noticed only in one plot in Kottayam and three plots in Idukki district.

Table.5 Incidence of virus diseases of vanilla in Kottayam and Idukki districts during summer 2003-2004.

Name of districts	Name of taluks	No. of plots showing Symptom Type No.1	No. of plots showing Symptom Type No.2	No. of plots showing Symptom Type No.3
Kottayam	Kanjirapally	8	4	0
	Kottayam	6	2	0
	Changanacherry	7	3	1
Idukki	Todupuzha	8	5	2
	Peerumedu	8	0	0
	Devikulam	7	2	1

Symptom Type No.1

Symptom Type No.1
Plate No. 1

Plate No. 2
Symptom Type No. 2

Plate No. 3
Symptom Type No. 3

So further study such as per cent disease incidence and disease index were calculated for vanilla mosaic disease for each plot during summer and rainy season of 2003-04, the results of which are given in the following tables (Tables 6-9).

4.1.1 Disease Incidence

The per cent incidence of vanilla mosaic virus disease was estimated in the three taluks each of Kottayam and Idukki districts during summer and rainy seasons of 2003-04 and the data are presented in tables 6and 7. The mean per cent disease incidence were 6.683 and 8.205 during summer season in Kottayam and Idukki districts while it were 5.325 and 4.900 respectively during the rainy season. There was no significant difference with respect to the per cent disease incidence between taluks in each district and also between districts during summer as well as rainy season. However the mean per cent disease incidence was found to be reduced from 6.683 during summer season to 5.325 in the rainy season in Kottayam district while it reduced from 8.205 in summer season to 4.900 in rainy season in Idukki district.

4.1.2 Disease Index

The intensity of vanilla mosaic disease was assessed by estimating the disease index in Kottayam and Idukki districts during the two seasons of 2003-04 and the data are presented in tables 8 and 9. The mean disease index recorded for both the districts were 2.553 and 2.976 during summer season and 1.573 and 1.554 during the rainy season in Kottayam and Idukki districts respectively. No significant difference could be observed with respect to disease index between taluks in the same district as well as between the two districts surveyed. The disease index also showed a declining trend in rainy season in both the districts. (Table 8 and 9)

Table 6 Per cent incidence of vanilla mosaic virus in Kottayam and Idukki districts during summer season of 2003-04

Sl No.	District	Per cent incidence in taluks			Mean (District)
		T_1	T_2	T_3	
1	D_1	6.115 (2.667)	5.751 (2.598)	8.302 (3.050)	6.683 (2.772)
2	D_2	8.704 (3.115)	7.586 (2.930)	8.348 (3.057)	8.205 (3.034)

Values in the parenthesis are the corresponding transformed means

F value between districts- 0.60
F value with in D_1 - 0.34
F value with in D_2 - 0.05

CD between districts- 0.679
CD between taluks - 1.176

Kottayam (D_1)

D_1T_1- Kanjirapally
D_1T_2- Kottayam
D_1T_3- Changanacherry

Idukki (D_2)

D_2T_1 - Todupuzha
D_2T_2 - Peerumedu
D_2T_3 - Devikulam

Table 7 Per cent incidence of vanilla mosaic virus in Kottayam and Idukki districts during rainy season of 2003-04

Sl No.	District	Per cent incidence in taluks			Mean (District)
		T_1	T_2	T_3	
1	D_1	5.313 (2.513)	3.817 (2.195)	7.044 (2.836)	5.325 (2.515)
2	D_2	4.301 (2.302)	4.186 (2.277)	6.326 (2.707)	4.900 (2.429)

Values in the parenthesis are the corresponding transformed means

F value between districts- 0.079
F value with in D_1 - 0.74
F value with in D_2 - 0.42

CD between districts- 0.610
CD between taluks - 1.057

Kottayam (D_1)

D_1T_1- Kanjirapally
D_1T_2- Kottayam
D_1T_3- Changanacherry

Idukki (D_2)

D_2T_1 - Todupuzha
D_2T_2 - Peerumedu
D_2T_3 - Devikulam

Table 8 Vanilla mosaic disease index in Kottayam and Idukki districts during summer season of 2003-04

Sl No.	District	Disease index in taluks			Mean (District)
		T_1	T_2	T_3	
1	D_1	2.126 (1.768)	2.322 (1.823)	3.264 (2.065)	2.553 (1.885)
2	D_2	3.164 (2.041)	2.785 (1.946)	2.983 (1.996)	2.976 (1.994)

Values in the parenthesis are the corresponding transformed means

F value between districts- 0.35
F value with in D_1 - 0.53
F value with in D_2 - 0.05

CD between districts- 0.354
CD between taluks - 0.613

Kottayam (D_1)

D_1T_1- Kanjirapally
D_1T_2- Kottayam
D_1T_3- Changanacherry

Idukki (D_2)

D_2T_1 - Todupuzha
D_2T_2 - Peerumedu
D_2T_3 - Devikulam

Table 9 Vanilla mosaic disease index in Kottayam and Idukki districts during rainy season of 2003-04

Sl No.	District	Disease index in taluks			Mean (District)
		T_1	T_2	T_3	
1	D_1	1.512 (1.585)	1.154 (1.468)	2.100 (1.740)	1.573 (1.604)
2	D_2	1.432 (1.560)	1.350 (1.533)	1.900 (1.703)	1.554 (1.598)

Values in the parenthesis are the corresponding transformed means

F value between districts- 0.001 CD between districts- 0.27
F value with in D_1 - 0.48 CD between taluks - 0.473
F value with in D_2 - 0.30

Kottayam (D_1) Idukki (D_2)

D_1T_1- Kanjirapally D_2T_1 - Todupuzha
D_1T_2- Kottayam D_2T_2 - Peerumedu
D_1T_3- Changanacherry D_2T_3 - Devikulam

4.2 SYMPTOMOTOLOGY

The symptom produced by the most commonly occurring vanilla virus disease was studied in the field during the survey in Kottayam and Idukki districts. It was further investigated on vanilla plants in the green house using the infected planting material brought from the field. The disease symptom was characterized by development of chlorotic areas intermingled with green areas which gradually develops to mosaic pattern on leaves. As the disease progresses necrotic spots develop on these areas with leaf crinkling distortion and drastic reduction in leaf lamina, which results in drying of leaves and vines occasionally.

However vanilla plants upon artificial inoculation with infected sap failed to produce any symptom of the disease. When the virus was artificially inoculated on *C. amaranticolor,* which is the local lesion host of the virus, the symptom initially started as chlorotic specks, which later developed into well defined chlorotic spots. Gradually these spots turned brown and necrotic. (Plate No 4)

4.3 TRANSMISSION

4.3.1 Transmission through planting materials

Data on the study on the extent of transmission of vanilla mosaic virus disease through the infected planting material is given in Table 10. It is observed that 100% transmission was obtained when infected cuttings were used as planting material. There was no incidence of the disease when healthy cuttings were used as planting material. Hence the chief mode of transmission of the disease is through infected planting material.

Table.10 Transmission of vanilla mosaic virus disease through infected planting material

	Healthy			Diseased		
Sl No.	No. Planted	No. Infected	Per cent infection	No. Planted	No. Infected	Per cent infection
1	10	0	0	10	10	100

4.3.2 Mechanical transmission

Sap transmission studies on young leaves of vanilla plants using infected sap extracted from naturally infected vanilla plants using four different buffers failed to transmit the virus to young healthy vanilla plants.

Results of the mechanical transmission of vanilla mosaic virus to *C. amaranticolor* using 4 different buffers are presented in Table 11. The results indicated that when citrate buffer (pH6.2) was used for transmission, 10 out of 15 leaves inoculated with the virus showed typical chlorotic spots characteristic to the local lesion symptom of the virus with an average of 3.3 local lesions. Transmission study using tris buffer (pH 7.2) resulted in the development of local lesion symptom on 9 leaves out of 15 with an average of 2.08 local lesions on the tested leaves. When sodium phosphate buffer (pH 7.2) was used for the transmission all the inoculated leaves exhibited intense chlorotic spotting. On the infected leaves an average of 8.5 local lesions were developed. In the case of sodium borate buffer (pH 8.0) five leaves were infected out of 15 and showed clear local lesion symptoms of the virus. The average no of local lesions per inoculated leaves was 2.2

Among the different buffers used 0.1 M sodium phosphate buffer pH (7.2) gave more efficient transmission with an average of 8.5 local lesions and hence selected for all subsequent studies.

4.3.3 Insect transmission

The result of insect transmission studies using *A. gossypii, A. craccivora* and *B. tabaci* are presented in Table.12. None of the insects tested were able to transmit the vanilla mosaic virus on healthy vanilla test plants.

4.3.4 Graft and bud transmission

Although graft union was not established it was observed that through wedge grafting a per cent transmission of 46 and through approach grafting a per cent transmission of 33.3 was observed for vanilla mosaic virus to healthy vanilla test plants. (Table13)

Table.11 Mechanical transmission of vanilla mosaic virus using different buffers

Sl. No	Buffer used	No. of leaves inoculated	No. of leaves infected	Average no. of local lesions
1	Citrate buffer	15	10	3.3
2	Tris buffer	15	9	2.08
3	Phosphate buffer	15	15	8.5
4	Borate buffer	15	5	2.2

Table.12 Transmission of vanilla mosaic virus using different insects

Sl. No.	Name of the insect	No. of plants inoculated	No. of plants infected	Per cent transmission
1	*A. gossypii*	10	0	0
2	*A. craccivora*	10	0	0
3	*B. tabaci*	10	0	0

Similarly bud union also failed to establish on vanilla plants. But the virus could be transmitted through budding using leaf axil as bud of infected plants on healthy vanilla test plants. A per cent transmission of 26.6 was obtained for bud transmission studies. (Table 13).

Table .13 Graft and Bud transmission of vanilla mosaic virus

Sl.No.	Type	No. of plants tested	No. of plants infected	Per cent transmission
1	Wedge grafting	15	7	46.0
2	Approach grafting	15	5	33.3
3	Budding	15	4	26.6

4.4 PHYSICAL PROPERTIES

4.4.1 Dilution end point (DEP)

The data on the study of determining DEP of the virus indicated that infectivity of the sap was retained up to 10^{-4} dilution. (Table 14). When 10^{-4} dilution was used, 6 out of 10 leaves showed clear local lesions while no test plant was infected when 10^{-5} dilution was used for inoculation. 10^{-3} dilution gave local lesions on all the leaves inoculated with an average of 6.6 local lesions. The average number of local lesion per leaf inoculated were highest (8)at minimum dilutions which was found to be gradually reduced to zero as the sap was serially diluted.

4.4.2 Thermal inactivation point. (TIP)

Study on the TIP of the virus showed that the virus retained its infectivity up to 60°C with an average of 1.2 local lesions on inoculated leaves. The sap lost complete infectivity when it was subjected to treatment at 65°C. Up to 50 °C an average of 6.6 local lesions could be obtained beyond that it was gradually reduced and become zero at 65 °C. Similarly at lower temperatures (30°C) the average number of local lesions per leaf were higher (8.2) which was decreased to zero as the temperature to which the virus exposed was increased. (Table 15).

Table 14 Dilution end point of vanilla mosaic virus

Dilution	No. of leaves inoculated	No. of leaves infected	Average no. of local lesions
10^{-1}	10	10	8
10^{-2}	10	10	7.6
10^{-3}	10	10	6.6
10^{-4}	10	6	1.6
10^{-5}	10	0	0
10^{-6}	10	0	0

Table.15 Thermal inactivation point of vanilla mosaic virus

Temperature °C	No. of leaves inoculated	No. of leaves infected	Average no. of local lesions
30	10	10	8.2
35	10	10	7.9
40	10	10	7.9
45	10	10	7.5
50	10	10	6.6
55	10	8	4.3
60	10	5	1.2
65	10	0	0
70	10	0	0
75	10	0	0

4.4.3 Longevity *in vitro*

The results of the study on longevity *in vitro* of vanilla mosaic virus at room temperature and refrigerated conditions are presented in Tables 16 and 17.

It was observed that there was no loss of infectivity when the infected sap was immediately used for inoculation at room temperature. Average of 8.1 local lesions were noted on test plants. On 24 hours of storage at room temperature the infectivity was reduced giving an average of 0.7 local lesions on inoculated leaves. On 48 hours of storage at room temperature the infectivity of the virus was completely lost. The average number of local lesions per leaf was also found to be reduced from 8.1 of fresh sap to 0.7 at 24 hours.

Upon storage under refrigerated conditions the infectivity of the sap was retained up to 48 hours and an average of 0.7 local lesions were obtained. At 72 hours of storage in refrigerated conditions the sap lost its infectivity completely.

4.5 HOST RANGE

Out of the 12 species of plants tested for studying the host range of vanilla mosaic virus, only two species gave positive results out of which *N. glutinosa* produced systemic type of symptoms and *C. amaranticolor* exhibited local lesion symptoms. (Table 18, Plate 4 and 5)

On *N. glutinosa* the initial symptom expression was light chlorosis followed with slight leaf crinkling and inward cupping of leaves. The local lesion symptom on *C. amaranticolor* developed initially as light chlorotic specks, which gradually turned yellow round spots, and later became necrotic.

Plate No. 4
Systemic Infection of Vanilla Mosaic Virus on *Nicotiana glutinosa*

Plate No. 5
Local lesion symptom of Vanilla Mosaic Virus Disease on *Chenopodium amaranticolor*

Table.16 Longevity *in vitro* of vanilla mosaic virus at room temperature (30 °C)

Time in Hours	No. of leaves inoculated	No. of leaves infected	Average no. of local lesions
0	10	10	8.1
24	10	5	0.7
48	10	0	0
72	10	0	0
96	10	0	0
120	10	0	0

Table.17 Longevity *in vitro* of vanilla mosaic virus at refrigerated condition (10 °C)

Time in hours	No. of leaves inoculated	No. of leaves infected	Average no. of local lesions
0	10	10	8.1
24	10	10	2.8
48	10	6	0.7
72	10	0	0
96	10	0	0
120	10	0	0

Table.18 Host range of vanilla mosaic virus

Family and Plant	Symptoms	
	Local lesion	Systemic
Amaranthaceae-*Gomphrena globosa*	Nil	Nil
Apocyanaceae- *Catharanthus roseus*	Nil	Nil
Chenopodiaceae-		
Chenopodium amaranticolor	Local lesion	Nil
Chenopodium quinoa	Nil	Nil
Solanaceae-		
Datura stramonium	Nil	Nil
Datura metel	Nil	Nil
Nicotiana glutinosa	Nil	Systemic
Nicotiana benthamiana	Nil	Nil
Nicotiana tabacum	Nil	Nil
Capsicum annum	Nil	Nil
Lycopersicon esculentum	Nil	Nil
Orchidaceae-*Dendrobium spp.*	Nil	Nil

4.6 PATHOPHYSIOLOGY

4.6.1.Total chlorophyll

Results of the analysis of plant sample for determining the chlorophyll content in vanilla mosaic virus infected and healthy vanilla plants are shown in Table19. It was observed that the total chlorophyll content was greatly reduced in infected vanilla as compared to healthy ones. The mean total chlorophyll content was 0.939mg/g leaf tissue in infected plant as against 1.25mg/g in healthy plants. Similar trend was observed when the plant samples were analyased for chlorophyll a and b. Mean chlorophyll a content was only 0.856 mg/g of leaf tissue as compared to 1.053 mg/g of leaf tissue in healthy plants. Chlorophyll b content also gave a similar trend as it was only 0.083 mg/g of leaf tissue in infected as compared to 0.197 mg/g of leaf tissue in healthy.

Table.19 Changes in chlorophyll content of vanilla leaves (Expressed as mg/g of leaf tissue) in response to vanilla mosaic virus infection.

	Total chlorophyll		Chlorophyll a		Chlorophyll b	
Sl No.	Healthy	Infected	Healthy	Infected	Healthy	Infected
1	1.24	0.937	1.048	0.855	0.192	0.082
2	1.27	0.941	1.063	0.857	0.207	0.084
3	1.23	0.935	1.043	0.854	0.187	0.081
4	1.26	0.943	1.058	0.858	0.202	0.085
Mean	1.25	0.939	1.053	0.856	0.197	0.083

4.6.2.Total phenols

The mean total phenol content on healthy vanilla leaves was 9.5 µg/g leaf tissue while it was found to be increased to 13.0 µg/g leaf tissue on infected leaf samples. (Table 20)

Table.20 Changes in total phenol content of vanilla leaves (Expressed as μg/g leaf tissue) in response to vanilla mosaic virus infection.

Sl.No	Healthy	Infected
1	9.6	13.2
2	9.3	12.9
3	9.5	13.1
4	9.6	12.8
Mean	9.5	13.0

4.6.3.Total carbohydrates

The data on the analysis of total carbohydrate content in vanilla mosaic virus infected and healthy plants are shown in Table 21. It was observed that mean total carbohydrate content was 23mg/g leaf tissue in healthy plants which was greatly reduced (12 mg/g leaf tissue) in infected plants.

Table.21 Changes in total carbohydrate content of vanilla leaves (Expressed as mg/g leaf tissue) in response to vanilla mosaic virus infection.

Sl.No	Healthy	Infected
1	23.4	12.3
2	22.8	11.7
3	23.0	12.1
4	22.8	11.9
Mean	23.0	12.0

4.6.4.Total proteins

Analysis of leaf samples for the total soluble protein content indicated that there was drastic increase in the protein content from 11.5 mg/g of healthy leaf sample to 21.0 mg/g of infected leaf sample (Table.22)

Table.22 Changes in total protein content of vanilla leaves (Expressed as mg/g leaf tissue) in response to vanilla mosaic virus infection.

Sl.No	Healthy	Infected
1	11.8	21.3
2	11.5	21.0
3	11.2	20.8
4	11.5	20.9
Mean	11.5	21.0

4.6.5.Defense related enzymes

4.6.5.1 *Peroxidase (PO) activity*

The peroxidase enzyme activity in healthy and vanilla mosaic disease infected vanilla leaf samples was analyzed and the result is presented in Table 23. The change in enzyme activity observed in diseased samples was 0.048 as compared to 0.031 in healthy samples. The difference in change is 1.6 times higher in diseased samples than in healthy ones.

Table.23 Changes in peroxidase activity of vanilla plant (Expressed as change in optical density value/gram/minute) in response to vanilla mosaic virus infection.

Sl.No	Healthy	Infected
1	0.032	0.050
2	0.031	0.048
3	0.029	0.047
4	0.031	0.047
Mean	0.031	0.048

4.6.5.2. *Polyphenol oxidase (PPO) activity*

The change in polyphenol oxidase activity in healthy and vanilla mosaic disease affected samples is illustrated in Table 24. It was observed that the change in enzyme activity in diseased samples was 0.21 while that of healthy plants was 0.066. The difference in change is 3.5 times higher in diseased samples as compared to healthy ones.

Table.24 Changes in polyphenol oxidase activity of vanilla plant (Expressed as change in optical density value/gram/minute) in response to vanilla mosaic virus infection.

Sl.No	Healthy	Infected
1	0.069	0.21
2	0.066	0.19
3	0.062	0.22
4	0.067	0.22
Mean	0.066	0.21

4.6.5.3 *Phenylalanine ammonia lyase (PAL) activity*

Phenylalanine ammonia lyase activity was found to be increased to 8.3 µg/g leaf tissue in infected sample as compared to 4.0 µg/g leaf tissue of healthy leaves of vanilla. (Table 25)

Table.25 Changes in phenylalanine ammonia lyase activity of vanilla plant(Expressed as µg/g of leaf sample) in response to vanilla mosaic virus infection.

Sl.No	Healthy	Infected
1	4.3	8.5
2	3.9	8.3
3	3.7	8.1
4	4.1	8.3
Mean	4.0	8.3

4.7 CHARACTERIZATION OF VANILLA MOSAIC VIRUS

4.7.1 Electrophoretic analysis of protein by SDS-PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis.)

The Electrophoretic analysis of protein by SDS-PAGE of healthy and diseased leaf samples of vanilla indicated that a single major poly peptide band of molecular weight 37KDa was observed in diseased sample in addition to the identical bands observed in healthy and diseased leaf samples of vanilla. (Plate No. 6)

4.7.2 Purification of vanilla mosaic virus

Partial purification of vanilla mosaic virus was successfully done as per the procedure of Khurana *et al.* (1987). The virus was purified from the young leaves collected from the vanilla mosaic virus infected plants maintained in the glass house, which was used for inoculating rabbit for antiserum production.

4.7.3 Production of antiserum

Antiserum was produced in New Zealand white rabbit against vanilla mosaic virus by giving four intra muscular injections at weekly intervals with the partially purified virus with an equal volume of Freunds incomplete adjuvant. Antiserum was separated from the blood and used for immuno diagnosis.

4.7.3.1 *Determination of antibody titre.*

The leaf samples from infected plants gave positive reactions with antiserum produced against vanilla mosaic virus in all dilutions tested. In all non cross absorbed dilutions the antisera gave positive reaction but as there was interference with healthy protein the absorbance value of diseased samples was not distinguishable from healthy samples. In 1:250 dilution which was non cross absorbed the absorbance value for healthy is 1.94 and for diseased was 2.21. The best dilution for the detection of the virus was 1:500, which was cross-absorbed which gave an absorbance value of 0.158 for healthy and 0.856 for diseased sample. The absorbance value for diseased is 5.4 times as that of healthy sample. So 1:500 dilution which wascross absorbed gave more positive accurate result. (Table 26) (Plate No.7)

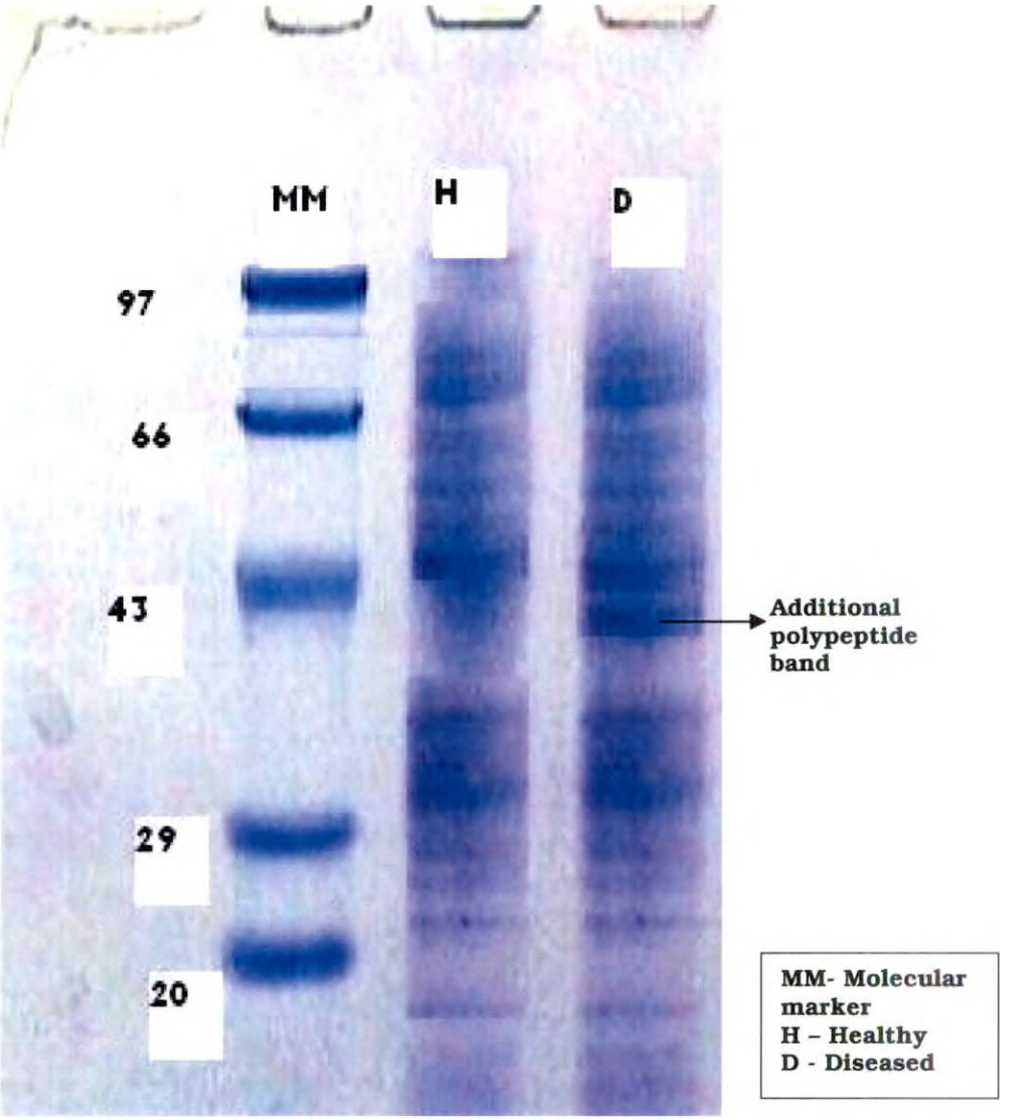


Plate No. 6

SDS – PAGE analysis of healthy as well as vanilla mosaic virus infected samples

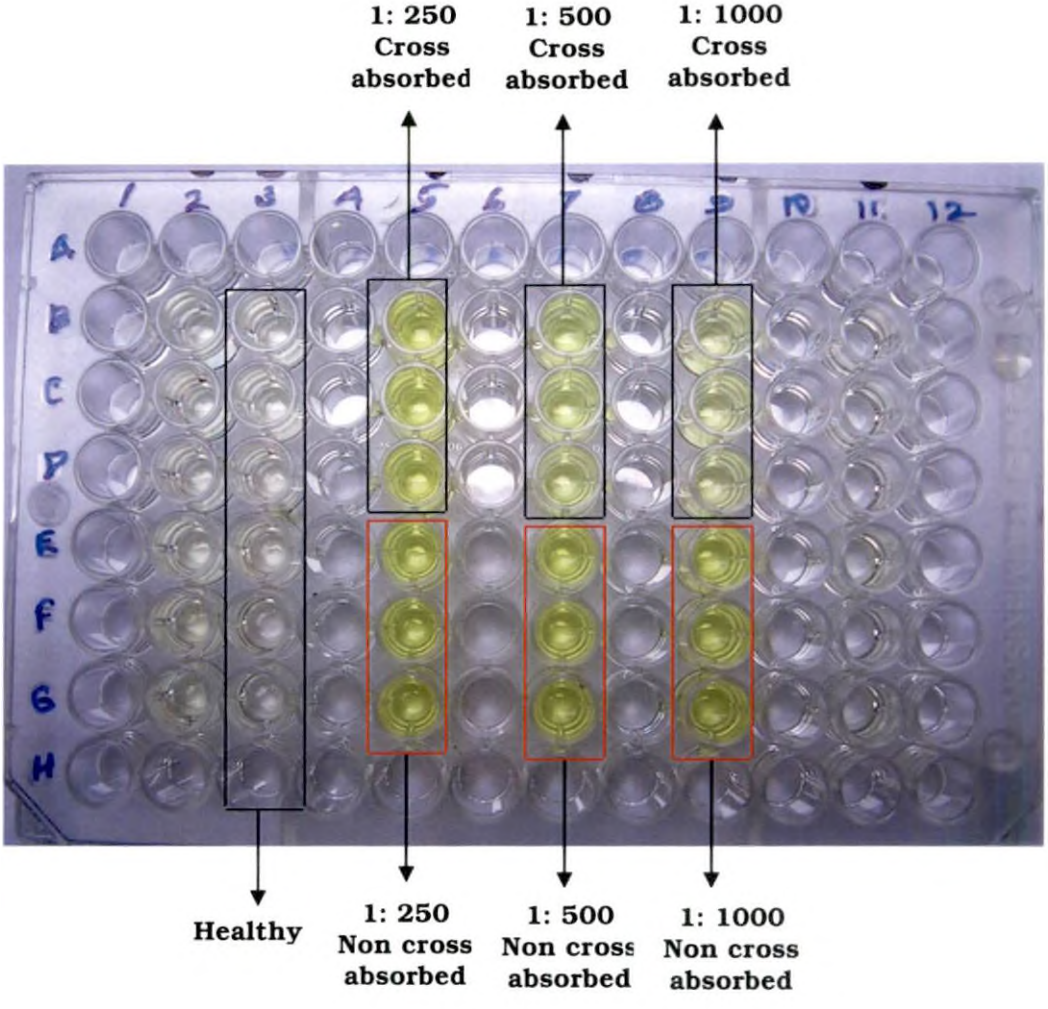


Plate No. 7
Reaction of Vanilla Mosaic Virus infected as well as Healthy vanilla samples to the developed antiserum in DAC - ELISA

4.7.3.2 *Immunological studies.*

4.7.3.2.1 Microprecipitin test

The test was found to be successful for detection of vanilla virus in clarified sap. The infected plant extract was allowed to react with vanilla mosaic virus specific antiserum raised in rabbit (which was cross absorbed) using micro precipitin test. A white precipitate was obtained on slides where as it was absent for healthy plant sap.

4.7.3.2.2.DAC ELISA- (Direct antigen coating enzyme linked immunosorbent assay)

Antibody produced against partially purified vanilla virus was used for DAC ELISA. Plant sap was prepared from both healthy and infected young vanilla leaves. Titre of the antiserum used was 1:500. The absorbance was measured at 405 nm in an ELISA reader. The result of the experiment revealed that the antiserum developed specific to vanilla mosaic virus gave high reactivity towards the virus isolate. The absorbance value of 0.170 was obtained for healthy sample while it was 0.877 for the infected sample. The reading of the infected sample was 5.2 times more than the reading of healthy sample. (Table 27)

Table.26 Determination of vanilla mosaic virus antibody titre using DAC ELISA.

Treatment		Absorbance at 405 nm		
		1:250	1:500	1:1000
Healthy	Cross absorbed	0.163	0.158	0.155
	Non cross absorbed	1.94	1.81	1.60
Diseased	Cross absorbed	2.07	0.856	0.033
	Non cross absorbed	2.21	2.08	1.96

Table.27 Reaction of vanilla mosaic virus to developed antiserum in DAC ELISA.

Treatment	Absorbance at 405 nm
Healthy	0.170
Diseased	0.877

DISCUSSION

5. DISCUSSION

Cultivation of vanilla is assuming importance in India since it fetched very remunerative price in world market. New areas were brought under cultivation in Kerala in recent years. Widespread occurrence of virus diseases was found to be a major yield limiting factor for this crop. Hence the present investigation was undertaken to identify the incidence of various virus diseases in vanilla and to characterize and diagnose the widely occurring virus disease by undertaking studies on symptomatology, methods of transmission, physical properties, host range and serology.

5.1 SURVEY

Survey conducted to evaluate the extent and severity of incidence of various virus diseases in vanilla identified the occurrence of three major virus diseases in Kerala. These virus diseases were categorized based on symptomatology as (a) those produced Symptom Type No.1 (b) those produced Symptom Type No.2 and (c) those produced Symptom Type No.3.

The survey further showed that the most widely occurring virus disease was the one which produced the Symptom Type No.1 followed by Type2 and Type 3. (Table 5). (Figure 1and 2). The characteristic symptom of the virus which produced Symptom Type No.1 was mosaic with leaf crinkling and hence it was named as Vanilla Mosaic Virus Disease. (VMVD).

The survey also revealed that the extent of vanilla mosaic virus disease incidence was 70% in the plots surveyed in Kottayam district and 76.6 % in the plots surveyed in Idukki district while the incidence of Symptom Type No.2 were 30 and 23.3% and Symptom Type No.3 were 3.3 and 10 % in Kottayam and Idukki districts respectively. Hence the survey clearly showed that the widely prevalent virus disease of vanilla in the areas surveyed in Kerala was vanilla mosaic virus disease (VMVD).

Fig. 1 Incidence of virus diseases of vanilla in Kottayam district during summer 2003-04.

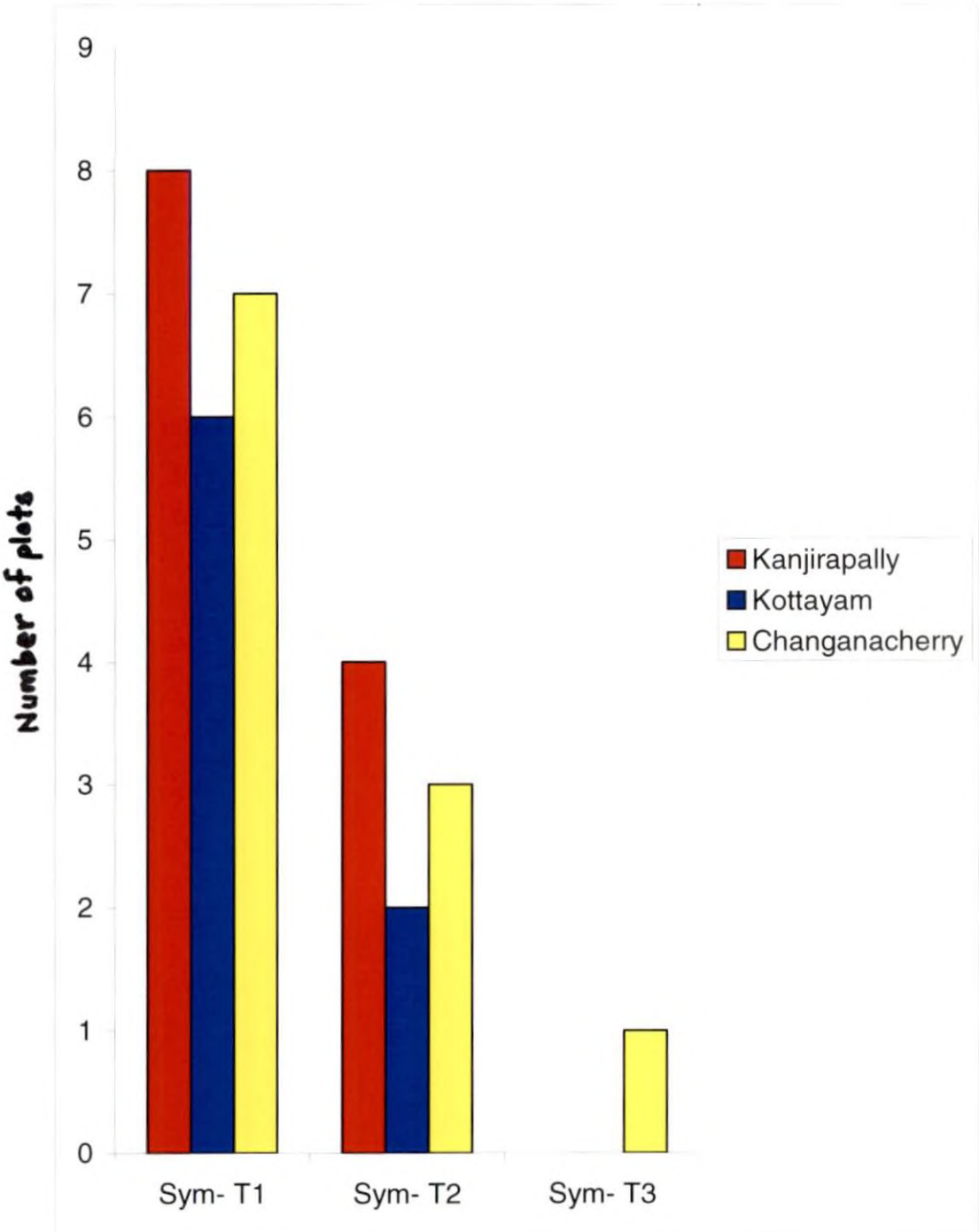
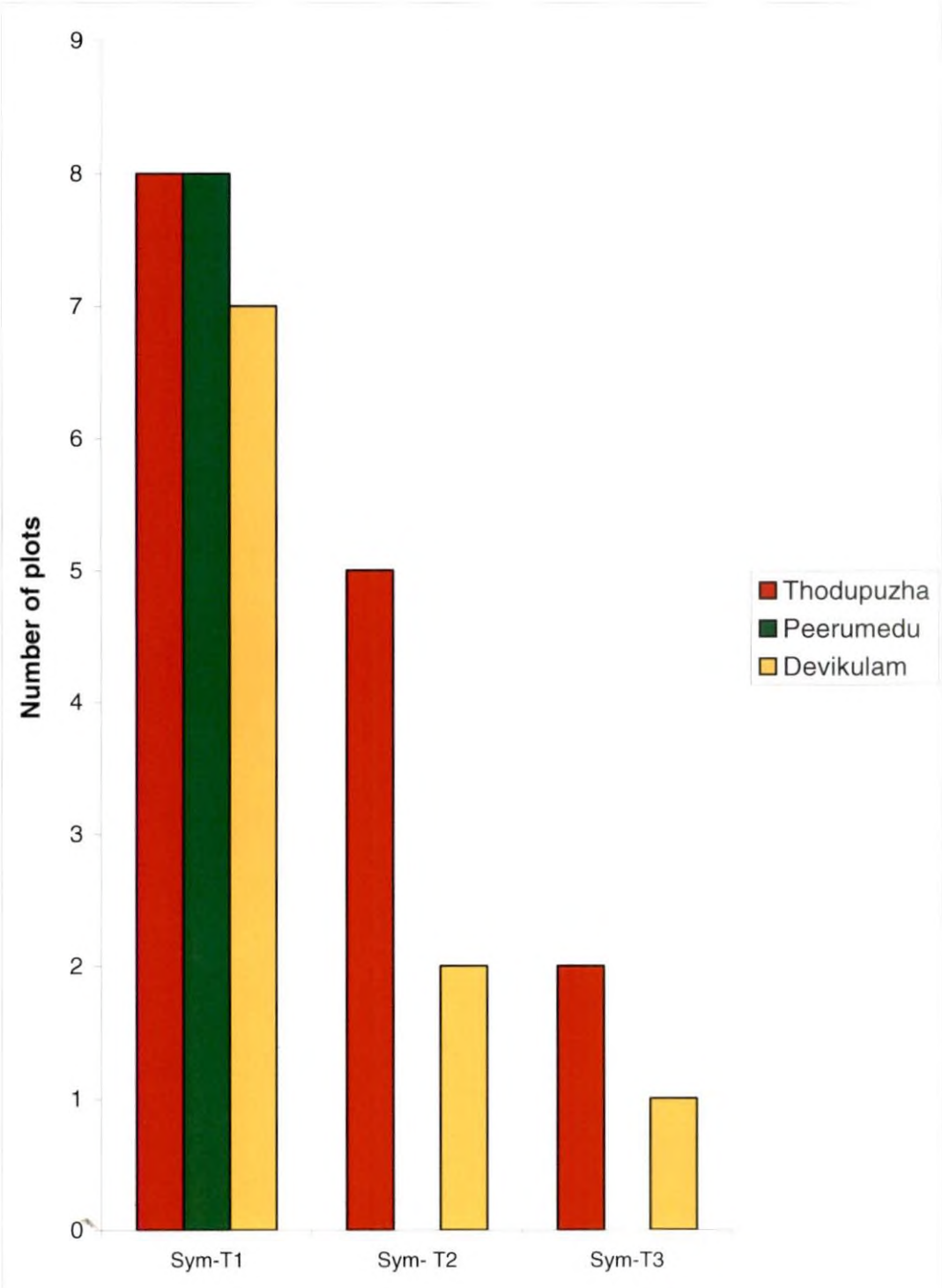


Fig. 2 Incidence of virus diseases of vanilla in Idukki district during summer 2003-04.



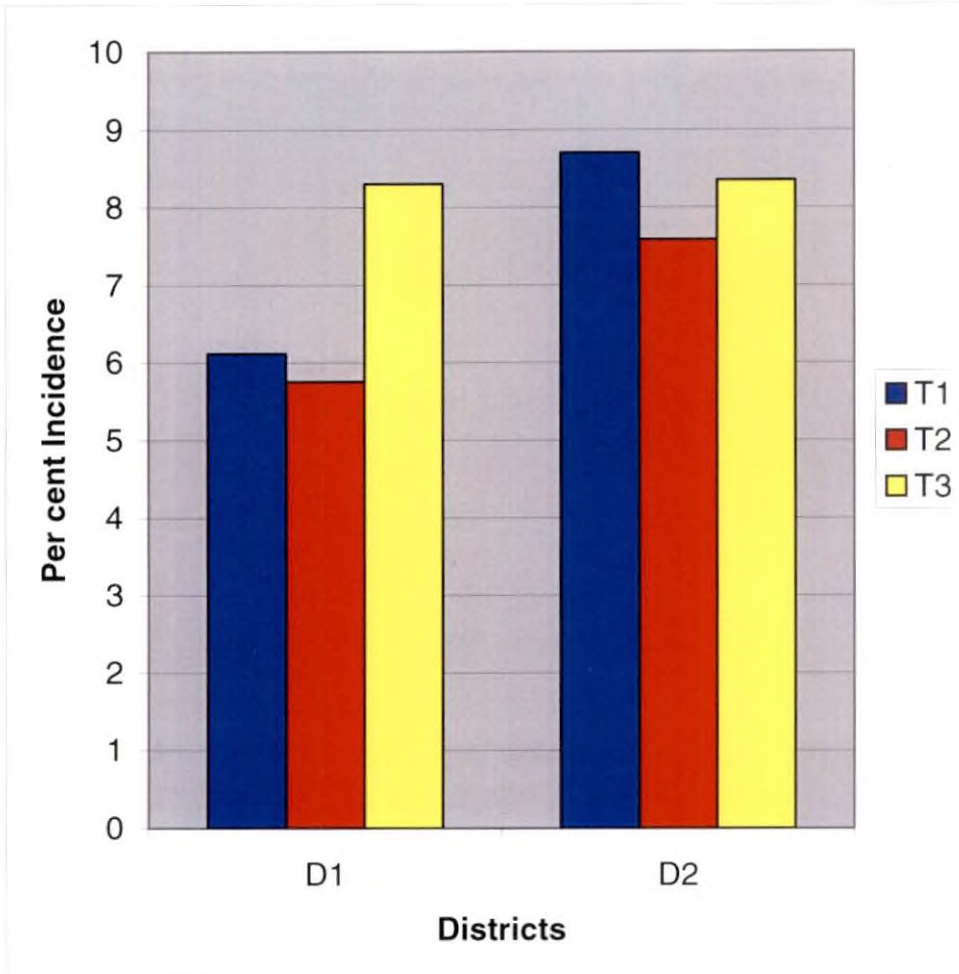
A similar virus disease showing chlorotic spots or streaks parallel to venation on the leaves with wavy margin and reduced leaf size was reported by Thomas (2002) from Kerala. A mosaic disease of vanilla exhibiting mild mosaic or mottling with chlorotic specks or streaks mainly on leaves and rarely on stem with leaf blistering and mild distortion was reported by Sudharshan *et al.* (2003) from Karnataka which is also similar in occurrence and symptomatology to vanilla mosaic virus in the present study.

Results of the survey on the extent of vanilla mosaic virus disease incidence indicated that the mean per cent disease incidence varied from 6.683 in Kottayam district to 8.205 in Idukki district during summer season while it was 5.325 and 4.900 respectively during the rainy season. (Table 6 and 7; Figure 3 and 4) The study clearly indicated that the vanilla mosaic virus disease incidence is prevalent in both the districts even though the extent of incidence in the areas surveyed were less than 10%. The present study further showed that the per cent disease incidence showed a declining trend during rainy season in both the districts but the reduction was greater (59.7%) in Idukki district. Evaluation of disease index which is a measure of disease severity indicated that the mean disease index varied from 2.976 to 2.553 during summer season and from 1.554 to 1.573 during rainy season in Idukki and Kottayam districts respectively.(Table 8 and 9 ; Figure 5 and 6). The study showed that the disease index is comparatively low in both the districts even though the observed per cent disease incidence were slightly higher. Similar to per cent disease incidence disease index also showed a declining trend during the rainy season.

A detailed survey conducted by Pone (1988) indicated that vanilla viral infection was as high as 50% in some fields in Tonga.

The survey conducted by Bhat *et al.* (2003) in Kerala and Karnataka also showed similar results. They reported the occurrence of vanilla virus disease in both the states and varied from a mean per cent incidence ranging from 0-10 with high per cent incidence of 60 % in few gardens surveyed. Another survey conducted by Bhat *et al.* (2004a) in India reported the occurrence of vanilla mosaic disease ranging from 0-5%.

Fig 3. Per cent incidence of vanilla mosaic virus in Kottayam and Idukki districts during summer season of 2003-04



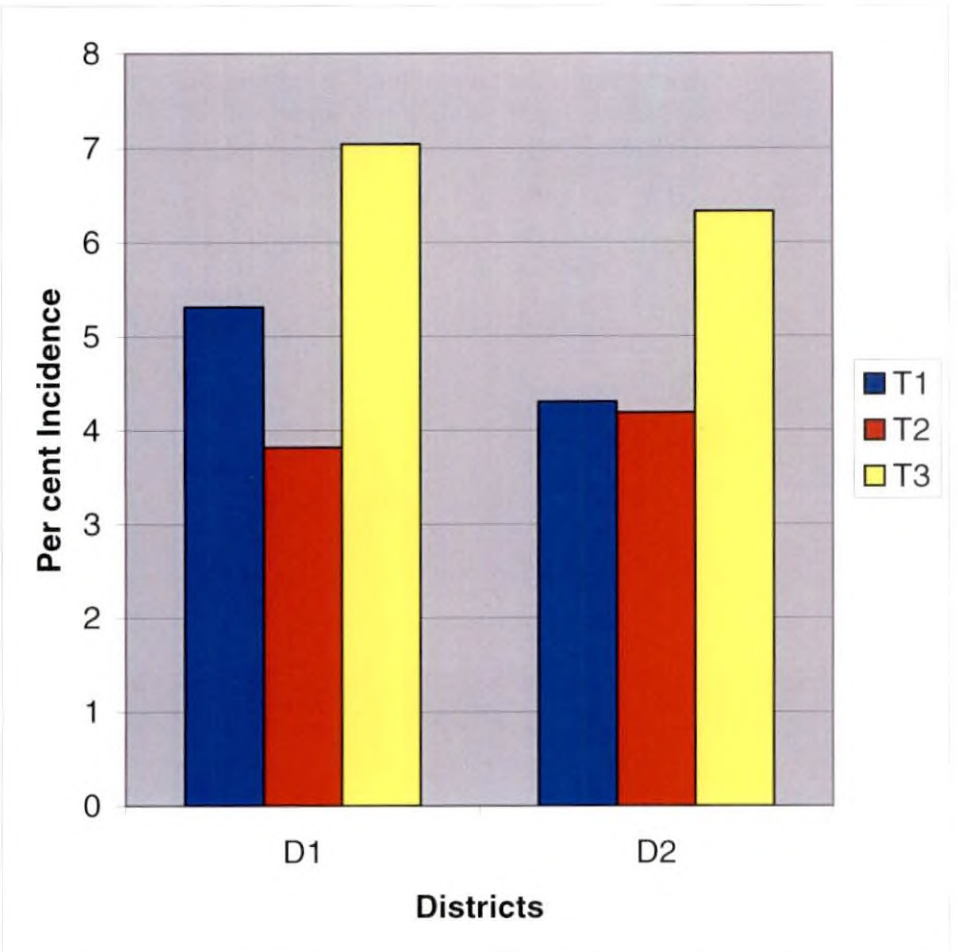
Kottayam (D_1)

D_1T_1- Kanjirapally
D_1T_2- Kottayam
D_1T_3- Changanacherry

Idukki (D_2)

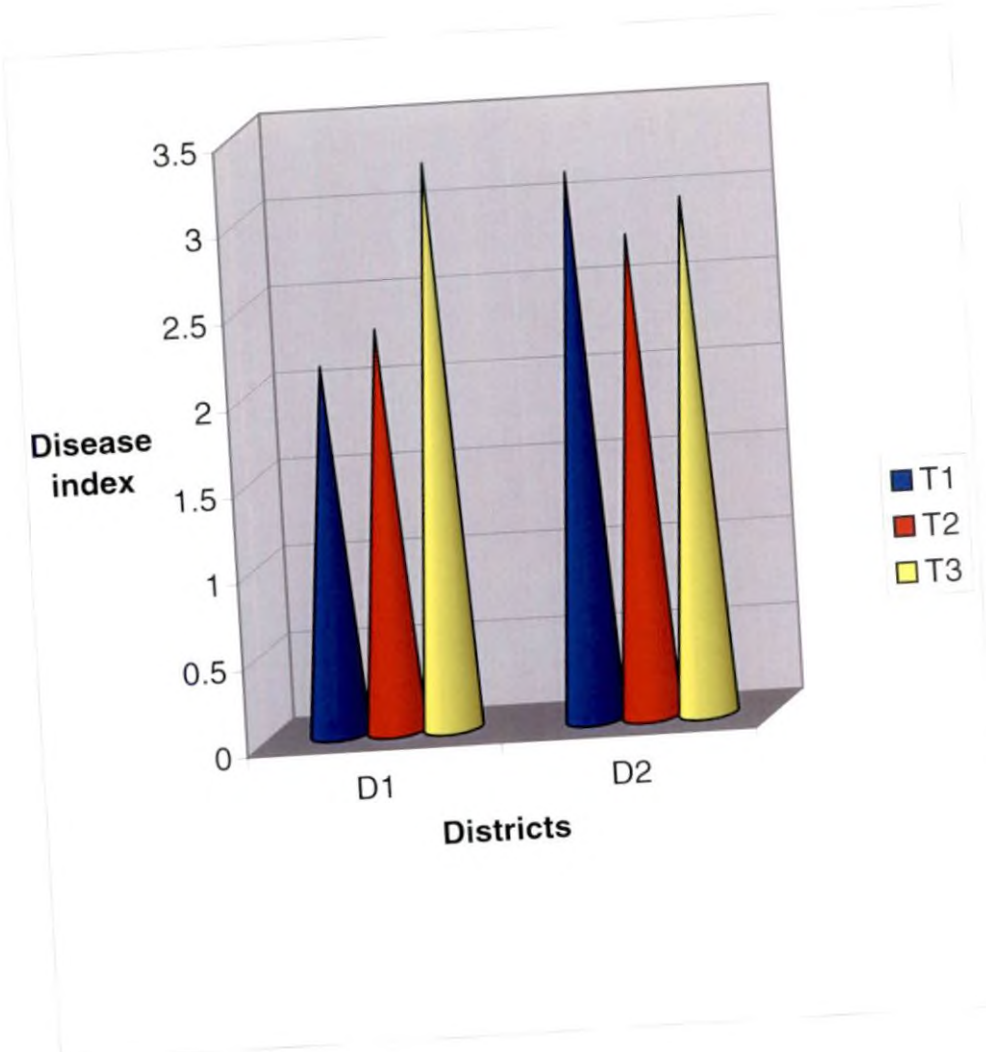
D_2T_1 - Todupuzha
D_2T_2 - Peerumedu
D_2T_3 - Devikulam

Fig 4. Per cent incidence of vanilla mosaic virus in Kottayam and Idukki districts during rainy season of 2003-04



Kottayam (D_1)	Idukki (D_2)
D_1T_1- Kanjirapally	D_2T_1 - Todupuzha
D_1T_2- Kottayam	D_2T_2 - Peerumedu
D_1T_3- Changanacherry	D_2T_3 - Devikulam

Fig 5 Vanilla mosaic disease index in Kottayam and Idukki districts during summer season of 2003-04



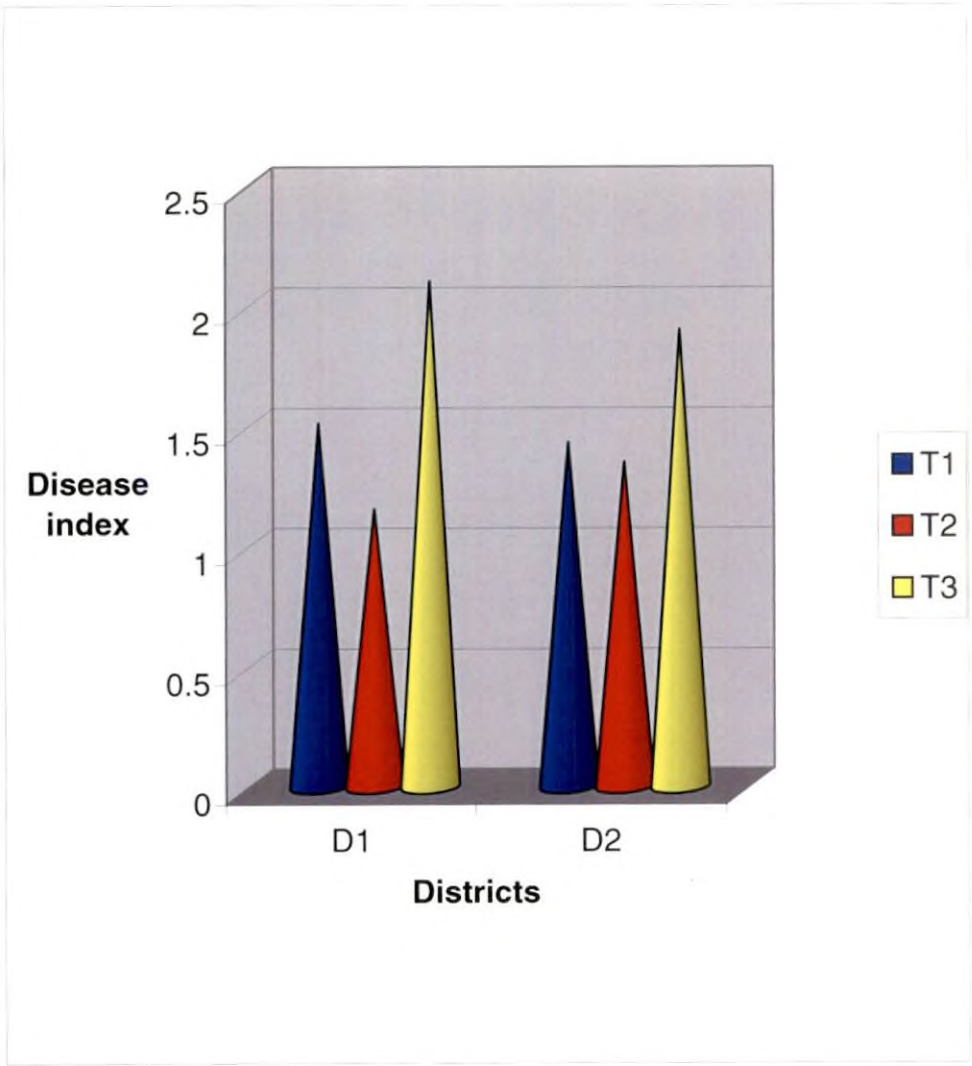
Kottayam (D_1)

D_1T_1- Kanjirapally
D_1T_2- Kottayam
D_1T_3- Changanacherry

Idukki (D_2)

D_2T_1 - Todupuzha
D_2T_2 - Peerumedu
D_2T_3 - Devikulam

Fig 6 Vanilla mosaic disease index in Kottayam and Idukki districts during rainy season of 2003-04



Kottayam (D_1)

D_1T_1- Kanjirapally
D_1T_2- Kottayam
D_1T_3- Changanacherry

Idukki (D_2)

D_2T_1 - Todupuzha
D_2T_2 - Peerumedu
D_2T_3 - Devikulam

The results of the survey indicated that the per cent vanilla mosaic disease incidence and disease index are at low level in both the districts. It may be noted that the survey was conducted at a time when more areas were brought under vanilla cultivation aggressively with high expectation of income generation. So it may be possible that even though the present level of vanilla mosaic virus incidence is at low ebb, it may assume serious nature in the years to come. Hence the survey points to urgent eradication of the virus infected vanilla plants in infected gardens.

5.2 SYMPTOMATOLOGY

The symptoms produced by the vanilla mosaic virus disease in the field comprised of development of mosaic pattern on leaves in the form of irregular chlorotic areas intermingled with green areas. Gradually necrotic spots developed on these areas with leaf crinkling, distortion and leaf size reduction. A *potyvirus* causing mosaic and severe malformation of leaves was described on *V. tahitensis* and *V. pompona* in French Polynesia by Wisler *et al.* (1987) and was tentatively named as vanilla mosaic virus (VanMV).

Incidence of a similar mosaic virus disease was reported from India by Sudharshan *et al.* (2003). He described the symptoms as appearance of mild mosaic mottling with chlorotic spots or streaks mostly on leaves and rarely on stem, in association with leaf blistering and mild distortion. The mosaic symptoms produced by the vanilla mosaic virus in the present study is similar to those mentioned above. However development of necrotic spots in the affected areas is quite distinct which show similarity to vanilla necrosis virus (VNV) reported by Pearson and Pone (1988) and Pearson *et al.* (1993). Hence further elaborate study is required for determining the relationship of vanilla mosaic virus disease to those already been reported.

5.3 TRANSMISSION

When diseased vanilla cuttings were used as planting material 100 per cent transmission was obtained. Hence the chief mode of spread of the disease in field is through the use of infected planting materials which was reported to be the

principal mode of transmission of all the viruses reported to occur in vanilla. (Bhat *et al.,* 2003).

The study on the sap transmissibility of the virus using four different buffers indicated that the virus could not be mechanically transmitted to young vanilla plants. Wisler *et al.* (1987) were unable to mechanically transmit VanMV to nine plant species including *V. pompona.* Hence it is believed that vanilla mosaic virus in the present study may be related to VanMV described by Wisler *et al.* (1987). However the virus could be transmitted successfully to *C. amaranticolor,* which produced characteristic chlorotic local lesions on the inoculated leaves.

Among the four different buffers used for transmission to *C. amaranticolor,* maximum transmission with intense local lesion (average of 8.5 local lesions) was obtained with 0.1 M sodium phosphate buffer (pH7.2). (Table.11) *C. amaranticolor* was recognized as a standard local lesion host for many of the plant viruses. It produced distinct local lesion symptoms when inoculated with other vanilla mosaic virus diseases such as VNV, by Pearson *et al.* (1990) and CMV by Madhubala *et al.* (2005).

Since the virus was not transmissible to vanilla plants by sap transmission, but produced distinct local lesions on *C. amaranticolor,* this plant was used as the host for maintenance of virus culture and all subsequent studies.

None of the insects tested (*A. gossypii, A. craccivora* and *B. tabaci.*) were able to transmit the virus to vanilla test plants in spite of repeated attempts. Attempts to transmit Tongan isolate of vanilla mosaic virus from *V. fragrans* to *N. clevelandii* with *A. gossypii* and *M. persicae* and the French Polynesian isolate from *V. pompona* to *N. clevelandii* with *M. persicae* were unsuccessful due to the aphids failing to probe tough vanilla leaves. (Pearson *et al.,* 1990). Vanilla is not a natural host of aphids and white flies and generally do not feed on the plant. In addition considering the thick cuticular surface of vanilla leaves the extent of aphid transmission of vanilla mosaic virus may be very low under field condition. Moreover reports on experimental transmission of vanilla virus disease by insects

are very sparse. However in few cases such as transmission of vanilla *potyvirus* from infected to healthy *N. clevelandii* seedlings by *A. gossypii* and *M. persicae* has also been reported. (Pearson *et al.*,1990).

Although successful graft or bud union could not be established between healthy and infected vanilla plants it was observed that vanilla mosaic virus was transmitted to healthy plants by wedge grafting (46%), approach grafting (33.3%) and budding (26.6%) (Table13). Such experimental transmission of virus diseases by grafting and budding have been demonstrated in several viral diseases up on contact of sap between healthy and diseased portions of test plants even though union could not be established. Sastry *et al.* (1978) reported the successful transmission of banana bunchy top virus by core grafting under laboratory codition. Bhat *et al.* (2003) reported that some viruses infecting vanilla such as *Tobamovirus* and *Potexvirus* could be transmitted through contact between plants, cutting knives and other implements used in agricultural operations. This observation highlighted the significance of utmost caution to be taken while pruning and pollination activities which are routine practices followed in vanilla plantations.

5.4 PHYSICAL PROPERTIES

Study on the physical properties of the vanilla mosaic virus revealed that the virus has a dilution end point between10^{-4} and10^{-5}, thermal inactivation point between 60 and 65 °C, longevity *in vitro* at room temperature between 24 and 48 hours and at refrigerated conditions between 48 hours and 72 hours. It was also observed that progressive increase in dilution and temperature and prolonged storage tended to reduce the per cent infectivity of the virus progressively. Information on the physical properties of virus infecting vanilla is at present lacking and hence no comparison could be made with any of the previously reported vanilla virus disease. But the vanilla mosaic virus in the present study is showing similarity in TIP and LIV to *Cucumis virus* 1 reported by Johnson and Grant (1932). It is also showing similarity in TIP and DEP to CMV reported by Shawkat and Felga (1979).

It can be assumed from the physical properties of the virus that it is a moderately stable virus under different physical conditions. (Table 14,15,16,17)

5.5 HOST RANGE

Among the 12 different plant species belonging to four different families tested for the host range of vanilla mosaic virus only *N. glutinosa* produced systemic type of infection and *C. amaranticolor* exhibited local lesion symptoms. The symptoms produced on *N. glutinosa* were mild chlorosis, slight leaf crinkling and inward cupping on leaves, while the local lesions on *C. amaranticolor* were very distinctly chlorotic initially and later become necrotic. (Plate No.4 and 5)

The unusually narrow host range of the virus under study indicated that it may be a strain of VNV reported by Pearson *et al.* (1990) which have been reported to have narrow host range. This inference require further confirmation since the virus resembled VanMV in terms of symptomatology and transmission and resembled VNV on host range.

5.6 PATHOPHYSIOLOGY

Physiological changes in vanilla infected with vanilla mosaic virus was studied and the changes in the content of total chlorophyll, total carbohydrates, total protein, total phenols and activity of defense related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase were analyzed.

In the present study the content of total chlorophyll, chlorophyll a and chlorophyll b were found to be reduced as a result of vanilla mosaic virus infection. The reduction in chlorophyll content upon virus attack is due to the diversion of plastid proteins into virus reported by Bawden (1954) or due to normal cell enzymes that attack chlorophyll (Goodman *et al.* 1967). Reduction in total chlorophyll, chlorophyll a and chlorophyll b due to virus infection was reported by several workers in other crops (Johri and Padhi, 1985 ; Shukla *et al.*, 1992 ; Sarma *et al.*, 1995 ; Dantre *et al* ., 1996 ;Thind *et al.*, 1996)

Ramiah (1978) and Ahmed *et al.* (1986) found that reduction in chlorophyll content might be due to increased chlorophyllase activity of the virus infected plants.

The content of phenols in plants was found to be related with disease resistance. Increase in total phenols was noted in vanilla mosaic infected plants compared to healthy ones in the present study. Increase in total phenols in infected plants were reported by many scientists. (Srivastava and Tiwari 1998; Sarma *et al.,* 1995). Jeeva (2001) reported that sweet potato feathery mottle virus infection on sweet potato contributed to an increase in the activity of hexose monophosphate (HMP) shunt pathway which produced intermediaries required for synthesis of phenolic compounds. Farkas *et al.* (1960) were of the opinion that the activation of phenol oxidizing enzymes was less in host due to systemic virus infection so that the phenol level is high during infection. Total phenol was reported to be high in virus infected leaves of many plants (Chakraborthy *et al.*, 1994; Sarma *et al.*, 1995; Dantre *et al.*, 1996)

The total carbohydrate content was found to be decreased in vanilla mosaic virus infected plants. Such reduction in virus infected plants was due to retarded photosynthesis and rapid utilization of starch and non reducing sugars as substrates for increased respiration (Goodman *et al.*, 1967). Reduction in carbohydrate content in host plant in response to pathogen attack has also been reported by Goodman *et al.* (1967). Narayanasamy and Ramakrishnan (1966) reported decreased carbohydrate content in pigeon pea sterility mosaic virus infected pigeon pea. According to them this was to provide the substrate for accelerated respiration.

The total protein content of vanilla mosaic infected vanilla plants were found to be higher when compared to healthy ones in the present study. Increased protein content due to virus infection has been reported by Sarma *et al.* (1995). According to Uritani (1971) the increased protein content in virus affected plants might be due to the production of new PR proteins. Investigations on enhanced protein content in virus infected plants were also reported by Singh and Singh

(1984). Mali *et al.* (2000). Reported that the increase in total protein content of virus infected plants might be due to an increase in virus concentration as well as due to production of other non viral proteins during active virus synthesis.

Defense related enzymes are reported to act as important factors in the induction of disease resistance. (Dasgupta ,1988). The changes in the activities of PO, PPO and PAL were high in vanilla mosaic infected vanilla plants compared to healthy ones. The increased peroxidase (PO) activity was due to enhanced respiration during infection (Pantanelli ,1912). Farkas *et al.* (1960) reported an increased in PPO content in virus infected plants with necrotic lesions. Since there was necrotic lesions associated with vanilla mosaic virus disease, this may be due to increased PPO activity. Goodwin and Mercer (1972) found that the enzyme PAL served as the precursor for synthesis of phenols. Since vanilla mosaic infected plants contained more amount of phenol, the increased level of PAL might be the response of vanilla plants to vanilla mosaic virus infection. Similar results were obtained by Zaid *et al.* (1992) in Carnation etched ring virus and found that the substantial increase in the level of PAL activity along with increased phenolic content in response to infection showed good correlation and suggest that the virus infection has altered the phenyl propanoid pathway which lead to the accumulation of phenols. Gomathi *et al.* (1993) reported the increased activity of PO, PPO and PAL enzyme activity in banana infected with banana streak mosaic virus.

5.7 CHARACTERIZATION OF THE VIRUS

Electrophoretic analysis of virus proteins by SDS- PAGE revealed that the infected samples contained an additional major polypeptide band of molecular weight 37 KDa in addition to the identical bands observed in healthy and diseased leaf samples. Wang and Pearson (1992) purified vanilla mosaic virus from *V.tahitensis* and in SDS- PAGE it produced a single major polypeptide band of 34 KDa. Pearson *et al.* (1990) also done SDS PAGE for the vanilla necrosis virus and found that the *potyvirus* produced a single major polypeptide band of 32.7 KDa. In the present study the virus is showing similarities to the above mentioned

potyviruses since the polypeptide band was found to have a molecular mass of 37 KDa.

Partial purification of vanilla mosaic virus was done as per the procedure of Khurana *et al.* (1987). Attempts to purify virus infecting vanilla such as vanilla mosaic virus (VanMV) (Wang and Pearson, 1992), vanilla necrosis virus (VNV) (Pearson *et al.*, 1990) and Cucumber mosaic virus (CMV) (Farreyrol *et al.*, 2001) were successfully carried out. However in the present study the virus could only be partially purified because vanilla contains considerable amount of mucilage and phenolic compounds, which interferes with the virus extraction and purification. The partially purified virus was used for the production of antiserum in New Zealand White rabbit.

Antiserum was successfully produced against vanilla mosaic virus using the partially purified virus preparation in New Zealand white rabbit. vanilla mosaic virus antiserum was produced in a similar manner by Pearson *et al.* (1990) and Wang and Pearson (1992).

The titre of the antiserum was evaluated and found to be between 1:500 and 1:1000. (Table 26). Perusal of literature did not reveal such studies on vanilla mosaic virus. Pearson *et al.* (1990) produced antiserum against vanilla mosaic *potyvirus*, which has a homologous titre of 1:1024. Grisoni *et al.* (2004) used 1:1000 dilution of polyclonal antibody for detection of *potyviruses* in *V. tahitensis*.

Use of disease free planting materials is a primary procedure for reducing the incidence of virus diseases in cultivated crops. Proper diagnostic techniques for the detection of invisible pathogens such as viruses using serological tests is a key practice in vegetatively propagated crops. In the present study a rapid and easy serological diagnostic test employing the microprecipitin test was done. A clear white precipitate was formed when the antiserum was mixed with virus infected plant sap, which can be routinely practiced for rapid detection of the virus in planting material.

ELISA is one of the quickest and reliable techniques to detect and characterize a virus. In the present study DAC-ELISA was conducted using polyclonal antibodies developed against partially purified vanilla mosaic virus. The antiserum developed gave good results at 1:500 dilutions, which was cross-absorbed. In DAC- ELISA the absorbance value at 405nm.were higher for infected plants (0.877) compared to healthy one (0.170). (Table 27). This was in conformity with the findings of Wang and Pearson (1992). They observed a mean absorbance of 0.061 for healthy and 1.027 for vanilla mosaic virus infected samples at 405nm. Pearson *et al.* (1993) also got similar results for vanilla mosaic virus infected samples.

SUMMARY

6. SUMMARY

The present investigation was taken up to study the extent of incidence of viral diseases of vanilla in Kerala and to characterize the most widely occurring vanilla virus disease. Survey conducted in Kottayam and Idukki districts indicated that viruses producing three distinct disease symptoms occur in these areas which were initially categorized as a) Symptom Type No.1, b) Symptom Type No.2 and Symptom Type No.3. The survey revealed that the most widely occurring virus disease was the one, which produced the Symptom Type No.1, the characteristic symptoms of which were mosaic pattern on leaves upon which necrotic spots develop later with leaf crinkling, distortion and reduction in leaf size and hence named it as vanilla mosaic virus disease. (VMVD).

The mean per cent disease incidence of vanilla mosaic virus disease varied from 6.683 in Kottayam district to 8.205 in Idukki district during summer season while it was 5.325 and 4.900 respectively during the rainy season. The mean disease index varied from 2.976 to 2.553 during summer season and from 1.554 to 1.573 during rainy season in Idukki and Kottayam districts respectively. Data further indicated that both disease incidence and disease index showed a declining trend during rainy season.

The conspicuous symptoms of vanilla mosaic virus disease was the development of chlorotic areas intermingled with green areas on the leaves which gradually developed into mosaic pattern upon which necrotic spots developed later with leaf crinkling, distortion and leaf size reduction.

Attempts to transmit the virus by sap transmission to vanilla test plants failed. Mechanical transmission of virus with different buffers showed that the virus could be transmitted very effectively to *C. amaranticolor* , the local lesion host of the virus, which produced distinct chlorotic spots on the inoculated leaves. Most efficient transmission was obtained with 0.1M sodium phosphate buffer (pH7.2)

None of the insect vectors tested could transmit the virus. Even though graft and bud union could not be established the virus could be transmitted to vanilla test plants by wedge grafting, approach grafting and budding.

Study on the physical properties of the virus indicated that, it has a dilution end point between10^{-4} and10^{-5}, thermal inactivation point between 60and 65°C and longevity *in vitro* at room temperature between 24 and 48 hours and under refrigerated conditions between 48 and 72 hours.

The virus could only be transmitted to *N. glutinosa* systemically and to *C. amaranticolor* with local lesions

Biochemical analysis of total chlorophyll and total carbohydrates of infected and healthy plant samples showed that there was a drastic reduction in total chlorophyll, chlorophyll a, chlorophyll b and total carbohydrate in the virus infected samples. The total soluble protein and phenol content of vanilla plants were found to be greatly enhanced in response to infection by vanilla mosaic virus disease. Estimation of activities of defense related enzymes oh healthy and virus infected vanilla plants indicated that the activities of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase were found to be increased in response to vanilla mosaic virus disease incidence.

Characterization studies by subjecting the vanilla mosaic virus by SDS-PAGE analysis revealed that the infected samples contained an additional major polypeptide band of molecular weight 37 KDa in addition to the identical bands to healthy samples, based on which it was presumed that the virus in the present study is similar to vanilla mosaic virus reported by Wang and Pearson (1992) and vanilla necrosis virus by Pearson *et al.* (1990)

The virus was partially purified and antiserum was successfully produced using the partially purified virus in New Zealand white rabbit. The titre of the antiserum was determined and was found to be between 1:500 and 1:1000. An easy and very rapid method of diagnosis of vanilla mosaic virus disease was developed by employing microprecipitin test in the present study. Similarly DAC-ELISA was also conducted using polyclonal antibodies developed against the partially purified virus, which showed very good results at 1:500 dilutions. Both these tests can be employed for rapid detection of vanilla mosaic virus disease in the field.

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* Originals not seen

APPENDICES

APPENDIX -I

Buffers used in sap transmission

1. 0.1M Sodium phosphate buffer (pH 7.2)

A. 0.1M sodium phosphate monobasic anhydrous - 5.99g/500ml
B. 0.1M sodium phosphate dibasic dihydrate - 8.89g/500ml

28ml of A mixed with 72ml B

2. 0.1 M sodium borate buffer (pH 8.0)

A. Boric acid (0.2 M) 1.237g/100ml
B. Borax Na2B4O7 10 H2O 1.097 g/100ml

3ml of A mixed with 7 ml of B diluted to total of 20ml.

3. 0.1M Citrate buffer (pH 6.2)

A. 0.1M Citric acid 2.1 g/100ml
B. 0.1M Sodium citrate 2.9 g/100ml

1.6ml of A mixed with 18.4ml of B

4. 0.1M Tris buffer (pH 7.2)

A. Tris 24.23g/1000ml
22.5ml of 0.2N HCl mixed with 25 ml of Tris, diluted to a total of 50ml.

APPENDIX -II

Preparation of stock dye solution for protein estimation

100mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95 % ethanol and 100 ml of concentrated orthophosphoric acid was added. The volume was made up to 200ml with water and kept at 4°C. The working dye was prepared just before use by diluting the stock solution to five times with water.

APPENDIX -III

0.1M Sodium phosphate buffer (pH 6.5)

A. 0.2M sodium phosphate monobasic anhydrous - 27.8g/1000ml
B. 0.2M sodium phosphate dibasic dihydrate - 53.65g/1000ml

68.5 ml of A mixed with 31.5 ml B diluted to a total of 200ml.

APPENDIX –IV

0.1M Borate buffer (pH 8.8)

A.0.2 M Boric acid - 12.4g/1000ml
B.0.05M borax solution - 19.05g/1000ml

50ml of A mixed with 30ml of B diluted to a total of 200ml.

APPENDIX -V

Protein denaturing solution

10M urea -80ml
1M $NaH_2PO_4.2H_2O$ (pH8) -5ml
1m Tris (pH 8) -1ml
5M sodium chloride -2ml
Make up volume to 100ml by adding 12 ml of distilled water.

APPENDIX –VI
0.01 M phosphate buffer (pH 7.0)

A. 0.2M sodium phosphate monobasic anhydrous - 2.78g/1000ml
B. 0.2M sodium phosphate dibasic dihydrate - 5.37g/1000ml

39 ml of A mixed with 61 ml B diluted to a total of 200ml.

APPENDIX –VII

Buffers for DAC-ELISA

1.Phosphate buffered saline (PBS-pH 7.4)

Sodium chloride	- 8.0g
Potassium di hydrogen phosphate	-0.2g
Disodium hydrogen phosphate	-1.1g
Potassium chloride	-0.2g
Sodium azide	-0.2g
Water	-1000ml
Tween-20(0.05%)	-0.5ml

2.Coating buffer (pH 9.6)

Sodium carbonate	- 1.59g
Sodium bicarbonate	- 2.93g
Sodium azide	- 0.2g
Water	- 1000ml

3. Substrate solution (pH 9.8)

Diethanolamine	- 97ml
Sodium azide	- 0.2g
Water	-800ml
Add HCl to give pH 9.	

ABSTRACT

VIRAL DISEASES OF VANILLA (*Vanilla planifolia* Andrews) IN KERALA

By

ABHILASH DINAKAR

Abstract of the
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ABSTRACT

Preliminary survey conducted in Kottayam and Idukki districts of Kerala indicated the incidence of three virus diseases in vanilla and were categorized as those producing a) Symptom Type No.1 b) Symptom Type No.2 and c) Symptom Type No.3. The most widely occurring virus disease was the one which produced Symptom Type No.1, the characteristic symptoms of which were mosaic pattern on leaves up on which necrotic spots developed later with leaf crinkling, distortion, and leaf size reduction. The virus is named as Vanilla mosaic virus disease (VMVD). The disease was prevalent in the two districts surveyed. The mean per cent disease incidence varied from 6.683 to 8.205 during summer season. The mean disease index varied from 2.553 to 2.976 during summer season .The virus was not sap transmissible to vanilla test plants but was easily transmissible to *Chenopodium amaranticolor*, exhibiting chlorotic local lesions in which highest per cent transmission was obtained using 0.1M sodium phosphate buffer (pH7.2). The disease was not transmitted by insects such as *Aphis gossypii, A. craccivora* and *Bemisia tabaci*. Hundred per cent transmission was obtained when infected cuttings were used as planting material. The virus was found to be transmitted to vanilla test plants by wedge and approach grafting and budding even though perfect graft and bud union did not establish. The virus has a dilution end point between 10^{-4} and 10^{-5}, thermal inactivation point between 60and 65°C and longevity *in vitro* at room temperature between 24 and 48 hours and under refrigerated conditions between 48 and 72 hours. The virus could be transmitted only to *Nicotiana glutinosa* systemically and to *C.amaranticolor* with local lesions. Drastic reduction in total chlorophyll, chlorophyll a, chlorophyll b and total carbohydrate were observed in virus infected plant samples while total soluble protein and phenol content were greatly enhanced. Peroxidase, polyphenol oxidase and phenylalanine ammonia lyase activities were found to be increased in response to virus infection. SDS PAGE analysis revealed the presence of an additional major polypeptide band of 37 KDa in infected samples in addition to the identical bands. The virus was partially purified and antiserum was successfully produced with a titre between 1:500 and 1:1000. A rapid method of diagnosis of vanilla mosaic virus disease was developed employing microprecipitin and DAC- ELISA tests.