Characterization, host range and management of *Papaya ringspot virus* (PRSV)

By

ATHEENA HARISH

(2016-11-009)

THESIS

Submitted in partial fulfilment of the requirement of the degree of

Master of Science in Agriculture

(PLANT PATHOLOGY)

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2018

DECLARATION

I hereby declare that the thesis entitled **"Characterization, host range and management of *Papaya ringspot virus* (PRSV)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Vellanikkara

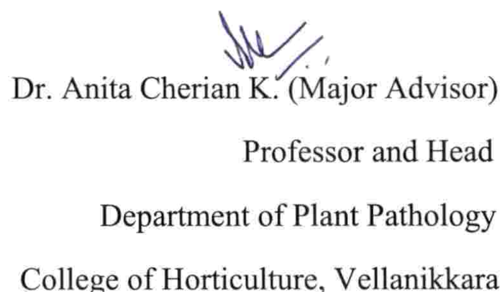
Date: 23-10-2018

ATHEENA HARISH

(2016-11-009)

CERTIFICATE

Certified that the thesis entitled **"Characterization, host range and management of *Papaya ringspot virus* (PRSV)"** is a record of research work done independently by **Ms. Atheena Harish** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

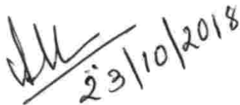


Vellanikkara
23-10-2018

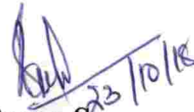
Dr. Anita Cherian K. (Major Advisor)
Professor and Head
Department of Plant Pathology
College of Horticulture, Vellanikkara

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Atheena Harish (2016-11-009)**, a candidate for the degree of Master **of Science in Agriculture**, with major field in **Plant Pathology**, agree that the thesis entitled **"Characterization, host range and management of *Papaya ringspot virus* (PRSV)"** may be submitted by **Ms. Atheena Harish** in partial fulfilment of the requirement for the degree.



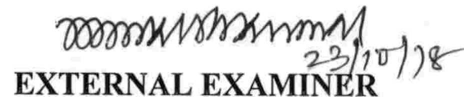
Dr. Anita Cherian K.
(Chairman, Advisory Committee)
Professor and Head
Department of Plant Pathology
College of Horticulture, Vellanikkara



Dr. Beena S.
(Member, Advisory Committee)
Professor
Department of Plant Pathology
College of Horticulture, Vellanikkara

Dr. M.R. Shylaja
(Member, Advisory Committee)
Professor and Head
CPBMB
College of Horticulture, Vellanikkara

Dr. Girija D.
(Member, Advisory Committee)
Professor and Head
Department of Agricultural Microbiology
College of Horticulture, Vellanikkara



EXTERNAL EXAMINER
Dr. T. Makeshkumar
Principal Scientist
ICAR - CTCRI
Thiruvananthapuram

ACKNOWLEDGEMENT

The last two years have been a period of intense learning for me, not only in the scientific arena, but also on a personal level. Writing this thesis has had a big impact on me. The completion of this thesis would not have been possible without the support and encouragement of several people. I would like to take this opportunity to thank everyone who have assisted me throughout my research work in innumerable ways.

First and foremost, I would like to express my heartful and deep sense of gratitude to my major advisor, **Dr. Anita Cherian K.**, Professor and Head, Department of Plant Pathology, for her expert guidance in all aspects of my research program with unparalleled enthusiasm and energy. I gratefully remember the unfailing patience, kind consideration and motherly approach she gave me during the course of study. For her limitless support and unwavering dedication, I am eternally grateful.

I express my sincere thanks to my advisory committee member, **Dr. Beena S.**, Professor, Department of Plant Pathology for her excellent guidance and constructive criticisms. I am also thankful to her for helping me to make necessary corrections in my thesis and enrich my ideas.

I am deeply indebted to **Dr. M.R. Shylaja**, Professor and Head, CPBMB, member of my advisory committee, for her well-timed guidance and help rendered during the conduct of my experiment. I am also thankful to her for providing me the facilities at CPBMB to carry out the molecular studies in my thesis.

I also take this opportunity to express my gratitude to **Dr.Girija D.**, Professor and Head, Department of Agricultural Microbiology, member of my advisory committee for her valuable suggestions extended during the research work.

I am deeply obliged to my teachers **Dr. Sainamole Kurien P., Dr. Reshmy Vijaraghavan** and **Dr. Deepa James** from the Department of Plant Pathology for their help and constant support during thesis work.

My sincere thanks to all the teaching and non- teaching staff of Department of Plant Pathology for their cooperation.

I am also grateful to my beloved teacher, Dr. **K.Umamaheswaran**, Professor, Department of Plant Pathology, College of Agriculture, Vellayani, for his valuable advice given during my research work. I am also thankful to him for providing facilities

at Advanced Centre for Plant Disease Diagnosis, Vellayani to conduct serological studies in my thesis work.

My heartfelt thanks to **Dr. Deepu Mathew**, *Assistant Professor, CPBMB, for his whole hearted co-operation and technical advice provided to me during my molecular characterization studies. I would also like to thank* **Dr. Donald James**, *Assistant Professor, CPBMB for generously offering assistance as I carried out my research work. I wish to express my sincere thanks to* **Dr. Binu S.** *and* **Dr.Marriet** *for their valuable suggestions.*

I would like to express my gratitude to **Dr. S. Krishnan**, *Associate Professor and Head, Department of Agricultural Statistics, for his valuable assistance, help and guidance during the statistical analysis of the data.*

I would like to acknowledge in depth to **Dr. C. George Thomas**, *Associate Dean, College of Horticulture, for his valuable suggestions for thesis writing.*

The financial assistance provided by **Kerala Agricultural University** *for the conduct of research work is deeply acknowledged.*

I wish to express my sincere thanks to the librarian, **Dr. A.T. Francis**, *the staff of COH Library and Central Library, Kerala Agricultural University for providing the facilities to collect the necessary literature needed for my thesis work. I am also thankful to the staff of Computer Centre, for the prompt help in the preparation and compilation of the manuscript.*

My heartfelt thanks to **Dr. Mani Chellappan**, *Professor and Head, Department of Agricultural Entomology, for providing me with facilities for capturing good photographs from the field.*

I wish to express my sincere thanks to **Dr. M.T. Thomas**, *Professor, Department of Botany, St. Thomas College for his timely assistance to conduct histopathological studies.*

I sincerely thank my seniors, Mr. Mustafa, Mrs. Milsha, Mrs. Sanju, Mrs. Sumbula, Ms. Amrutha, Ms. Deepa, Ms. Nusrath and Mr. Basil who helped me in several ways for the completion of my thesis work. A special thank you to Ms. Darsana for her generosity in clearing my doubts and openness in sharing her experiences which helped me enrich my ideas. I wish to express my thanks to my loving juniors, Reshma, Chinju, Saleena, Anjali, Alan and Sohaib.

With pleasure, I wish to convey my heartfelt thanks to my dear friends Doncy, Rahila, Laya, Aswathi and Divya for their great understanding, help and support they gave me during the difficult times of my research.

I also want to thank my batchmates, Roshni, Niranjana, Faiza, Midhuna, Pooja, Sreelakshmi, Shibana, Shilpa, Nimisha and Shahnaz for all the help they extended during the research work. A special thank you to Naveen and Anjana, Department of Plant Pathology, College of Agriculture, Vellayani for assisting me with immunological studies inspite of their busy schedule.

I am in dearth of words to express my gratitude to my loving mother, Hyrunnisa, for her constant support, blessings, boundless patience and selfless love without which I would never have completed this venture. A special thank you to my aunt, Shahida, for her unmatched energy and enthusiasm in helping me to get my work done. I can never forget to thank my uncle Samith, who has always encouraged me to move forward when my steps faltered. I am also thankful to my sister Agnetha, my brother-in-law Moniz and my fiancé Naveed for their immense love and support.

Finally, I want to dedicate this thesis to my beloved late father, Harish, who taught me to persevere and who always believed in my ability to be successful in the academic arena. Even though he is gone, his belief in me has made this journey possible.

Atheena Harish

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE NUMBER
1	INTRODUCTION	1 - 3
2	REVIEW OFLITERATURE	4 - 31
3	MATERIALS AND METHODS	32 - 48
4	RESULTS	49 - 83
5	DISCUSSION	84 - 104
6	SUMMARY	105 - 109
7	REFERENCES	i - xxiv
	APPENDICES	
	ABSTRACT	

LIST OF TABLES

Table no.	Title	Page No.
1	Geographical distribution of papaya ringspot disease	**5**
2	Distribution of papaya ringspot disease in India	6
3	List of plants used for host range studies	36
4	Reagents used for cDNA synthesis	41
5	Details of reported primers used for amplification	41
6	Components of reaction mixture used for PCR	42
7	Details of selected nuclear inclusion b and coat protein sequences of PRSV isolates used for phylogenetic analysis	45-46
8	Treatment details of pot culture experiment	48
9	Incidence and severity of papaya ringspot disease in different locations	51
10	Histopathological changes in leaf due to virus infectiom	57
11	Transmission of PRSV through mechanical inoculation	61
12	Transmission of PRSV through seeds	61
13	Host range of PRSV	62
14	DAC- ELISA: Absorbance value at 405 nm	64
15	Yield and absorbance ratio of total RNA isolated from leaf samples	66
16	Quantity and quality of cDNA	66

17	Effect of dilutions of template cDNA on RT-PCR amplification	67
18	Standardization of annealing temperature for RT- PCR	67
19	Per cent disease severity at weekly intervals	78
20	Per cent reduction of disease over control after final treatment application	79
21	Ranking of different treatments	80
22	Effect of treatments on biometric parameters	81
23	Influence of different treatments on virus titre of papaya plants	82
24	Effect of treatments on chlorophyll content of leaves	83

LIST OF FIGURES

Figure No.	Title	After Page No.
1a	Nucleotide sequence of partial nuclear inclusion b (NIb) and coat protein (CP) gene of PRSV isolate VK-1 (1249 bp)	**68**
1b	Nucleotide sequence of partial NIb and CP gene of PRSV isolate KR-2 (1204 bp)	68
1c	Nucleotide sequence of partial NIb and CP gene of PRSV isolate ML-3 (1185 bp)	68
1d	Nucleotide sequence of partial NIb and CP gene of PRSV isolate PZ-4 (1209 bp)	68
1e	Nucleotide sequence of partial NIb and CP gene of PRSV isolate PT-5 (820 bp)	68
2a	Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate VK-1	68
2b	Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate KR-2	68
2c	Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate ML-3	68
2d	Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate PZ-4	68
2e	Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate PT-5	68
3a	Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate VK-1	69

3b	Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate VK-1	69
4a	Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate KR-2	69
4b	Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate KR-2	69
5a	Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate ML-3	70
5b	Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate ML-3	70
6a	Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate PZ-4	70
6b	Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate PZ-4	70
7a	Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate PT-5	71
7b	Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate PT-5	71
8	Phylogenetic analysis of partial NIb and CP gene of PRSV isolates from different locations	72
9	Per cent reduction in disease severity over control after final application	79
10	Plant height and mean stem girth after final treatment application	81
11	Chlorophyll content after final treatment application	83

LIST OF PLATES

Plate No.	Title	After Page No.
1	Locations of purposive sampling surveys	33
2	Mechanical inoculation of the virus	34
3	General view of site of pot culture experiment	47
4	Maintenance of PRSV culture	51
5a - 5c	Symptoms under natural conditions: on leaves	53
5d	Complex of symptoms on leaves	53
5e	Symptoms under natural conditions: on petiole	53
5f	Symptoms under natural conditions: on stem	53
5g-5h	Symptoms under natural conditions: on fruits	53
5i	General symptoms on infected plants	54
6a - 6c	Symptoms under artificial conditions	55
7a - 7c	Histopathological changes in leaf due to virus infection	57
8a	Host range of PRSV: Symptoms on *Chenopodium amaranticolor* and snake gourd	58
8b	Host range of PRSV: Symptoms on bitter gourd	59
8c	Host range of PRSV: Symptoms on cucumber	59
8d	Host range of PRSV: Symptoms on pumpkin	60
9	Electron micrographs of PRSV	64
10	Detection of PRSV by DAC-ELISA	64
11	Total RNA isolated from leaf samples	67
12	Standardization of annealing temperature	67
13	Gel profile of PCR products	68
14	Test plants after final application of *Bougainvillea* leaf extract 10% (T7) and *Pseudomonas fluorescens* - 2% (T3)	81

LIST OF APPENDICES

Appendix No.	Particulars
I	Composition of buffer for mechanical transmission
II	Composition of reagents used for DAC- ELISA
III	Composition of buffer and dyes used for gel electrophoresis
IV	Composition of buffer and dye used for gel electrophoresis of PCR products
V	List of laboratory equipments used for the studies
VI	Weekly air temperature data recorded during pot culture experiment

Introduction

1. INTRODUCTION

Papaya (*Carica papaya* L.), is a dicotyledonous crop belonging to the family Caricaceae. It is a fast growing perennial herb with an economic life not more than three years. It is a powerhouse of nutrients and a good source of calcium, vitamins and proteolytic enzymes and can be considered as a multifaceted plant with anti- cancerous, anti- inflammatory, anti-diabetic and anti-oxidant effects (Yogiraj *et al.*, 2014). Realising such nutritional and medicinal values of papaya, its cultivation has significantly increased across the globe. India is the largest producer of papaya with a share of 44 per cent in global production (FAOSTAT, 2014). However, the major challenge faced in profitable cultivation is the incidence of many fungal and viral diseases. Of these, papaya ringspot disease caused by *Papaya ringspot virus* (PRSV) have devastating effects on the economic yield of the crop. In the last decade, the occurrence of papaya ringspot disease has become a major constraint in papaya cultivation and has resulted in economic loss upto 100 per cent (Tennant *et al.*, 2007).

PRSV is one of the most serious pathogens infecting papaya (Fermin and Gonsalves, 2003). It belongs to the genus *Potyvirus* which includes most economically devastating plant viruses. The members of this genus are sap and aphid transmissible. Two strains of PRSV exist of which type P infects papaya and cucurbits wheras type W infects only cucurbits. PRSV-P was thought to have arisen by mutation from PRSV-W. PRSV- P has been isolated from almost all papaya growing regions of the world including the Caribbean, tropical Asia, Africa, Central and South America and South Pacific (Tennant *et al.*, 2007)

In India, the incidence of PRSV was first reported by Capoor and Varma (1948). Since then, it has spread to different locations of the country irrespective of the agroclimatic conditions. In south India, PRSV was not reported until 1995.Within a

span of five years, it had spread throughout south India causing significant yield loss in papaya. Now, it is the most widespread and damaging viral disease affecting papaya production causing heavy economic loss to papaya growers not only in the country, but throughout the world. The widely grown variety 'Red Lady' is found to be highly susceptible and about 86 per cent disease incidence has been reported (Chavan *et al.,* 2010.). The disease has now become a major limiting factor for successful cultivation of papaya in India. The incidence reported in different states of the country ranges from 3 to 100 per cent and 35-66 per cent disease incidence was reported in Kerala (Verma *et al.*, 2007). Considering, the importance of this emerging disease, this project was undertaken to characterize the virus and develop suitable management strategies against the disease.

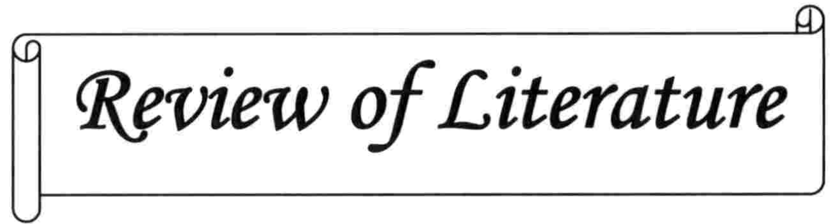
Papaya plants of all stages are vulnerable to the disease. The fruits of the infected plants become small, deformed, and unfit for consumption rendering them unmarketable. In the recent years, the incidence of disease has become very severe and this could be attributed to the secondary spread through the insect vectors. The role of collateral hosts in the wide spread of the disease also needs to be suspected. A detailed study on the symptomatology of the disease and host range of PRSV will help in the early diagnosis and management.

Despite the scope for commercial cultivation and export of papaya fruits, the disease is a major limitation. In case of papaya ringspot, early detection and diagnosis of infection is of prime importance because symptomless hosts may carry the viral inoculum and spread disease in a short period of time. Detection of the pathogen which remain latent in the plant could be done through various nucleic acid and protein-based indexing techniques which have been developed in virus indexing laboratories across the world. Considering this aspect, the serodiagnostic approaches for detection of PRSV were undertaken.

Coat protein mediated resistance has been successfully employed for developing transgenic papaya (Gonsalves, 1998). However, the distinct nature of coat protein gene necessitates molecular characterization of PRSV isolates of a particular location in order to determine the divergence of PRSV isolates (Hema and Prasad, 2004). This would help in the development of a location specific transgenic papaya. A perusal of literature also revealed that not much work has been done on the characterization of PRSV isolates in Kerala. Availability of more sequences of coat protein gene of PRSV from new locations in the Indian subcontinent would help in better assessment of sequence divergence within the PRSV population. Hence, molecular characterization of local isolates of PRSV was proposed in the project which will provide detailed knowledge regarding the nucleotide and amino acid sequences and aid in a comparative study with other isolates and help in development of suitable management strategies in the future.

Several defense inducers, anti-viral principles from botanicals and mircrobial formulations are reported as useful for the management of plant diseases especially viral diseases. Hence, the present study was undertaken to evaluate the effects of different treatments in reducing the severity of papaya ringspot disease.

Therefore, the project was proposed with the main objective of biological and molecular characterization of the virus, immunological studies and management of papaya ringspot disease.


Review of Literature

2. REVIEW OF LITERATURE

Papaya (*Carica papaya* L.) is one of the most important fruit crops that is cultivated widely in the tropics and subtropics. India is the largest producer of papaya with a share of 44 per cent in global production (FAOSTAT, 2014). During the last decade, the area under papaya cultivation has dramatically increased due to liberalization and introduction of superior Taiwanese and Hawaiian hybrid varieties. But, the major setback subsequent to the introduction of such varieties has been the occurrence of papaya ringspot disease which resulted in severe yield loss in papaya. The disease is caused by *Papaya ringspot virus* (PRSV) which belongs to genus *Potyvirus* and family *Potyviridae*. The virus is transmitted mechanically through sap (Bayot *et al.*,1990) as well as by insect vectors like aphids *viz.*, *Aphis gossypii*, *A. craccivora* and *Myzus persicae* in a non-persistent manner (Kalleshwaraswamy *et al.*,2007).

2.1 OCCURRENCE AND GEOGRAPHICAL DISTRIBUTION OF PRSV

During the last few decades, over 20 plant virus species are reported as potential pathogens of papaya (Tennant *et al.*, 2007). Of these different viruses, PRSV has been identified as the most destructive one across the globe, both in tropical and subtropical regions (Prowidentii, 1996). The origin of the disease outbreak is not known. It was first identified and reported by Lindner (1945) from Hawaii and since then, it has been reported from different parts of the world. Later, it was documented as the major challenge for papaya production in many tropical and subtropical regions including South and Central America, Asia, Africa and Carribean Islands (Tripathi *et al.*,2008). The global distribution and occurrence of the disease have been thoroughly reviewed and enlisted in Table 1.

Nationally also, PRSV poses a major threat to papaya cultivation throughout the country by rendering the orchards economically unproductive. The virus was

reported to occur in every region of the country where papaya was grown, irrespective of the agroclimatic conditions resulting in severe crop losses (Lokhande and Moghe, 1992; Hussain and Varma, 1994)

Table 1: Geographical distribution of papaya ringspot disease

Sl. No.	Year of report	Country/ region	Reference
1.	1945	Hawaii	Lindner,1945
2.	1948	India	Capoor and Varma,1948
3.	1966	Colombia	Torres and Giacometti,1966
4	1969	Brazil	Costa *et al.*,1969
5	1986	Philippines	Opina,1986
6.	1987	Mexico	Alvizo and Rojkind,1987
7.	1991	Malaysia	Wahab, 1991
8.	1991	Australia	Thomas and Dodman,1993
9.	1992	Nepal	Shrestha and Albrechtsen,1992
10.	1995	Japan	Maoka *et al.*1995
11.	1997	China	Xiao *et al.*,1997
12.	1998	Sri Lanka	Perera *et al.*,1998
13.	2002	Papau New Guinea	Davis *et al.*,2002
14.	2003	Bangladesh	Jain *et al.,*2004a
15.	2004	Pakistan	Ali *et al.*,2004
16.	2005	French Polynesia and Cook Islands	Davis *et al.*,2005
17.	2005	Cyprus	Papayiannis *et al.*,2005
18.	2006	Cote d Ivoire	Diallo *et al.*,2007
19.	2011	Egypt	Omar *et al.*,2011
20.	2015	South Africa	Ibaba *et al.*, 2015
21.	2016	Argentina	Maderos *et al.*, 2016

In India, the disease was first reported as papaya mosaic in Poona, Bombay Province (Capoor and Varma, 1948). Although it was called 'papaya mosaic', the symptoms described indicated that the causal agent was PRSV. Subsequently, it was reported from Madhya Pradesh (Garga,1963) and later reported as papaya ringspot in Uttar Pradesh (Khurana and Bhargava,1970). Surekha *et al.* (1977) reported the occurrence of the virus in Udaipur of Rajasthan. In south India, PRSV was reported only in 1995 in Karnataka (Byadgi *et al.*, 1995). Later, Philip and Nair (1999) reported the disease from Vellayani, Kerala. PRSV was reported to have spread within a short period of time of 5-6 years throughout south India (Verghese, 2001). The distribution of the disease in India is summarized in Table 2.

Table 2: Distribution of papaya ringpsot disease in India

Sl. No.	Year of report	State	Reference
1.	1948	Bombay Province	Capoor and Verma,1948
2.	1955	Bihar	Mishra and Jha,1955
3.	1963	Madhya Pradesh	Garga, 1963
4.	1970	Uttar Pradesh	Khurana and Bhargawa,1970
5.	1977	Rajasthan	Surekha *et al.*,1977
6.	1980	Maharashtra	Yemevar and Mali, 1980
7.	1995	Karnataka	Byadgi *et al.*,1995
8.	1999	Kerala	Philip and Nair, 1999
8.	2002	Andhra Pradesh	Gourgopal and Jain,2002
9.	2005	Tamil Nadu	Sharma *et al.*,2005
10.	2007	Kerala	Verma *et al.*,2007

2.1.1 Disease Incidence and Economic Impact

PRSV is a very serious pathogen and 70 per cent yield loss was observed due to disease incidence (Barbosa and Paguio, 1982).Wan and Conover (1983) surveyed 30 papaya plantations in Southern Florida and an incidence upto 100 per cent was recorded. Wahab (1991) reported that out of 74 hectares of field surveyed in Johore, Malaysia, PRSV incidence was recorded in 26 hectares. Papaya ringspot disease severely limited the production of papaya in many countries of South East Asia *viz.*, Philippines, Taiwan, Thailand and Vietnam. It also affected other regions including Indian Subcontinent, Africa, South and Central America (Kiritani and Su Hong, 1999).

In India, Lokhande and Moghe (1992) reported the occurrence of PRSV on different cultivars during different seasons. The incidence ranged from 75 to 100 per cent and was found to be high during rainy season. Lakshminarayan Reddy (2000) surveyed homesteads and commercial orchards in and around Bangalore and reported an incidence from 75 to 100 percent. Singh *et al.* (2003) reported PRSV incidence ranging from 48 to 100 per cent in all the districts of Eastern Uttar Pradesh while Vimla *et al.* (2005) conducted a survey in commercially cultivated papaya orchards of Eastern U.P., and reported 95 per cent disease incidence with 70 per cent yield loss. The disease incidence reported in different states *viz.*, Bihar, Maharashtra, Uttar Pradesh, Karnataka, Kerala and West Bengal were 100, 3 to 100, 74 to 90, 60, 35 to 66 and 40 per cent respectively (Verma *et al.*, 2007). Singh and Shukla (2009) reported an average yield loss of 61.3 per cent in the infected plants. The economic value of the infected plant was reduced up to 38.7 per cent. Gonsalves *et al.* (2010) stated that the plants infected at young stage remained stunted and did not produce any economic yield.

Chavan *et al.* (2010) conducted a screening of eight commercial papaya cultivars and reported the lowest disease incidence (13.2 %) in the cultivar Madhubala followed by cultivars CO2 (39.8 per cent) and Pusa Nanha (44.8 %) when compared to the widely grown papaya Red Lady which showed the maximum disease incidence

(86 %). A survey on disease incidence revealed that the commonly cultivated varieties *viz.*, Sunrise Solo, Red Lady, Surya and Coorg Honey Dew were susceptible to PRSV. Among the viruses infecting papaya, PRSV is found to be very serious causing yield loss up to 70 per cent (Reddy *et al.*, 2011).

Out of 15 surveyed districts of Assam, highest PRSV incidence of 74.3% was noticed in Goalpara district followed by 70% in Kokrajhar district and the lowest in Nagaon district (28.0%) (Talukdar *et al.*, 2013).

2.2. SYMPTOMATOLOGY OF PAPAYA RINGSPOT DISEASE

Capoor and Varma (1948) reported the occurrence of mosaic disease in papaya which expressed symptoms similar to PRSV infection. The symptoms included reduction in size of leaf lamina, malformed fruits with circular or concentric water soaked lesions all over the fruit surface. Jensen (1949) reported that the initial symptom of the disease appeared on young leaves as puckering of leaf tissue between veins and veinlets resulting in upward curling of leaves followed by chlorotic mottling and blistering on leaf surface. In severe cases, leaves become highly distorted. Moreover, the PRSV infection was typically characterized by the production of circular or concentric water soaked lesions all over the fruit surface. PRSV-P isolates differed in the severity of symptoms produced. Symptom expression is highly influenced by environmental conditions and were more severely expressed during cooler months (Gonsalves and Ishii, 1980).

The symptoms like chlorotic ringspots, mosaic pattern, leaf distortion, shoestring effect on leaves, oily rings on stem, petioles and fruits, malformed double fruits, stunting of plants and failure in production of flowers and fruits in later stages of infection were reported by many workers (Torres and Giacometti,1966; Khurana and Bhargava,1970; Surekha *et al.*,1977; Almeida and Carvalho,1978; Yemewar and

Mai,1980; Lana,1980; Kitajima *et al.*,1987; Lokhande and Moghe ,1992; Thomas and Dodman,1993; Hussain and Varma,1994; Dahal *et al.*,1997; Gonsalves,1998).

Kunkalikar (2003) studied the symptoms on mechanically inoculated papaya var. Surya and Solo Sunrise and reported that the incubation period of the virus was 15 days. Initially, the leaves turned pale green and later developed chlorotic spots. Subsequently, the entire leaf produced mosaic symptoms. In advanced stages, the leaves exhibited severe distortion leading to shoestring effect. Stem started tapering within the canopy and developed rosette appearance. On the stem surface, concentric rings and oily streaks were formed which extended up to the base of the petioles. Singh *et al.* (2003) reported similar symptoms on the foliage along with thickening of veinlets.

The development of circular and concentric water soaked lesions and necrotic rings with solid central spots on mature green fruits were the most characteristic symptom of the disease (Rao *et al.*, 2008). Singh and Shukla (2011) reported that PRSV infection induced ringspot, distortion and mottling symptoms on the foliage, stunting, fragile roots and deteriorated fruit quality and quantity. Plants showed crowding of apical leaves and denuded appearance due to reduced fruiting, defoliation and severe stunting. Furthermore, on studying the influence of inoculation time on severity of the disease, it was observed that symptom expression was severe under low temperatures but the symptoms were masked during high temperatures resulting in mild incidence of the disease.

Apart from chlorotic spots and puckering symptoms on leaves, Krishnapriya (2015) also recorded vein banding symptom. Additionally, the production of flowers and fruits in the infected plants were drastically reduced and the fruits were disfigured with development of bumps.

Singh *et al.* (2017) described the nature of ringspots which appeared on the skin of infected fruits. Size of these necrotic spots ranged from 3- 20 mm in diameter.

The number of ringspots in a single fruit varied from a few to more than 200 depending upon the size of fruits and the disease severity. Furthermore, they stated that maximum number of ringspots were observed on the side where the fruit was exposed to the sun. These spots coalesce with each other and turned to distorted ringspots on most of the fruits.

2.3. HISTOPATHOLOGY OF PAPAYA RINGSPOT VIRUS DISEASE

Histopathological studies of PRSV infected leaves conducted by Kunkalikar *et al.* (2007) revealed distortion of palisade parenchyma. They also reported that the spongy parenchyma cells lost their normal round shape and showed complete disintegration. Singh and Shukla (2012) stated that there was extensive deformation of parenchyma tissues in leaf, poor differentiation of vascular tissue, disintegration of chloroplasts, increased number of calcium oxalate crystals in stem, reduced size of xylem cells in roots. They also reported that the lactiferous tubes remain unaffected.

2.4. BIOLOGICAL CHARACTERIZATION OF THE VIRUS

2.4.1. Transmission Studies

2.4.1.1. Mechanical Transmission

Capoor and Varma (1948) proved sap transmissibility of PRSV under insect-proof glass house conditions. The symptoms first appeared as necrotic spots on leaf lamina of newly developing leaves in about 20 days following inoculation. Furthermore, the use of 600 mesh fine carborundum powder as an abrasive gave a higher percentage of infection and the disease symptoms also appeared earlier compared to plants which were inoculated without the use of an abrasive.

Conover (1962) reported mechanical transmission of *Papaya distortion ringspot virus* to papaya. Similarly, many researchers have reported the mechanical transmission of PRSV to papaya (Zettler *et al.*, 1968; Story and Halliwell, 1969; Lima

and Gomes, 1975, Yemewar and Mali, 1980; Bayot *et al.*, 1990; Yeh *et al.*, 1984 and Thomas and Dodman, 1993).

Kunkalikar (2003) proved mechanical transmission of PRSV with 0.5 M phosphate buffer (pH: 7.5) from infected papaya plants to healthy test plants. 100 per cent transmission was recorded with an incubation period of 18 days. The results were confirmed with DAC- ELISA. Reddy *et al.*, (2007) established successful transmission of PRSV through mechanical inoculation under glass house conditions using 0.1M potassium phosphate buffer (pH: 7.0) to healthy test plants.

Similar results on 100 per cent mechanical transmission was reported but with 0.1 M sodium phosphate buffer (pH: 7.2) with an incubation period of 18-22 days (Dhanam *et al.*,2011) and 0.1 M sodium phosphate buffer (pH: 7.0) with an incubation period of 20 days (Krishnapriya, 2015).

2.4.1.2. Seed Transmission

Capoor and Varma (1948) investigated the transmission of the virus through seeds. Out of 450 seeds from a diseased fruit sown, 340 germinated and produced only healthy seedlings. Wang *et al.* (1978) reported that PRSV is not transmitted through seeds. Prasad and Sarkar (1989) also failed to establish transmission of PRSV through seeds.

On the contrary, Bayot *et al.*(1990) reported that 2 out of 1355 seedlings *viz.*, 0.15 per cent from the fruit of an infected tree exhibited symptoms caused by PRSV. Moreover, they stated that the transmission is dependent on weather factors, variety of papaya and the assay methods used.

Dahal *et al.* (1997) reported that none of the seeds collected from infected papaya fruits produced diseased seedlings. Kunkalikar (2003) conducted seed transmission studies on different varieties of papaya *viz.*, Surya, Sunrise solo, Taiwan, Solo, Red Lady and local varieties of which none of them exhibited seed transmission.

Additionally, seed transmission studies carried out by Reddy *et al.* (2007) revelaed that the virus was not seed borne. Laney *et al.* (2012) reported that black locust isolates of PRSV and WMV are seed borne. Krishnapriya (2015) recorded no seed transmission from seeds of infected fruits upto 60 days after sowing.

2.4.2. Host Range Studies

Capoor and Varma (1958) have reported that the Maharashtra isolate of PRSV could infect seven cucurbitaceous hosts *viz. Lagenaria siceraria, Trichosanthes anguina, Cucumis sativus, Luffa acutangula, Cucurbita moschata, C. pepo* and *C. maxima*. Conover (1962) reported mechanical transmission of *Papaya distortion ringspot virus* to cucumber, muskmelon, summer squash and water melon. Zettler *et al.* (1968) successfully proved that cucurbitaceous plants are hosts of PRSV.

Story and Halliwell (1969) mechanically transmitted *Distortion ringspot virus* to papaya and *Cucurbita pepo* L. but not to *Chenopodium amaranticolor* and *Vinca rosea*. Similarly, other research workers reported the transmission of PRSV from infected papaya to healthy papaya and cucurbits (Lima and Gomes,1975; Yemewar and Mali, 1980 and Yeh *et al*,1984). Yeh and Gonsalves (1984) reported that PRSV could induce local lesions in *Chenopodium quinoa*. In contrast, Opina (1986) and Ramos (1987) failed to induce local lesions on *C. quinoa*. Bayot *et al.*(1990) stated that these conflicting observations are probably due to differences in virus strain, environmental factors and the strain of the host used in the study.

The members of Leguminoceae, Solanaceae, Mahaceae, Amaranthaceae, Asteraceae, Cruciferaceae, Euphorbiaceae, Chenopodiaceae, Labitaceae, Caesalpinaceae, Fabaceae and Convulvulaceae were not susceptible to PRSV (Lakshminarayan Reddy, 2000). Kunkalikar (2003) established successful transmission of PRSV from infected papaya plants to healthy test plants belonging to Cucurbitaceae *viz., Momordica charantia, Cucurbita pepo, C. moschata Cucumis sativus, Luffa acutangula* and *Trichosanthes anguina*. However, transmission of PRSV

to *Nicotiana tabacum*, *Solanum melongena* and *Helianthus annuus* could not be established.

The most commonly observed symptom of PRSV-W strain were leaf distortion, blisters and shoe string effect on zucchini and mosaic or yellow mosaic , blisters and leaf distortion on other cucurbits. Disease incidence ranged from 85-100 per cent in zucchini, 4- 100 per cent in cucumber, 4 – 100 per cent in pumpkin and 10 – 100 per cent on bottlegourd, choyote and watermelon (Dahal *et al.*, 1997). Tripathi *et al.* (2008) reported that PRSV has a limited number of hosts belonging to the families Caricaceae, Chenopodiaceae and Cucurbitaceae.

In contrast to this, Kelaniyangoda and Madhubashini (2008) reported that *Cucumis melo, Cucumis sativus, Nicotiana tabaccum, N. glutinosa, Gomphrena globosa* and *Chenopodium amaranticolor* did not take up PRSV inection up to 3 weeks after mechanical inoculation. This was also confirmed through indirect ELISA. However, *Chenopodium quioa* produced local lesions following three weeks after inoculation and gave a positive ELISA reading.

The biological indexing and electron microscopic studies conducted by Chavan *et al.* (2010) revealed *Cucumis melo* L., *Alternanthera sessilis, Datura metel, Xanthium indicum, Cassia tora* and *Physalis minima* as off season weed hosts of PRSV. Dhanam *et al.* (2011) conducted host range studies on different cucurbitaceous plants *viz.*;ashgourd, bittergourd, bottlegourd, ridgegourd, snakegourd, cucumber and zucchini. Among them, zucchini alone exhibited chlorotic lesions on leaves within 18-20 days after inoculation. None of the other cucurbits showed any symptom. Similar studies conducted on *Chenopodium amaranticolor* and *C. quinoa* revealed that the former expressed chlorotic local lesions on leaves within 20-22 days of inoculation.

Singh *et al.* (2017) reported that members of Cucurbitaceae viz., *Cucumis melo*, *C. sativus*, *Cucurbita moschata*, *C. pepo*, *Luffa acutangula*, *L.* cylindrica, *Lagenaria siceraria* and *Momordica charantia* are hosts of PRSV and expressed mosaic mottling

symptoms on inoculation. They also stated that members of Solanaceae and Leguminosae are not susceptible to PRSV. *Chenopodium amaranticolor* and *C. quinoa* expressed necrotic local lesions on PRSV inoculation.

2.4.3. Morphology of Viral Particles

Kulkarni (1970) purified three viruses of papaya by density gradient centrifugation. Electron microscopy revealed rod shaped particles of 750 x 12 nm. Lana (1980) obtained purified virus from papaya using density gradient centrifugation. The virus particles measured 791 x 12 nm. Chen (1984) observed filamentous particles of PRSV measuring 700 - 750 nm when negatively stained in leaf dip preparation. In the sections, cellular inclusions in the form of pinwheels, tubular and short laminated aggregates were observed. Hussain and Varma (1994) identified PRSV in electron microscope which revealed flexuous particles measuring 780 x 12 nm.

Examination of infected samples used in ELISA tests by electron microscopy and immunosorbent electron microscopy (ISEM) confirmed the presence of typical potyvirus flexuous particles of size 700-800 nm long which reacted strongly to PRSV-W antiserum (Dahal *et al.,* 1997). Kunkalikar (2003) reported similar observations regarding the morphology of virus particles. PRSV particles of leaf dip preparation and decoration were flexuous rods of size 750 x 12nm. Tripathi *et al.* (2008) reported that the virions are filamentous, non-enveloped and flexuous measuring 760-800 x 12 nm, confirming the morphological features of *Potyviridae.*

Flexuous rod shaped virus particles were observed under electron microscopy by Dhanam *et al.* (2011) in infected leaf samples collected from field as well as artificially inoculated plants. In ISEM, the virus particles got decorated with PRSV specific polyclonal antibodies.

2.5. PROTEIN BASED DETECTION OF PRSV

Gonsalves and Ishii (1980) detected the presence of Hawaiian isolate of PRSV using DAC - ELISA. The ELISA readings were found to be comparitively higher when the molarity of the phosphate buffer was increased (>0.2 M). Bayot *et al.* (1990) could detect PRSV in both papaya seeds and seedlings using polyclonal antibodies and monoclonal antibodies. Kuan *et al.*, (1999) studies 27 isolates of PRSV showing different symptoms. PRSV- P and PRSV- W were serologically indistinguishable as determined by polyclonal antibodies but were found to be distinguishable when determined by monoclonal antibodies. Kunkalikar (2003) conducted DAC - ELISA on symptomatic leaves, skin of diseased fruit and from stem. The skin of diseases fruit and stem showed mild positive reaction indicating the low concentration of virus. However, strong positive reaction was shown from the leaves. Cruz *et al.* (2009) detected PRSV by direct ELISA using AGDIA kit and indirect ELISA using polyclonal antibodies. Krishnapriya (2015) standardized the detection of Vellayani isolate of PRSV using DAC - ELISA.

2.6. MOLECULAR CHARACTERIZATION OF PRSV

Papaya ringspot virus belongs to the genus *Potyvirus*, family *Potyviridae*. The virus consists of non- enveloped flexuous filamentous particles which consists of a positive sense, single-stranded, unipartite RNA genome encapsidated by the genome-encoded coap protein (CP). The genomic RNA of PRSV is 10326 nucleotides long followed by a tract of poly A sequence. An open reading frame starting at nucleotide 86 and ending at nucleotide 10120 encoodes a polyprotein of 3344 aminoacids from which all the proteins of the virus are derived. As with other potyviruses, several proteins are produced through a combination of co-translational, post-translational, auto-proteolytic and transproteolytic processing by three virus-encoded endoproteases, P1, HC-Pro and Nia (Yeh and Gonsalves, 1985; Yeh *et al.*, 1992). About 21 isolates

were recorded worldwide so far and were grouped into two types; Type W (PRSV – W) infects only cucurbits and Type P (PRSV – P), which infects both papaya and cucurbits (Gonsalves, 1998).

The name and size of the viral genes listed in the order of their occurrence (5` to 3`) in the PRSV genome are: P1(63k), helper component (HC-Pro,52k), P3(46k), cylindrical inclusion protein(CI,72k), 6K(6k), nuclear inclusion protein a (NIa, 48k), nuclear inclusion protein b (NIb,59k) and coat protein (CP, 35k) (Yeh and Gonsalves, 1985; Quemada *et al.*,1990; Yeh *et al.,*1992;Wang and Yeh,1997).

Comparison of CP gene sequences is one of the most reliable way of studying the variability among the virus isolates. Coat Protein Mediated Resistance (CPMR) has been successfully used to confer resistance to a wide range of viruses including PRSV (Gonsalves, 1998). Previous studies indicate that coat protein genes of PRSV strains are very distinct in geographic origin and pathogenicity (Bateson *et al.*, 1994). A transgenic papaya with CP genes specific to the PRSV-P strains existing in a particular region needs to be developed for effective control of the disease in that region. However, in India the strains responsible have not been adequately characterized at the molecular level. Therefore, a region – specific molecular characterization is necessary in order to know the nucleotide and amino acid sequeces of the CP gene and to determine the degree of variation from other isolates (Hema and Prasad, 2004).

2.6.1. Reverse Transcription- Polymerase Chain Reaction

PCR is an *in vitro* method for amplification of target nucleic acid sequences. DNA plant viruses (caulimo, gemini and badnaviruses) could be detected directly by PCR; however for detecting RNA viruses, RNA needs to be converted to complementary DNA (cDNA) by reverse transcription prior to PCR. The cDNA provided suitable DNA target for subsequent amplification and this modified technique

for detection of RNA plant viruses was called RT-PCR (Reverse Transcription-Polymerase Chain Reaction) (Henson and French, 1993).

A coat protein gene fragment of PRSV of size 950 bp was amplified by RT-PCR (Quemada *et al.*, 1990). Bateson *et al.* (1994) used synthetic primers MB11 and MB12, homologous to the part of PRSV-W (Aust- DB1) coat protein gene sequence to amplify the coat protein gene of two Australian field isolates and three Asian field isolates. Jain *et al.* (1998) characterized P and W isolates of PRSV from India at molecular level by cloning and sequencing the 3' terminal regions comprising a part of the nuclear inclusion b (NIb) gene, the complete coat protein (CP) and the untranslated region (UTR) which has a size of 1.7 kb. Kunkalikar (2003) developed immunocapture RT-PCR for detection of PRSV and yielded a PCR product of size 550bp.

Hema and Prasad (2004) used RT- PCR technique to amplify the CP region of a south Indian isolate which yielded a product of 900 bp. Jain *et al.* (2004b) also carried out RT-PCR to amplify coat protein region of PRSV isolates from multiple locations in India. The product size of the amplicon varied from 840- 858 nucleotides, encoding protein of 280-286 amino acids.

Ha *et al.* (2008) designed two pairs of novel degenerate primers from sequences within the potyviral CI and HC-Pro-coding region, and these were highly specific to the memebers of the genus *Potyvirus*. RT-PCR using the novel primers NIb2F and NIb3R was proved as a routine diagnostic assay for detection of all the major groups within the genus *Potyvirus* (Zheng *et al.*, 2010).

Srinivasulu and Sai Gopal (2011) cloned and sequenced the 3` terminal region of about 1.7 kb comprising a part of nuclear inclusion b NIb gene, complete coat protein (CP) and 3` untranslated region (3'UTR) of seven PRSV isolates from south India.

Similarly, RT-PCR of symptomatic papaya leaves in Coimbatore yielded an amplicon of 850 bp corresponding to the coat protein gene of the virus (Dhanam *et al.*, 2011). Krishnapriya (2015) standardized molecular detection and characterization of PRSV in papaya. RT-PCR amplification of the CP gene using gene specific primers resulted in the amplification of 220 bp fragment.

Usharani *et al.* (2012) used duplex PCR to simultaneously detect PRSV and *Papaya leaf curl virus* from papaya. Tuo *et al.* (2014) used multiplex RT-PCR with a mixture of three specific primer pairs to amplify three distinct fragments of 613 bp from the P3 gene of PRSV, 355 bp from the CP gene of PLDMV and 205 bp from the CP gene of PapMV.

2.6.2. Sequence Diversity of PRSV Isolates

In the case of Papaya ringspot virus, most studies have been focused on examining sequence variation in the CP gene from PRSV isolates. Earlier, studies carried out in USA and Australia (Quemada *et al.*, 1990; Bateson *et al.*, 1994) suggested that there was little sequence variation in the CP gene of PRSV isolates.

Later, Bateson *et al.* (2002) studied the evolution and molecular epidemiology of PRSV-P by sequencing CP genes of both PRSV P and W type isolates from Vietnam, Thailand, India and Philippines. On comparison with sequences of 28 other isolates, they found that mutation together with long distance movement had contributed for variation in isolates and evolution of type P from W. Phylogenetic analyses of PRSV also showed its closest known relative, *Moroccan watermelon mosaic virus*, indicate that PRSV might have originated in Asia, particularly in the Indian subcontinent.

The homology of twelve Brazilian isolates of PRSV were studied by Lima *et al.* (2002). The CP gene from these isolates have shared an average homology of 97.3 per cent at the nucleotide level among Brazilian isolates. When compared to 27 isolates from outside the Brazil in a homology tree, these isolates showed clustering with Australian, Hawaiian, Central and North American isolates, with an average degree of homology of 90.7 per cent among them.

Recent sequence data of PRSV CP genes of isolates from India indicate greater sequence variation among the local populations of PRSV in other countries. CP sequence diversity at the amino acid (10%) and nucleic acid (14%) levels were highest among the Asian populations of PRSV isolates (Jain *et al.*, 2004b).

Hema and Prasad (2004) compared the CP gene of south Indian strain (INP-UAS) of PRSV with 74 other sequences reported from different geographical isolates. They concluded that the variation and distinctiveness in the N terminal region from the previously reported isolates, was linked to its geographical location. The CP gene of INP- UAS isolate had a deletion of 24 nucleotides, corresponding to 8 amino acids in this region in contrast to P-IND isolate (Jain *et al.*, 1998) which showed a deletion of 4 amino acids in the N terminal region of CP. Furthermore, comparison of sequences corresponding to the CP N-terminal regions of PRSV isolates revealed two lineages. Among them, all the American isolates (USA, Mexico, Brazil, Puerto Rico), Australian isolates, isolate P-IND from India and Sri Lanka formed one lineage whereas the rest of South East Asian and Western Pacific isolates (China, Indonesia, Thailand, Vietnam, Taiwan, Japan) formed another. However, the south Indian strain INP-UAS did not fall into any of these two lineages but formed a separate and distinct group outside the major cluster.

Bag *et al.* (2007) studied the sequence diversity in the coat proteins of 28 PRSV isolates originating from different locations in India. There was heterogeneity in CP gene length (275-289 amino acids) among the isolates from central, eastern, northern, southern and western India (up to 23%). Among all the isolates, KA4, INU-01 and AP2 from southern India were found to be unique. Maximum heterogeneity was observed in southern isolates (up to 23 %), followed by central (upto 11%), eastern and northern (upto 10%) and western (upto 7%) isolates. Lack of relationship between variability and geographical origin of the isolates was also found.

The complete sequencing of New Delhi Indian isolate of PRSV (PRSV-DEL) from IARI was done by Parameswari *et al.* (2007). Comparative sequence analyses revealed that the PRSV-DEL shared 83–89 per cent and 90–92 per cent overall sequence identities at the nucleotide and amino acid levels respectively, with other PRSV isolates. Maximum sequence identity at the amino acid level (92%) was observed with isolates from the America forming one cluster, followed by 90 to 91 per cent identity with Asian isolates, forming a distinct cluster.

Srinivasulu and Sai Gopal (2011) compared the coat protein nucleotide and deduced amino acid sequences of seven isolates from different locations of south India and also with 22 other PRSV isolates from different geographical locations in the Indian subcontinent. The sequence comparison revealed greater sequence divergence upto 18.4 per cent and 15 per cent at nucleotide and amino acid level respectively within Indian PRSV populations. All south Indian isolates were clearly separated from isolates of other geographical regions and formed a major group in phylogenetic trees and the clustering pattern of the isolates did not correlate well with their geographical origins.

Castillo *et al.* (2011) stated that nucleotide sequences of CP gene of Indian isolates had the highest level of diversity and this may suggest an ancestral Indian

origin. Omar *et al.* (2011) reported that the Egyptian isolates of PRSV grouped together in a distinct clade. Comparison with PRSV sequences from the GenBank presented nucleotide identities in the range of 87.5 to 97.1 per cent.

Krishnapriya (2015) characterized the PRSV isolate from Vellayani, Kerala. Comparative nucleotide sequence alignment of the virus with the available databases from NCBI revealed a 99 per cent homology with PRSV isolate from Andhra Pradesh. It also shared 93 per cent homology with PRSV isolate biotype W from Brazil.

2.7. MANAGEMENT OF PAPAYA RINGSPOT DISEASE

2.7.1. Effect of various chemicals, nutrients, microbial formulations and plant extracts on incidence of PRSV

Salicylic acid (SA) is a signal required for the activation of systemic acquired resistance (SAR) (Malamy *et al.,* 1990). SAR is dependent on the production of SA in response to infection (Gaffney *et al.*, 1993) and is associated with the accumulation of pathogenesis-related proteins (PRs) both in the inoculated and in distant leaves (Ryals *et al.*, 1996, Van Loon and Kammen, 1970). Because exogenous application of SA induces both SAR and PRs, the latter are commonly taken as markers of the induced state. SAR is non-specific with respect to both the inducing and the challenging pathogen and therefore, a primary infection in plant caused by either fungi, bacteria or virus may lead to an enhanced systemic resistance against them (Kuc, 1982).

Root drench of papaya seedlings with BTH, an analogue of SA resulted in increased accumulation of a PR1 (a gene widely used as a marker for SAR) mRNA. This indicated that papaya has a SAR response which can be induced by BTH, making this chemical a valuable research tool and possible future field treatment (Qiu *et al.*, 2003). The disease was effectively controlled by SA treatment of 50 mg/L. The SA treatment in the form of spraying showed a considerable reduction in disease more than

70 per cent in the variety Solo (Zhang *et al.*, 2007). SAR induction is triggered, typically, by recognition of a specific incompatible pathogen but its effects can enhance plant resistance indiscriminately against a very broad range of both viral and non-viral pathogens (Palukaitis and Carr, 2008; Lewsey *et al.*, 2009).

Both spray and seed treatment of salicylic acid against tobamoviruses reduced the concentration of virus and this was confirmed through DAC- ELISA (Madhusudhan *et al.*, 2011).

Effects of viruside, 83-fatty acid and salicylic acid (SA) on PRSV and relative defensing enzyme activities in papaya leaves were studied by Zhang *et al.* (2009) by artificial PRSV inoculation. They reported that, both 83-fatty acid and salicylic acid could activate polyphenol oxidase (PPO), superoxide dismutase (SOD) and peroxidase (POD). Hence the PRSV resistance increased and the virus levels were controlled effectively.

The control efficiency of different defence inducers *viz.*, salicylic acid (SA) @ 50 mg/L, methyl jasmonate (MeJA) @ 0.05 mmol/L and chitosan (CTS) @ 1% were recorded by Lai *et al.* (2012) against PRSV by inoculating papaya plants after spraying inducers of different concentrations. Every inducer increased the resistance of PRSV, while the control efficiency of SA was superior (77.19%) to MeJA (53.11%) and CTS (22.95%).

Antoniw and White (1979) conducted preliminary investigations on the effect of aspirin against mosaic virus in tobacco. They observed that injection of plants with aspirin induced virus resistance and associated proteins and can therefore be considered as a potential antiviral chemical. Aspirin applied as root drenches or leaf injections (1.1mM) reduced the disease severity following challenge with *Tobacco necrosis virus* in tobacco. However, spray treatments were found to be ineffective (Dennis and Guest,

1995). Systemic resistance to tomato plants against *Ralstonia solanacearum* was induced following the application of acetyl salicylic acid or SA @ 0.25 mM and 0.5 mM and considerably reduced disease severity (Abd-el-Said *et al*., 1996)

Senaratna *et al*. (2000) studied the effects of SA and acetyl salicylic acid in bean and tomato plants grown from seeds treated with these chemicals. They observed that the application imparts multiple stress tolerance which is more consistent with the signaling role of these molecules. Similar results were obtained when the treatments were applied in the form of soil drenching.

El Mougy (2004) reported that application of acetyl salicylic acid gave a considerable reduction in the root rot disease of lupine and that it may be because of the action of aspirin as a plant defense inducer. Reddy *et al*. (2006) evaluated the efficacy of antiphytoviral chemicals *viz*., DHT [2,4-dioxo-hexahydro-1,3,5-triazine], acetylsalicylic acid [aspirin], salicylic acid and benzoic acid against *Urdbean leaf crinkle virus* (ULCV) in urdbean (*Vigna mungo*) and reported that, DHT significantly increased the incubation period whereas acetyl salicylic acid was found to be the least effective. Horotan and Apahidean (2015) stated that aspirin generates normal growth and resistance against fungal pathogens in tomato.

Rhizobacteria – mediated induced systemic resistance has been discussed by Van Loon *et al*. (1998) and it is stated that various strains of *Pseudomonas putida*, and *P. fluorescens* confer resistance in plants against *Cucumber mosaic virus* in cucumber and tomato; *Tobacco mosaic virus* and *Tobacco necrosis virus* in tobacco. Similarly, treatment of seeds and seedlings with *Pseudomonas fluorescens* was effective in reducing the incidence of *Bean common mosaic virus* on French bean (Kumar *et* al., 2005). A study was conducted by Barakat *et al*. (2012) on induction of systemic resistance in watermelon plants against *Watermelon mosaic virus*- 2, which is a

potyvirus whose genome structure is closely related to PRSV. The study revealed that bacterial and fungal inducers were more effective bioagents than plant oils to induce SAR in water melon plants. Seed inoculation with *Bacillus polymyxa*, *B. megaterium* and *Trichoderma harzianum* gave a reduction of infection by 29.10 per cent, 35.5 per cent and 42 per cent respectively.

The efficiency of two *Bacillus* species *viz.*, *B. firmus* and *B. subtilis* and a mixture of both was investigated by Abd El- Shafi and Yesser (2012) against ZYMV, a potyvirus in squash plants. A reduction in percentage of infection was observed when the two species were mixed and applied. Moreover, it showed an increase in the growth rate of plants.

Ruwanthi *et al.* (2014) demonstrated the influence of PGPR on disease incidence by PRSV. Five *Bacillus* spp. were isolated from the rhizosphere of papaya and these were applied to papaya plants with a concentration of 10^8 cfu/ml in the form of soil application followed by mechanical inoculation of PRSV and spraying on test plants already inoculated with PRSV. Isolate B2 Madampe (T5) was recorded as the most effective isolate giving considerable reduction in virus titre in plants which was validated through DAC- ELISA. Abdalla *et al.* (2017) reported that PGPR strains are superior to conventional chemicals for controlling PRSV- W in squash and soil drenching was recorded as the best method of application followed by root dipping and seed coating.

Lecanicillium spp., which are entomopathogenic fungi exhibit high pathogenic activity against aphids (Jackson *et al.*, 1985). Cherian (1998) reported that *Verticillium lecanii* is effective against whitefly, *Bemisia tabaci*, which are vectors of *Tomato leaf curl virus*. Yeon *et al.* (2008) reported 72 to 97 per cent mortality in field evaluation of *Lecanicillium* against green peach aphid, *Myzus persicae* which is a potential vector of PRSV.

A study was conducted by Rajinimala *et al.* (2009) on management of *Bitter gourd yellow mosaic virus* BGYMV using biotic and abiotic defense inducers *viz.*, *Pseudomonas chlororaphis*, *P. fluorescens*-A, *Bougainvillae spectabilis* leaf extract, Bion 200 ppm and insecticide monocrotophos in bittergourd plants. All the treatments, except *Pseudomonas* spp. were applied four times starting from 15 DAS at 15 days interval and the *Pseudomonas* spp was applied as seed treatment. The BGYMV was artificially inoculated at 15 days after sowing. The results revealed that the *B. spectabilis* treated plots recorded the least disease incidence of 33.33%. Other treatments recorded 66.66 % whereas control recorded 100 % incidence of BGYMV at 75 DAS.

Experiments carried out to manage PRSV disease by insecticides and mineral oil sprays were not successful because of large number of species of aphids acting as vectors and their non - persistent relationship with the virus (Horkness, 1967; Acosta, 1969). Bhargava and Khurana (1969) reported that spray of castor and light paraffin oils (as 1 % emulsion in water) caused acute phytotoxicity whereas mustard and coconut oils induced milder effect on 6 to 8 weeks old papaya seedlings, which recovered later. Transmission of the virus by *Aphis gossypii* from or to sprayed plants was reduced or prevented. Groundnut oil (2%) caused slight stunting and at one per cent totally inhibited aphid transmission upto 3 days without phytotoxicity.

Verma and Kumar (1980) reported that foliar sprays of leaf extract of *Mirabilis jalapa* caused a marked symptom suppression along with improved growth and flowering in tomato infected with *Tomato yellow mosaic virus* and *Tomato yellow mottle virus*; cucumber infected with *Cucumber mosaic virus* and urd bean infected with *Urd bean mosaic virus*.

Antiviral activity of *Mirabilis jalapa* containing a ribosome inactivating protein (RIP) called Mirabilis antiviral protein (MAP) against mechanically transmitted viruses (PVX, PVY, PoLRV and PSTVd) was studied (Vivanco *et al*., 1999). Root extracts of *M. jalapa* sprayed on test plants 24 h before virus or viroid inoculation inhibited infection by almost 100 per cent as corroborated by infectivity assays and the nucleic acid spot hybridization test.

Reddy *et al*. (2006) tested the efficacy of various plant extracts on the inhibiton of ULCV in urdbean plants mechanically inoculated with the virus and a maximum reduction in transmission of 30 per cent was observed in plants sprayed with *Mirabilis jalapa* leaf extract and 50 per cent reduction of transmission was observed in plants sprayed with leaf extracts of *Datura metel*, *Bougainvillae spectabilis*, *Boerrhavia diffusa* and *Azadirachta indica* as against 80 per cent reduction of transmission in control. Moreover, an increase in incubation period was recorded in all the leaf extract applications with the maximum increase of 8.8 days recorded in case of *Mirabilis jalapa* leaf extract.

Prasad *et al*. (2007) evaluated the antiviral properties of certain botanicals *viz*., *Azadirachta indica, Boerhaavia diffusa, Bougainvillea spectabilis, Clerodendrum inerme, Psidium guajava*, and *Thuja occidentalis* against potyviruses infecting cowpea. Maximum disease reduction upto 40 per cent was recorded in case of *B. diffusa* in field conditions at 0.75 percent concentration of extract. In spray treatment, *B. diffusa* and *B. spectabilis* reduced the disease incidence up to 13 and 12 per cent under screenhouse conditions, whereas *B. diffusa* and *C. inerme* reduced the disease incidence up to 31 and 32 per cent under field conditions. When plant extracts were mixed with BCMV-BlCMV inoculum and young seedlings inoculated, *B. spectabilis*, *C. inerme* and *M. jalapa* extracts reduced the disease incidence up to 42, 40 and 48 per cent respectively under screenhouse conditions as against 80 per cent in control.

Delaying the sowing date along with spraying the faba bean seedlings with six aqueous botanicals extracts under field conditions at fortnightly intervals was reported as a simple strategy for BYMV control in faba beans (Mahdy *et al.*, 2007). The systemic inhibitory effect after three sprays of botanicals *viz.,Chrysanthemum cinerariifolium* (leaves), *Clerodendrum inerme* (leaves), *Dianthus caryophyllus* (leaves), *Mirabilis jalapa* (roots), *Phytolacca americana* (leaves), *P. americana* (roots), *Schinus terebinthifolius* (fruits), and *S. terebinthifolius* (leaves) was recorded as 71,81, 89, 96, 97, 98, 85 and 68 percent respectively.

Elsharkawy and El- Sawy (2015) studied the effect of various plant extracts *viz., Plectranthus tenuiflorus, Azadirachta indica, Clerodendrum inerme, Schinus terebinthifolius* and *Mirabilis jalapa* as antiviral materials against *Bean common mosaic virus* (BCMV), a potyvirus in bean plants. The mixing of *P. tenuiflorus* extracts with BCMV inoculum achieved the highest reduction in BCMV infection (92%), while the mixing of *S. terebinthifolius* extracts with BCMV inoculum recorded the lowest reduction in BCMV infection (68%). Spray treatments of *P. tenuiflorus* and *M. jalapa* reduced disease incidence to 12 per cent and 17 per cent under greenhouse conditions, whereas under field conditions they reduced disease incidence to 17 and 23 percent respectively.

Humic acid (HA) is a compound, constructed on potassium-humates, which can be utilized successfully in many parts of plant production as a plant growth promoting or soil conditioner for enhancing natural resistance against plant diseases (Scheuerell and Mahaffee, 2006). Moreover, humic acid trigger the soil microorganisms (Qualls, 2004).

Humic acids in organic soils are known to inhibit the infectivity of TMV in tobacco (Polak and Pospisil, 1995). Application of HA consistently enhanced the

concentration of some antioxidants such as, β-carotene, superoxide dismutases, and ascorbic acid. These antioxidants may play an important role in the regulation of plant growth and development of disease resistance (Dmitrier *et al.*, 2003).

Kshirsagar (2014) investigated the influence of HA on incubation period of PRSV in papaya seedlings. The incubation period (in days) was recorded as 23, 27 and 26 in seeds pre- treated with 10 ppm, seedlings sprayed with 10 ppm HA 72 hours prior inoculation and seedlings sprayed with HA 72 after virus inoculation respectively.

Bondok and Thabet (2016) conducted an experiment where exogenous application of HA (at 0,1 and 2g/l) and a combination of HA and riboflavin, an antioxidant was done on tomato plants to induce systemic resistance against TMV. Based on the estimation of phenolic compounds in the inoculated plants, a combination of HA with riboflavin was considered a better treatment than HA alone. At that, Nagendran *et al.* (2017) reported that 0.2 per cent humic acid given as soil drenching can be practiced for managing mosaic disease in cucumber.

Additionally, Wu *et al.* (2016) reported that humic acid and fulvic acid extracted and purified from soil collected in green houses, where vegetables were grown, had antifungal properties too.

Use of insecticides and micronutrients was one of the strategies outlines by Verghese *et al.* (2001) for the management of papaya ringspot disease. Dar- Non-Wang and Ko (1975) reported that mosaic affected papaya developed deficiency of boron resulting in deformation of fruits which could be rectified by application of 0.25 per cent borax on diseased plants. A study conducted by Lokhande and Moghe (1992) on correlation between yield and different fruit quality traits on PRSV inoculated field grown papaya cv. Honeydew showed highly significant result with inhibited disease severity and improved fruit quality and yield with soil application of 200g N+100g

P/plant in four equal splits followed by foliar spray of 1% urea+0.2% boron+50ppm IAA @ 50, 100, 200 and 300ml spray solutions of each test chemical respectively applied at 90 days interval starting 15 days after transplanting. A combination of 0.5 per cent zinc sulphate and 0.1 per cent boric acid when sprayed in monthly intervals were found to improve the fruit yield and quality.

Jahir Basha (2002) reported that foliar application of boron at 0.2 per cent delayed the symptom expression of PRSV by 3 and 5 days with 70 and 60 per cent disease incidence, when applied before and after inoculation respectively. Under glass house and field condition, the same treatment applied at 15 days intervals showed promising effect with increased petiole length and reduced blister number, distortion of leaves, vein clearing of leaf and shoestring leaf, with mild mosaic symptoms in the seedlings of papaya cv. Solo.

Kunkalikar (2003) investigated the effect of combining insecticide with boron and spraying boron alone. It was observed that the disease incidence was seen 293 days after planting with a fruit yield of 9.12 kg fruit per plant on spraying a combination of phorate and boron whereas the disease incidence occurred 292 days later with a yield of 8.92 kg fruit per plant when boron alone was sprayed. Similarly, delayed occurrence of the PRSV disease incidence by 42 days after sowing was also recorded by Hemavati (2005) with application of boron @1.5 g/l which may be due to callose deposition along the lesion.

Manjunatha (2012) reported the effect of certain micronutrients and their combinations on the incidence of PRSV in papaya variety Red Lady. The percentage of PRSV incidence was recorded in plants which were given foliar sprays of zinc sulphate (0.5 %), iron sulphate (0.5 %) and borax (0.1 %) at 3, 6 and 9 months after transplanting. A gradual increase was seen in borax treated papaya plants and a disease

incidence of 43 percent was recorded 9 months after transplanting (MAT) and a similar trend was observed in the case of plants treated with iron sulphate and zinc sulphate with a maximum disease incidence of 41.33 and 46.33 per cent as against 48.67 percent in untreated control. However, combining the above three treatments in the same concentration except for zinc sulphate @ 0.25 per cent gave a much reduced disease incidence of 33.33 percent as against 48. 67 in untreated control.

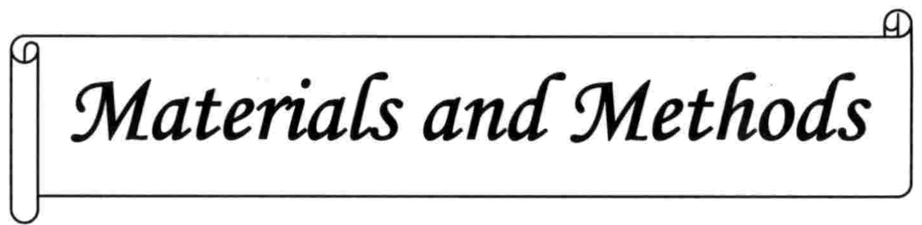
The effect of soluble silicic acid on PRSV incidence was also studied (Manjunatha, 2012) at different concentrations *viz.*, 5ml/l, 4ml/l and 3ml/l and the disease incidence of 25, 26.67 and 28.33 were recorded in each concentration, 9 MAT as against 48.67 in untreated control.

Lokesh (2014) studied the influence of micronutrients on PRSV incidence of papaya cv. Red Lady and reported that no significant difference was recorded among the treatments *viz.*, borax, zinc sulphate and their combination at different concentrations. However, the least incidence of PRSV was observed in plants applied with borax at 0.50% + ZnSO4 at 0.25% (26.50 %) whereas the maximum incidence was noticed in the untreated control (52.08%).

Furthermore, as a part of integrated disease management, Nagendran *et al.* (2017) recommended that a spray of boron 0.2 per cent, 0.5 per cent zinc or micronuritent mix @ 2ml/l combined with soil drenching with humic acid @ 5ml/ l against yellow vein mosaic disease in okra.

The effect of potassium silicate against many fungal diseases has been reported by researchers. However, a perusal of literature shows that not much has been studied about the effect of silicon/ silicate in controlling viral pathogens.

Zellner *et al.* (2011) observed that most of the tobacco plants treated with 0.1 mM potassium silicate did not exhibit levels of systemic *Tobacco ringspot virus* (TRSV) symptoms to the same extent as the controls, and plants grown in elevated levels of silicon showed a delay in *Tobacco ringspot virus* systemic symptom formation. It was also noticed that the foliar accumulation of silicon may be part of a defense response in tobacco to TRSV. Silicon supplementation in cucumber plants infected with *Cucumber mosaic virus* caused a shift in gene expression (Holz *et al.* 2014). Elsharkawy and Mousa (2015) found that silicon application to cucumber plants significantly reduced the severity of PRSV and its accumulation in leaves. The expression of the majority of various pathogen-related genes was meditated by silicon treatment.


Materials and Methods

3. MATERIALS AND METHODS

The present study on "Characterization, host range and management of *Papaya ringspot virus* (PRSV)" was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara during 2016-2018. The details of materials and methods are described in this chapter. The details of laboratory equipments used in the study are given in Appendix V.

3.1 SURVEY FOR PRSV INCIDENCE AND COLLECTION OF SAMPLES

Purposive sampling surveys were conducted at selected locations of Thrissur district, Kerala where papaya was grown commercially. The locations were, Vellikulangara, Muringoor, Koratty, Venginissery, Melur, Pazhayanur, Puthur and Vellanikkara (Plate 1). The observations on growth stage of the crop, the variety grown, total number of plants, number of infected plants, per cent disease incidence, per cent disease severity and symptoms expressed on different parts of plant were recorded. The per cent disease incidence at each location was recorded using the following formula:

$$\text{Per cent disease incidence} = \left\{ \frac{\text{Number of infected plants}}{\text{Total number of plants observed}} \right\} \times 100$$

Based on the severity of the symptoms, the vulnerability index (V) was calculated. The scoring was done as per the 0-5 scale developed by Bos (1982) as mentioned below:

0 = no symptom

1 = slight vein clearing, very little mottling of light and dark green colour in younger leaves

2 = mottling of leaves with light and dark green

3 = blisters and raised surfaces on the leaves

4 = distortion of leaves

5 = stunting of plant with negligible or no flowering and fruiting

Based on the scoring, vulnerability index (V) was calculated using the following equation,

$$V = \frac{(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5) \times 100}{n_t (n_c - 1)}$$

where, $n_0, n_1, .. n_5$ = number of plants in disease category 0 to 5 respectively

n_t = total number of plants

n_c = total number of categories

This index obtained was expressed as Per cent disease severity (PDS).

3.2. MAINTENANCE OF VIRUS CULTURE

The virus culture was maintained by repeated transfers of the virus inoculum collected from the field to healthy plants through mechanical inoculation using 0.1 M potassium phosphate buffer (pH 7.0) (Appendix I) and these were maintained in plant virology glass house under insect proof conditions. This was served as inoculum for further studies in the present investigation.

3.3. SYMPTOMATOLOGY OF PAPAYA RINGSPOT DISEASE

The types of symptoms expressed on different parts of the infected plant *viz.*, leaves, petiole, fruits and stem were documented in naturally occurring field conditions during the survey. The leaves showing typical symptoms were collected from the field and artificial inoculation with infectious sap was carried out on healthy papaya plants and maintained in insect proof glass house conditions. The symptoms produced on the inoculated plants and the incubation period of the virus *i.e.* the number of days to develop symptoms under artificial conditions were documented.

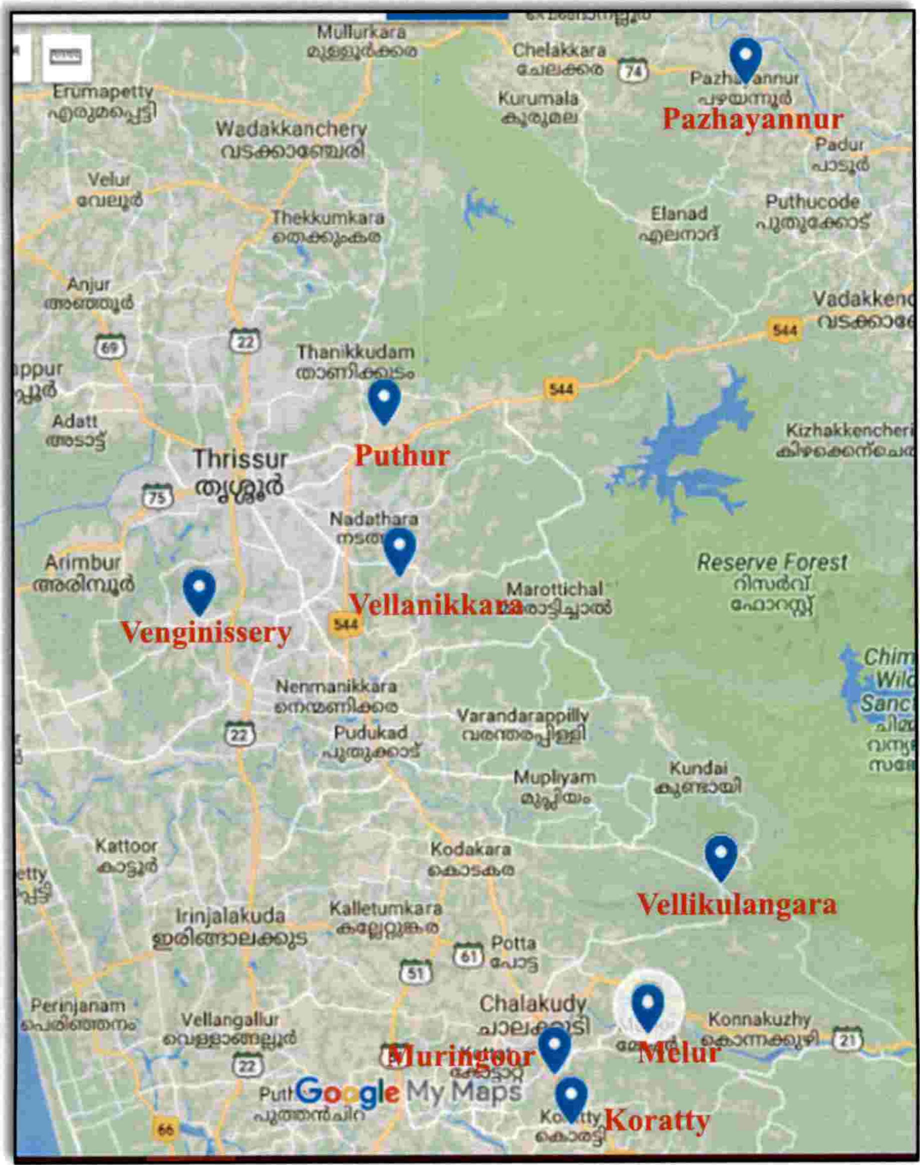


Plate 1: Locations of purposive sampling surveys

3.4. HISTOPATHOLOGY OF PAPAYA RINGSPOT VIRUS DISEASE

Thin sections of healthy and infected leaves showing different symptoms mainly, chlorosis, mottling, green island symptom and puckering were taken and observed under the microscope to study the histological changes brought about due to virus infection.

3.5. BIOLOGICAL CHARACTERIZATION OF THE VIRUS

3.5.1 Transmission studies

3.5.1.1. Mechanical Transmission

Virus infected papaya plants maintained in insect proof net house were used as source of virus inoculum. Sap transmission was conducted using 0.1 M potassium phosphate buffer (pH 7.0). Seedlings of papaya var. Red Lady were raised in polybags under insect proof net house conditions. The leaf sample was homogenized in a pre-chilled pestle and mortar using an equal quantity of buffer. The crude sap was filtered using a double layered muslin cloth. The papaya plants at six leaf stage were used as test plants and leaves were uniformly dusted with carborundum powder (600 mesh) prior to inoculation. The filtered sap was rubbed on the leaf surface carefully using cotton in one direction from petiole towards apex on the leaves of test plants which were uniformly dusted with carborundum powder (600 mesh). After five minutes, the test plants were washed with distilled water to remove excess inoculum and extraneous particles. The inoculated plants were then kept under insect proof conditions and monitored for symptom development. The procedure followed for mechanical inoculation is illustrated in Plate 2. The number of infected plants were recorded and the symptoms developed were documented upto 60 days after inoculation (DAI). The symptomatic plants were subjected to DAC – ELISA as described in section 3.6.1. to confirm the presence of the virus.

a. Infected leaf (inoculum)

b. Pre - chilled pestle and mortar, 0.1 M phosphate buffer and carborundum

c. Homogenization of infected leaf @ 1g/ ml of buffer

d. Filtered sap

e. Dusting the healthy leaf with carborundum

f. Swabbing leaf with filtered sap from petiole to apex

g. Washing inoculated leaf with distilled water

Plate 2: Mechanical inoculation of the virus

3.5.1.2. Seed Transmission

Seeds were collected from infected as well as healthy papaya fruits. The seeds were extracted, washed thoroughly under running water to remove the gelatinous material and air-dried for one week. These were sown in pro-trays filled with a mixture of vermicompost and coirpith in 1:1 ratio. The germinated seedlings were transplanted in polybags kept under insect proof conditions and monitored for symptom expression for up to 60 days. The seedlings raised from seeds of healthy and infected fruits were subjected to DAC- ELISA as described under section 3.6.1. to confirm the presence of virus.

3.5.2 Host range studies

The host range of PRSV was investigated by artificial inoculation of the virus to plant species belonging to different families including common weeds seen in and around papaya fields (Table 3). The plants were raised from healthy seeds in polybags containing soil and vermicompost in 1:1 ratio. Virus infected papaya plants maintained in insect proof net house were used as source of inoculum. Ten plants of each species were sap inoculated following the same procedure as in section 3.5.1.1.and monitored for symptom expression. An uninoculated plant was also maintained as control. The number of infected plants were recorded and plants were monitored for the development of symptoms for upto 60 DAI. The infection was further confirmed through DAC-ELISA test of the symptomatic plants as described under section 3.6.1. and by back inoculation to indicator plant *Chenopodium amaranticolor*.

Table 3: List of plants used for host range studies

Sl. No.	Host	Common Name	Family
1.	*Amaranthus polygamous*	Green amaranthus	Amaranthaceae
2.	*A. polygamous*	Red amaranthus	
3.	*Gomphrena globosa*	Globe amaranth	
4.	*Alternanthera sessilis* (weed)	Sessile joyweed	
5.	*Chenopodium amaranticolor*	Chenopodium	Chenopodiaceae
6.	*Momordica charantia*	Bitter gourd	Cucurbitaceae
7.	*Trichosanthes cucumerina*	Snake gourd	
8.	*Cucumis sativus*	Salad cucumber	
9.	*Cucurbita moschata*	Pumpkin	
10.	*Solanum lycopersicum*	Tomato	Solanaceae
11.	*Nicotiana tabaccum*	Tobacco	
12.	*Nicotiana glutinosa*	Tobacco	
13.	*Capsicum annuum*	Chilli	
14.	*Solanum melongena*	Brinjal	
15.	*Vigna unguiculata*	Cowpea	Fabaceae
16.	*Abelmoschus esculenthus*	Bhindi	Malvaceae
17.	*Musa sp.*	Banana	Musaceae
18.	*Synedrella nodiflora* (weed)	Cinderella weed	Asteraceae
19.	*Sphagneticola trilobata* (weed)	Singapore daisy	
20.	*Ageratum conyzoides* (weed)	Goat weed	
21.	*Cleome viscosa* (weed)	Tick weed	Cleomaceae

3.5.3. Electron microscopy

The morphological characters of the viral particles were studied by Electron microscopy (EM). The symptomatic leaves of papaya were collected from the infected plants maintained in the net house and despatched to Advanced Centre of Plant Virology, Department of Plant Pathology, I.A.R.I., New Delhi where facility is available.

The protocol followed for electron microscopy was leaf dip method. One centimeter square of infected leaf tissue was taken and crushed on a clean glass slide using 0.1 M phosphate buffer (pH: 7.0) containing one percent nicotine sulphate. A drop of extract was placed on the carbon coated grid of the electron microscope and allowed to stay for 2 minutes. This was followed by washing the grid with ten drops of distilled water and staining with 2 per cent uranyl acetate. The excess stain was removed by touching the edge of the grid with a piece of filter paper and it was examined under transmission electron microscope. The electron micrographs of the virus particles were taken on a plate film. The size and shape of the viral particles were documented.

3.6. DETECTION OF PRSV BY PROTEIN BASED METHODS

3.6.1. Direct Antigen Coating ELISA (DAC – ELISA)

Protein based detection method *viz.*, DAC – ELISA was validated. The experiment was carried out at Advanced Research Centre for Plant Disease Diagnosis, College of Agriculture, Vellayani. The infected leaf samples of papaya showing typical symptoms and healthy control were tested for virus infection by DAC – ELISA using primary antibody of PRSV obtained from DSMZ (German Collection of Microorganisms and Cell Culture), Germany. The protocol described by Huguenot *et al.* (1993) was followed for the detection.

One gram of infected young leaf was homogenized in 5 ml of coating buffer (carbonate buffer) (Appendix II) with a pinch of 2 per cent polyvinyl pyrolidine (PVP) in a pre-chilled mortar and pestle under chilled conditions. The healthy plant extract was prepared by using leaves of healthy plants to serve as control.

The homogenate was transferred to 2 ml centrifuge tubes and centrifuged at 5000 rpm for 10 min at 4°C. 100 µl of the supernatant was pipetted and dispensed into the wells of microtitre plate (with 96 wells, Tarsons Products Pvt. Ltd.). The treatments were replicated thrice. The plates were covered with aluminum foil and incubated at 37°C for 2 h. After incubation, the plates were washed with Phosphate Buffer Saline-Tween (PBS- T) (Appendix II) three times, each for a duration of 3 min using an ELISA plate washer (PW- 40, BIORAD) followed by gentle tapping on paper towel to remove the residual liquid.100 µl of blocking solution *viz.*, 5 per cent spray dried milk (SDM) (Appendix II) was added to the wells and incubated at 37°C for 2 h and washed again with PBS- T thrice, as mentioned in the previous step. The primary antibody was diluted in PBS- T Polyvinyl pyrrolidone ovalbumin (PBS-TPO) (Appendix II) in 1: 200 dilution. 100 µl of this diluted antiserum was dispensed into respective wells and incubated overnight at 4°C. The plates were washed again with PBS- T and then 100 µl of secondary antibody conjugated with alkaline phosphatase diluted in PBS – TPO (1:10,000) was added into the respective wells and incubated for 2 h at 37°C. The unbound secondary antibodies were washed thrice with PBS- T as done before. 100 µl of freshly prepared substrate *p*- nitrophenyl phosphate (Appendix II) was added to each well and incubated in dark at 37°C for 1 h for colour development. The absorbance value was recorded at 405 nm using an ELISA reader (Microplate Reader 680, BIORAD). The absorbance values of the test samples and healthy samples were compared and if the absorbance values of the test samples were twice more than that of healthy samples, the test samples were considered as positive for virus infection.

3.7. MOLECULAR CHARACTERIZATION OF PRSV ISOLATES

The experiments on molecular characterization of the virus were carried out at Centre of Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara. The leaves showing typical symptoms were collected from different papaya growing fields of Thrissur district (five locations) and brought to the laboratory in ice box for RNA isolation. The collection of samples was done during early morning and immediately kept in ice box.

3.7.1 Isolation of RNA

The total RNA of the infected sample was isolated using TRIzol reagent method (Sigma Aldrich). All the glasswares and micropipette tips used for RNA isolation were pre- treated overnight with 0.1 per cent diethyl pyrocarbonate (DEPC) (Appendix III) and double autoclaved at 121° C temperature and 15 lb pressure. The infected leaf sample collected from the field was wiped with RNase ZAP on both the sides. 100 mg of leaf sample was then ground to fine powder using liquid nitrogen with pestle and mortar along with a pinch of PVP and 50 μl β- mercaptoethanol. 1000 μl of TRIzol reagent was added to homogenize the powdered tissue. This was transferred to 2ml RNase free microcentrifuge tubes incubated until the homogenate became brown in colour. Once the homogenate became brown, it was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was turned to fresh 2ml centrifuge tubes. To this, 200 μl of chloroform was added and the contents were mixed well by shaking vigorously in a vortex mixer and incubated at room temperature for 10 min. Three separate phases were formed when centrifuged at 13,000 rpm for 15 min at 4°C. Out of the three layers, the colourless upper phase containing the RNA was carefully pipetted into a fresh 2 ml microcentrifuge tube and an equal amount of isopropanol was added to this followed by mixing slowly by inversion and incubated for one hour in ice. Then it was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 75 per cent ethanol by centrifugation at 7500 rpm for 10 min

at 4°C. Finally, the pellet was air dried and suspended in 20 µl of autoclaved DEPC water. Following this method, RNA was also isolated from leaf samples collected from healthy plants which served as control.

3.7.1.1 Gel documentation

Formaldehyde agarose gel (1 %) (Appendix III) was used to check the purity of RNA in 1X MOPS buffer (Appendix III). An aliquot of RNA sample (5 µl) mixed with 1 µl of loading dye was loaded in each of the wells of the gel. The electrophoresis was carried out at 50 V till the dye moved 5 cm from the well (BIO RAD Power Pac HV, USA). The gel was then visualized and the image was documented using BIORAD Molecular Imager (Gel Doc™ XR+).

3.7.1.2 Spectrophotometer analysis

The quantity and quality of RNA was determined by recording the concentration and the absorbance values of RNA samples using NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). The spectrophotometer was calibrated to blank (zero absorbance) with 1 µl of sterile DEPC water. Then, the concentration of the RNA samples (1 µl each) was recorded. The absorbance was recorded at 260 and 280 nm wavelength and purity was indicated by the ratio $A_{260/280}$.

3.7.2 Synthesis of First Strand Complementary DNA (cDNA)

The isolated RNA was used to synthesise complementary DNA using RevertAid First Strand cDNA synthesis kit (Catalogue No: K1621, Thermo Scientific) as per manufacturer's instructions. The components of the reaction mixture used are given in Table 4. The reagents were added into sterile nuclease free polypropylene PCR tubes on ice according to the sequential order indicated in Table 4. The contents were mixed gently and incubated at 42°C for 60 min in PCR machine (Applied Biosystems). This was followed by heating at 70°C for 5 min for termination.

Table 4: Reagents used for cDNA synthesis

Sl. No.	Name of the component	Volume (µl)
1.	RNA template	2
2.	Oligo $(dT)_{18}$ primer	1
3.	Water (nuclease- free)	9
4.	5X reaction buffer	4
5.	Ribolock RNase inhibitor 20 U/ µl	1
6.	10 mM dNTP mix	2
7.	Revert Aid M-MuLV RT (200u/ µl)	1
	Total volume	**20**

3.7.3. Standardization of Polymerase Chain Reaction (PCR)

3.7.3.1. Standardization of PCR conditions for reported primers

Primer pair specific to polyprotein gene of PRSV *viz*., partial nuclear inclusion b (NIb) gene, complete coat protein (CP) and 3' untranslated region (UTR) reported by Srinivasulu and Sai Gopal (2011) (Table 5) was used for characterization of the isolates of PRSV collected from Thrissur district.

Table 5: Details of the reported primers used for amplification

Primer	Sequence (5'-3')	Length	Tm value(°C)
PRSV – F	ATCACAATGTATTACGC	17 bp	48.1
PRSV – R	CTCTCATTCTAAGAGGCTC	19 bp	53.0

PCR was carried out in PCR thermo Cycler (Applied Biosystems) by using the reported primers. The dilution of cDNA was also standardized and the reaction was done at four different dilutions of cDNA *viz*., 1:1, 1:0.5, 1:0.25 and 1:0.1.The reaction

was carried out at 45, 46, 47, 48, 49 and 50^{0}C in order to standardize the annealing temperature using Veritiflex program in Applied Biosystems PCR Thermo Cycler. The best annealing temperature and dilution of cDNA was selected based on the quality of the band obtained during gel documentation.

The components of the reaction mixture used for PCR is presented in Table 6. After the preparation of the reaction mixture, these were mixed gently in a mini spinner and PCR was carried out.

Table 6: Components of reaction mixture used for PCR

Sl. No.	Component	Quantity (μl)
1.	Template cDNA (44.40 - 54.40 ng/μl)	2
2.	10X PCR buffer (with 1.5mM $MgCl_2$)	3
3.	dNTP mixture (2.5mM each)	3
5.	Forward primer	1
6.	Reverse primer	1
7.	*Taq* polymerase	1
8.	Water	19
	Total reaction volume	**30**

3.7.4. PCR amplification profile

Once the dilution of template cDNA and annealing temperature of reaction program was standardized, RT- PCR was carried out as follows:

Initial denaturation - 94° C - 5 min

Denaturation - 94° C - 30 sec

Annealing - 45° C - 45 sec } 35 cycles

Extension - 72° C - 2 min

Final extension - 72° C - 15 min

Control reaction with healthy samples was also carried out to differentiate the infected isolates and to confirm the presence of virus in them.

3.7.4.1.. Gel Documentation of PCR products

Once the reaction was over, the PCR products were analyzed on 1.2 per cent agarose gel prepared in 1 X TAE buffer (Appendix IV) containing 0.5μg/ ml ethidium bromide. 5 μl of PCR product was loaded into the well with 1 μl of tracking dye. 100 bp plus DNA ladder (Thermo Scientific, USA) was loaded in one well to compare the size of the PCR product. Electrophoresis was done at 80 V power supply with 1X TAE buffer as electrophoresis buffer till the dye moved 8 cm from the well. The amplified product was visualized and the image was documented using BIORAD Molecular Imager (Gel Doc^{TM} XR+).

3.7.5. Sequencing

The amplified PCR products were sent to Agri Genome, Kochi for purification and sequencing. The sequence information of the amplified gene fraction was further analyzed using various bioinformatic tools for the characterization of virus isolates.

3.7.6. *In silico* analysis of sequences

3.7.6.1. Homology search

The sequences generated in this study were compared with the PRSV sequences coding for NIb and CP regions available in the National Centre of Biotechnology Information (NCBI) database using BLAST tool. Nucleotide BLAST (BLASTn) was carried out for the viral sequences to find out the best aligned sequences available in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.7.6.2. Amino acid analysis

The amino acid sequences were deduced using ExPASy translate tool (http://web.expasy.org/cgi-bin/translate). The amino acid sequences were subjected to

protein blast in order to assess the identity percentage with reported sequences of PRSV isolates available on NCBI database.

3.7.6.3. Phylogenetic analysis

The nucleotide sequences of the five PRSV isolates generated in this study were aligned with gene sequences of 21 other PRSV isolates from different geographical regions in India and elsewhere whose sequences were retrieved from NCBI database (Table 7). The alignment of sequences was done using Clustal W program available in MEGA 7.0 software. The phylogenetic analysis of the aligned sequences was conducted using MEGA 7.0 software by constructing phylogenetic tree by Neighbor - joining method (Saitou and Nei, 1987).

Table 7: Details of selected nuclear inclusion b and coat protein sequences of PRSV isolates used for phylogenetic analysis

Sl. No.	Accession No.	Isolate Name	Host	Source	Reference
1.	DQ666640.1	KE-Ca	Papaya	Calicut	Srinivasulu and Sai Gopal, 2011
2.	DQ666641.1	TA- Ti		Tiruvallur	
3.	DQ666638.1	AP-Ko		Kovvur	
4.	DQ666639.1	KA-Gu		Gulbarga	
5.	AY839864.1	AP-Te		Hyderabad	
6.	AY839863.1	AP-Ra		Rly Kodur	
7.	AY839865.1	KA-Ho		Hospet	
8.	AF323637.1	PRSV-AP	Papaya	Andhra Pradesh	Roy,G. and Jain,R.K., unpublished data, 2000, unreferenced
9.	AF323638.1	PRSV-UP		Uttar Pradesh	
10.	KP161501.1	TN TDV SG1	Snake gourd	Tindivanam	Nagendran,K., Mohankumar,S., Keerthana,U., Naidu,R. and Karthikeyan,G.,unpublished data, 2014, unreferenced
11.	KP161500.1	TN METPUM1	Pumpkin	Mettur	
12.	KP161498.1	TN NGK Cucu2	Cucumber	Nagercoil	Nagendran,K., Mohankumar,S., Priyanka,R., Naidu,R.A. and Karthikeyan,G., unpublished data, 2014, unreferenced
13.	KP161494.1	TN NGK PUM1	Pumpkin	Nagercoil	
14.	KP161495.1	TN NGK PUM2	Pumpkin	Nagercoil	
15.	KP161499.1	TN UDU RG1	Ridge gourd	Udumalpet	

Table 7 Contd…

16.	LC331253.1		*Clitoria ternatea*	West Bengal	Sultana *et al.*, 2018
17.	JX661505.1	PK- Dar 3	Papaya	Pakistan	Naseem *et al.*, 2013
18.	JX024999.1	TJ 11			
19.	X67672.1	PRSV-HA	Papaya	China	Yeh *et al.*, 1992
20.	KX904879.1	Jinan	Zucchini	China	Cheng *et al.*, 2017
21.	AB044341.1	R175P	Cucumber	Taiwan	Maoka, T. and Noda,C., unpublished data, 2000, unreferenced

3.8. MANAGEMENT OF PAPAYA RINGSPOT DISEASE (PRSD)

The effect of different chemicals, plant extracts, micronutrients and microbial formulations against PRSD was evaluated under pot culture conditions. The experiment was conducted in an insect proof net house, Department of Plant Pathology, COH, Vellanikkara from October 2017 to January 2018. The most popular and widely grown variety Red Lady which was found to be very susceptible to the disease during the survey was selected to test the efficacy of different treatments in this experiment. The experimental details were as follows:

Design: Complete Randomized Design

No. of treatments: 14

No. of replications: 3

No. of plants per replication: 3

Variety: Red Lady

Potting mixture to fill the growbags were prepared by mixing soil, cowdung and coirpith in the ratio 1:1:0.5. One month old healthy seedlings of variety Red Lady were transplanted into these growbags. The general view of insect proof net house where the pot culture experiment was conducted is given in Plate 3.

One month after planting, once the plant established itself in the pot, the application of treatments was carried out. The methods of application followed for all treatments were foliar spraying and soil drenching. 1 week after the first application, the plants were subjected to challenge inoculation of PRSV by sap transmission as described in section 3.5.1.1. using the inoculum maintained in the insect proof net house. The second round of treatment application was done two weeks after the first application. Likewise, the application of treatments were carried out thrice at fortnightly intervals. The treatment details of pot culture experiment is given in Table 8. The plants were observed daily and monitored for symptom development.

The per cent disease severity was recorded at weekly intervals from the day of first appearance of symptoms. This was calculated based on the 0-5 scale developed by Bos (1982) as mentioned in section 3.1. The biometric characters like the plant height and mean girth of the stem were also recorded after every spray. The effect of different treatments on chlorophyll content was assessed using SPAD meter two weeks after the final application. Furthermore, following the final application, the apical leaves from each treatment were subjected to DAC- ELISA as mentioned under section 3.5 in order to record the effect of each treatment on the virus titre. The atmospheric temperature (maximum and minimum) prevailing at the time of experiment was recorded and tabulated at weekly intervals (Appendix VI).

Statistical analysis *i.e.* analysis of variance (ANOVA) was performed for the data obtained from this experiment using Web Agri Stat Package (WASP 2.0). Appropriate transformation of data was carried out as required (Gomez and Gomez, 1984).

Plate 3: General view of site of pot culture experiment

Table 8: Treatment details of pot culture experiment

Treatment	Treatment details
T1	Salicylic acid - 0.15 gL^{-1}
T2	Acetyl salicylic acid - 0.15 gL^{-1}
T3	*Pseudomonas fluorescens* - 2%
T4	PGPR mix II- 2%
T5	*Lecanicillium lecanii* - 2%
T6	*Mirabilis jalapa* - 10 % leaf extract
T7	*Bougainvillea spectabilis* - 10 % leaf extract
T8	Perfekt - 0.1%
T9	Micronutrient formulation Sampoorna - 1%
T10	Solubor - 1%
T11	Humic acid - 0.2%
T12	Potassium silicate -0.3%
T13	Solubor - 0.1%
T14	Untreated control

Results

4. RESULTS

The results of the project "Characterization, host range and management of *Papaya ringspot virus* (PRSV)" carried out during 2016- 2018 are presented in this chapter.

4.1. SURVEY FOR DISEASE INCIDENCE AND COLLECTION OF SAMPLES

During 2016 - 2018, purposive sampling surveys were carried out in different locations of Thrissur district where papaya was grown commercially. A total of 9 different fields were surveyed. During the survey, the growth stage of the crop,total number of plants, number of plants infected, per cent disease incidence and per cent disease severity were recorded. The results are given in Table 9. The predominant variety cultivated in the fields surveyed was Red Lady which was found to be highly susceptible. During the survey, the samples were also collected from infected plants and brought to the laboratory for further studies.

4.1.1. Assessment of disease incidence and disease severity

During the survey, distinct symptoms observed on different plant parts were documented which is detailed under section 4.3. Among the various fields surveyed, maximum per cent disease incidence (PDI) was recorded in Vellikulangara (99.60%) followed by 98 per cent in Koratty and Venginissery on variety Red Lady. The least disease incidence (25%) was recorded in one of the plots in Muringoor where the same variety was cultivated.

The per cent disease severity (PDS) was expressed by calculating the vulnerability index which was calculated in accordance to the 0-5 scale developed by Bos (1982) as mentioned under section 3.1. The highest PDS (96.67%) was recorded in Puthur followed by 65.7 per cent in Koratty and Venginissery. The PDS recorded in three different plots of Muringoor was 63.67, 46.33 and 39.33 per cent in plot 1, 2 and

3 respectively. Even though the PDI recorded at Vellikulangara was very high, the severity was found to be comparatively low indicated by a PDS of 48.67 per cent.

During the purposive sampling survey conducted at Melur, the PDI and the PDS recorded were 39.28 and 40 per cent respectively on variety Red Lady. At Pazhayanoor, the disease was found to be quite severe with a PDS of 55 per cent and 71.24 per cent of the plants observed were found to be infected.

A roving survey was also conducted in KAU main campus at Vellanikkara and a PDI of 81.8 per cent was recorded with a PDS of 58.18 per cent. Here, both Red Lady and another local variety were observed for symptoms. Both Red Lady and the local variety were infected with the virus.

4.2. MAINTENANCE OF VIRUS CULTURE

The leaf samples of the infected plants showing typical symptoms were collected in polythene covers during the survey and brought to the laboratory. This was used as a source of virus inoculum and was mechanically inoculated to healthy papaya plants maintained in insect proof plant virology glass house, Department of Plant Pathology. Repeated transfers to healthy plants of papaya variety Red Lady were conducted and the virus culture was maintained and used as a source of inoculum for further studies (Plate 4).

4.3. SYMPTOMATOLOGY

The symptoms of the disease expressed on different parts of the papaya plant *viz.*, leaf lamina, leaf veins, petiole, stem and fruits under natural conditions were documented during the survey. The symptoms expressed on healthy papaya seedlings following artificial inoculation were also documented.

Table 9: Incidence and severity of papaya ringspot disease in different locations

Sl. No.	Name of location	Cultivar	Total number of plants	Growth stage of the crop*	PDI	PDS
1.	Vellikulangara	Red Lady	500	H	99.60	48.67
2.	Muringoor Plot 1	Red Lady	300	H	85.00	63.67
3.	,Muringoor Plot 2	Red Lady	120	H	65.50	46.33
4.	Muringoor Plot 3	Red Lady	600	V,F, H	25.00	39.33
5.	Koratty	Red Lady	90	H	98.00	67.50
6.	Venginissery	Red Lady	75	H	98.00	67.50
7.	Melur	Red Lady	800	S, V, F, H	39.28	40.00
8.	Pazhayannur	Red Lady	450	V, F, H	71.24	55.00
9.	Puthur	Red Lady	80	V, H	97.50	96.67
10.	Vellanikkara	Red Lady and local variety	20	V,F,H	81.80	51.18

*S= Seedlings stage, V= Vegetative stage, F= Flowering stage, H= Harvesting

Plate 4: Maintenance of PRSV culture

4.3.1. Symptoms Observed Under Natural Conditions

Different types of symptoms were observed on the leaves under natural conditions.

Mottling was one of the most common symptoms observed. The symptom was equally prominent on old as well as young leaves. Here, the leaves were presented with an irregular distribution of dark and light green areas over the entire lamina (Plate 5a, Fig. A). The leaves also exhibited very small chlorotic spots of size ranging from 1 to 4 millimeter all over the leaf lamina in a scattered manner (Plate 5a, Fig. B). This symptom was seen both on old and young leaves. On some leaves, these spots were found to be coalesced giving an overall mosaic appearance to the leaves (Plate 5a, Fig. C). Depending on the severity of infection, the leaves showed either a dark green or pale green appearance. On plants with mild incidence, fewer chlorotic spots were observed on leaves which still appeared green. However, on severely affected plants, more spots were observed and the leaves turned yellowish green in colour. This type of symptom was documented on older leaves. Furthermore, these leaves were found to be more or less rugose in texture.

The infected leaves also showed wavy margins which were very conspicuous. This type of symptom was more pronounced on older leaves (Plate 5a, Fig. D). Additionally, such leaves also exhibited chlorotic mottling. This symptom was observed in severely stunted plants during the survey.

Another symptom observed both on young and old leaves was puckering. Here, the leaf lamina consisted of blisters or raised portions or areas with bulging of tissue (Plate 5b, Fig. A). This was observed as an initial stage of malformation of leaves. The old leaves exhibiting puckering symptom appeared either dark green or pale green and these were rough in texture compared to healthy leaves (Plate 5b, Fig. B).

Appearance of green islands was also clearly visible on leaves of PRSV infected plants. This symptom was predominantly expressed on older leaves as irregularly

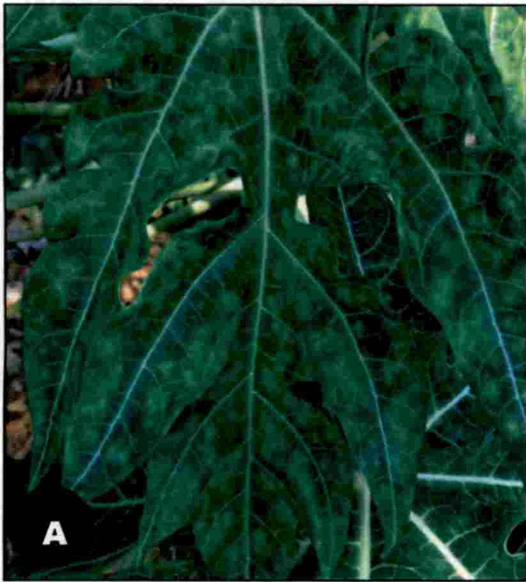
distributed areas of increased and decreased chlorophyll content. The leaves presenting this symptom had their lobes malformed (Plate 5b, Fig. C).

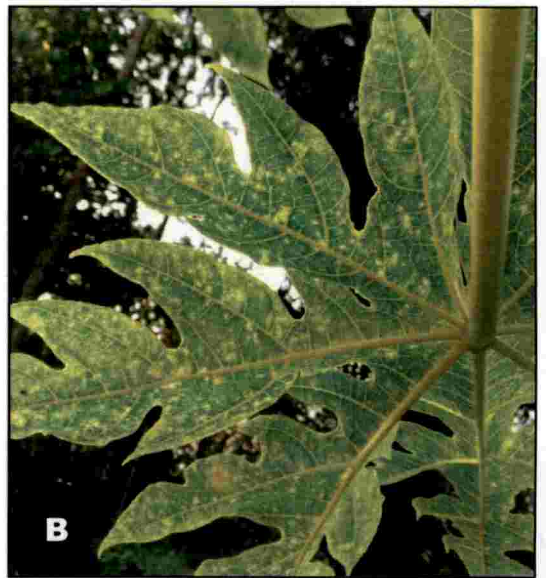
In papaya fields were high PDS was recorded, majority of the leaves were malformed to a greater extent. The veins of the malformed leaves were found to be very thick in comparison to the veins of healthy leaves. Moreover, those veins clustered together leading to the malformation of leaf lobes (Plate 5b, Fig. D).

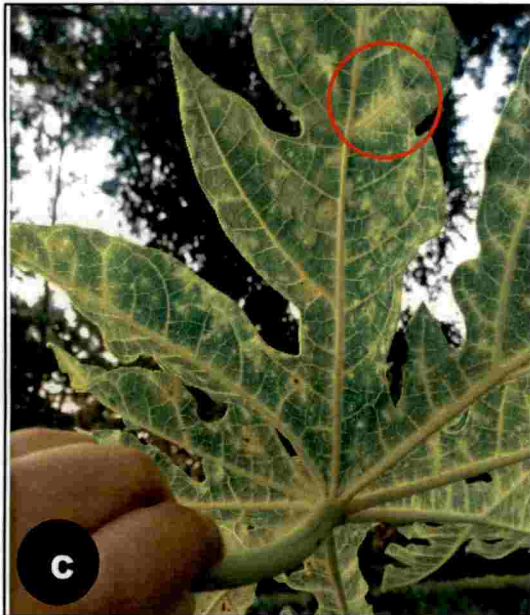
The malformation of the leaf was found to initiate from the central lobe (Plate 5c, Fig. A). The lamina of malformed leaves was highly reduced (Plate 5c, Fig. B). In severe cases, the leaf lamina was completely reduced which became extremely filiform giving a shoestring appearance (Plate 5c, Fig. C). The plants showing this symptom was more or less stunted. The newly emerging leaves of such plants were also found to be puckered and malformed (Plate 5c, Fig. D). In some cases, all the above mentioned symptoms were seen on the leaves as a complex leading to a severe reduction in yield (Plate 5d, Fig. A, Fig. B)

On the petioles of the infected leaves, elongated oily streaks were observed. The streaks were 2 to 4 cm in length and were congregated at the end of the petiole *i.e.* the region adjacent to the stem (Plate 5e, Fig. A).The petioles of the affected leaves also showed abnormal elongation leading to a twisted appearance (Plate 5e, Fig. B). On the upper half of the stem *viz.* the green region, oily irregular broken rings of diameter ranging from 2.5 to 3 cm were present in a scattered manner (Plate 5f).

The affected fruits showed typical ringspot symptom. These were either concentric or broken and the number ranged from few to many depending on the infection (Plate 5g, Fig. A). The size of the ringspot ranged from 8 mm to 1.5 cm. The ringspots remained on the fruit even after the fruits ripened. However, the ripening was uneven (Plate 5g, Fig. B). In some cases, instead of forming a ring, the fruits showed oily streak-like lesions in a disorganized fashion (Plate 5h, Fig.A). The severely affected fruits were completely distorted (Plate 5h, Fig. B) and were of no economic







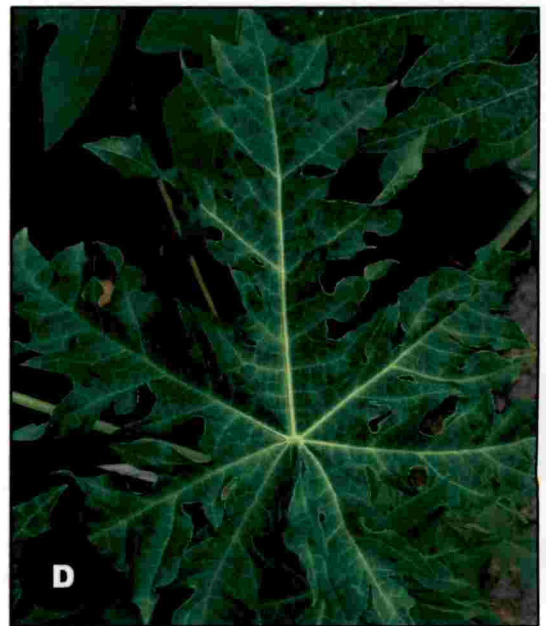


Plate 5a: Symptoms under natural conditions: On leaves (A) Mottling (B) Chlorotic spots (C) Chlorotic spots coalesce to produce mosaic appearance (D) Wavy margins on older leaves

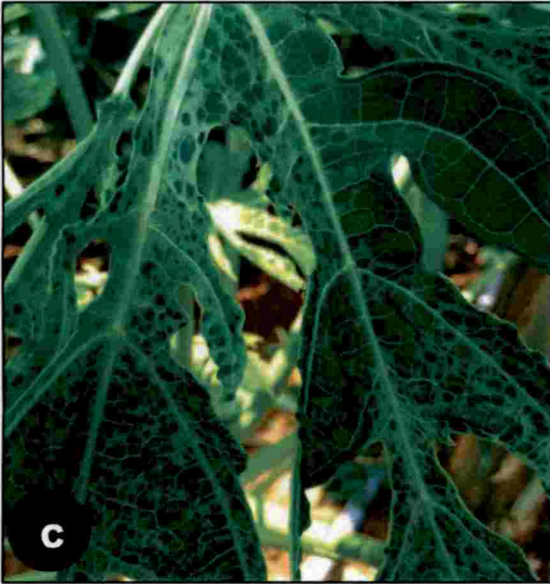


Plate 5b: Symptoms under natural conditions: On leaves (A) Puckering (B) Rugosity (C) Green islands (D) Vein thickening

Plate 5c: Symptoms under natural conditions: On leaves (A) Initiation of malformation from the central lobe (B) Completely malformed leaf (C) Shoestring symptom (D) Newly emerging malformed leaves

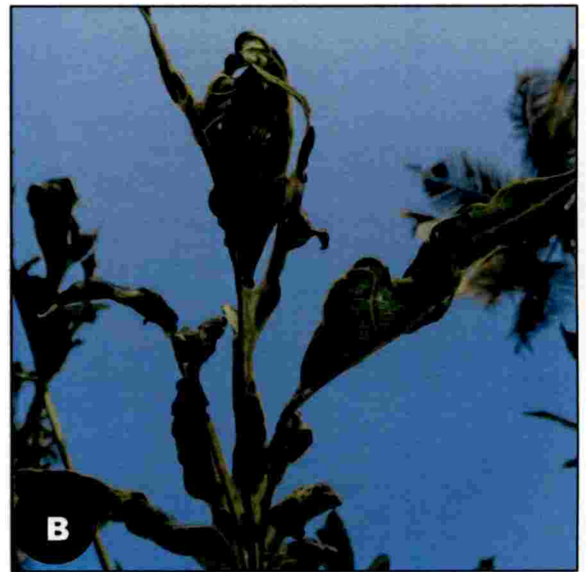
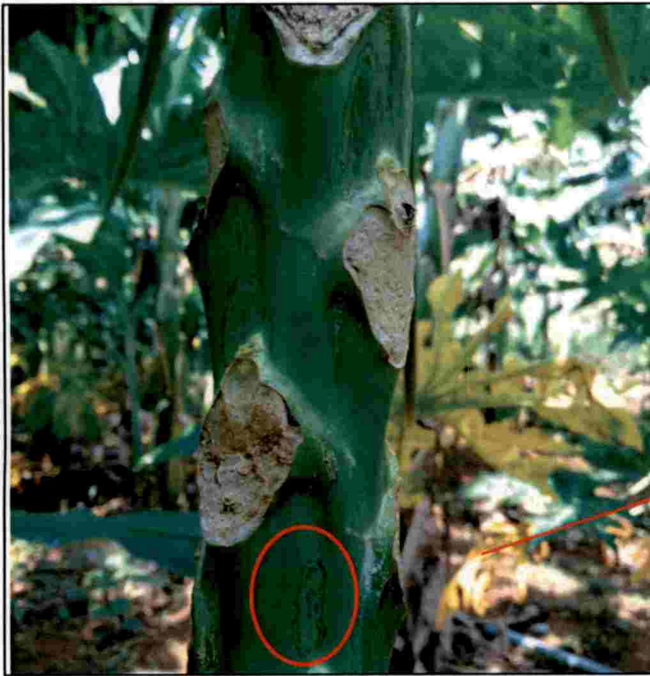


Plate 5d: Symptoms under natural conditions: Complex of symptoms on leaves (A) Vein thickening, puckering and leaf malformation (B) Puckering and shoestring symptom

Plate 5e: Symptoms under natural conditions: On petiole (A) Elongated oily streaks



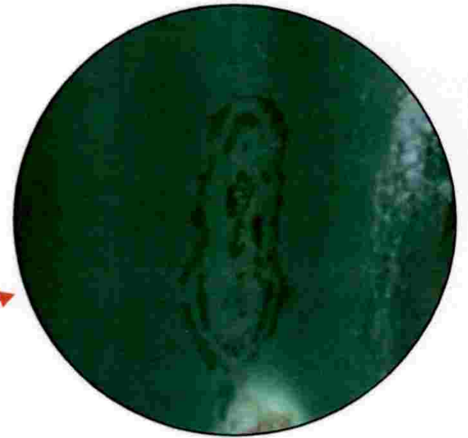


Plate 5f: Symptoms under natural conditions: on stem- oily irregular ring-like lesions

Plate 5g: Symptoms under natural conditions: On fruits (A) Oily ringspots on green fruits (B) Uneven ripening and oily ringspots

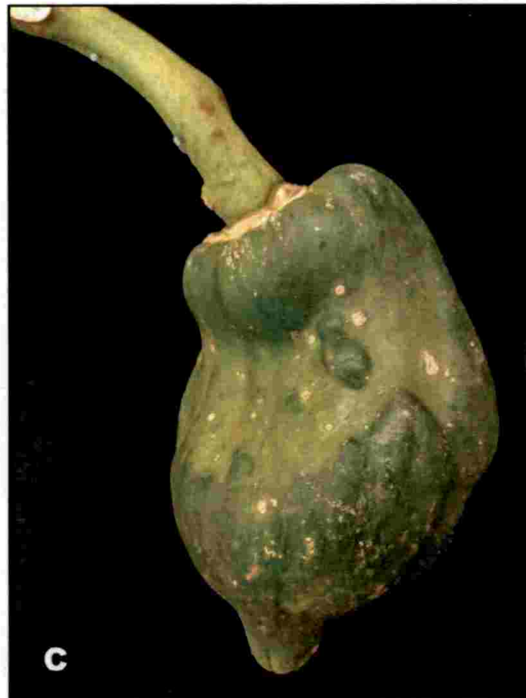


Plate 5h: Symptoms under natural conditions: on fruits (A) Oily streak-like lesions (B) Distortion (C) Bumps on fruits

value. Moreover, the surface of the affected fruits was rough and consisted of bumps (Plate 5h, Fig. C).

Tapering was observed in the crown region of the infected plants. The newly developing leaves in the canopy expressed rosetting (Plate 5i, Fig. A). In certain cases, the plants produced flowers and mature ripe fruits even though these were infected with the virus, which was evident from the oily ringspots on the fruits. Nevertheless, some plants failed to yield fruits which was accompanied by severe stunting (Plate 5i, Fig. B). Plants infected in seedling stage presented severe stunting and significant reduction in the girth of the stem (Plate 5i, Fig. C). In severely infected plants, the fruits produced did not develop into mature ones and showed high degree of distortion as already mentioned above. Under severe conditions, almost 100 per cent defoliation was observed giving a denuded appearance to the plant finally leading to its death (Plate 5i, Fig. D).

4.3.2. Symptoms Observed Under Artificial Conditions

The expression of symptoms following artificial inoculation of PRSV was studied on seedlings of papaya var. Red Lady in its primary leaf stage. The plants started expressing symptoms 14 days after inoculation (DAI). Initially, the leaves turned to pale green colour indicating initiation of chlorosis (Plate 6a, Fig. A) and in some cases, mild chlorotic spots were also seen as initial symptom (Plate 6a, Fig. B). Eventually, 20 DAI, the entire leaf turned chlorotic. On some leaves, vein clearing was also observed in leaves after the leaves turned pale green (Plate 6a, Fig. C). From 25 - 30 DAI, mottling was observed on leaves accompanied by initiation of malformation of leaves (Plate 6a, Fig. D). Wavy margins was also observed on mottled leaves (Plate 6b, Fig. A). Subsequently, puckering was observed on the leaves leading to severe distortion 30 to 35 DAI (Plate 6b, Fig. B). Malformation was observed on the leaves 55 to 60 DAI and it initiated from the central lobe (Plate 6b, Fig. C). Subsequently, the malformation progressed to other lobes leading to reduction in leaf lamina (Plate 6b,

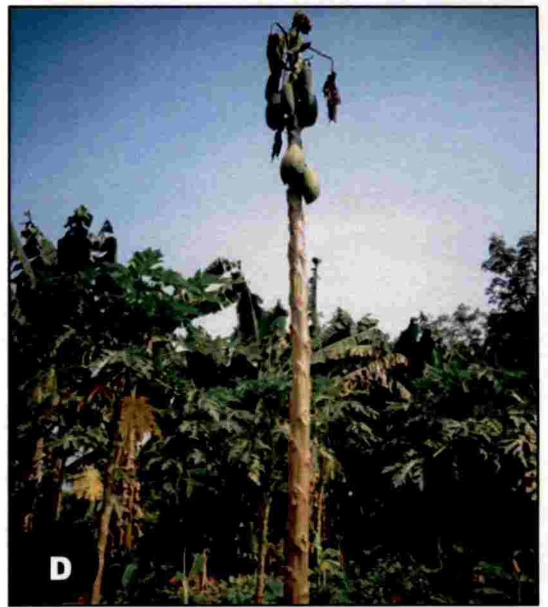


Plate 5i: General symptoms on infected plants (A) Rosetting of leaves in canopy (B) Stunting of infected plant (C) Infected seedling (D) Premature defoliation

Fig. D). Furthermore, the newly emerging leaves at this stage was also found to be malformed (Plate 6c, Fig. A). Complete reduction in leaf lamina giving a shoe string appearance was observed 75 to 80 DAI (Plate 6c, Fig. B). On the stem, oily spots of size 2 to 4 mm diameter were present (Plate 6c, Fig. C). This was observed about 60 to 70 DAI. The inoculated plants also showed stunting to a certain extent.

With the rise in temperature during summer season, a significant masking of symptoms was evident on the infected plants. The plants appeared healthy and the newly developing leaves did not show any signs of the disease. However, the fall in temperature soon after the monsoon started led to the reappearance of the symptoms which became very severe as they were earlier. Furthermore, artificial inoculation of the seedlings of the same variety and the same growth stage in summer when the atmospheric temperature was high, failed to express symptoms when maintained under same conditions. When a new set of seedlings were inoculated during the monsoon season when the atmospheric temperature was comparatively lower, the seedlings started expressing symptoms again exactly 14 DAI.

4.4. HISTOPATHOLOGY OF PAPAYA RINGSPOT VIRUS DISEASE

An attempt was made to study the histological changes in foliar tissue of papaya due to infection of PRSV. The microscopic examination of thin sections of leaves showing different symptoms was done and the results are given in Table 10. The microscopic observations revealed changes in the anatomy of the tissue. Comparative analysis was done by simultaneously observing the leaf section of healthy tissue.

In the leaves which presented chlorosis symptom, the parenchyma was disorganized and the chloroplasts were found to be completely disintegrated in contrast to the well-organized palisade parenchyma consisting of plenty of intact chloroplasts in the healthy leaf (Plate 7a, Fig. A, Fig. B). In leaves with green island symptom, plenty of chloroplasts were found to be suspended in some of the cells of the lower epidermis while less number were seen in some other cells. It also showed presence of

Plate 6a: Symptoms under artificial conditions (A) Mild chlorosis (B) Chlorotic spots (C) Vein clearing (D) Mottling

Plate 6b: Symptoms under artificial conditions (A) Wavy margins (B) Puckering (C) Initiation of malformation from central lobe (D) Progression of malformation to other lobes

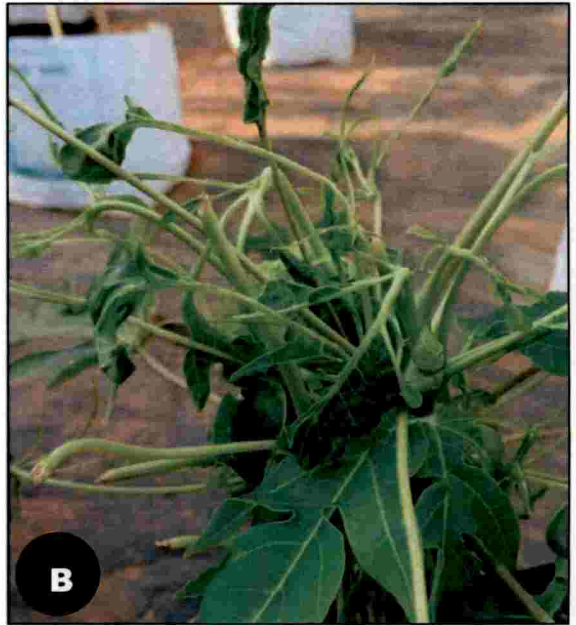


Plate 6c: Symptoms under artificial conditions (A) Malformation of newly developing leaves (B) Shoestring symptom (C) Oily spots on the stem

druses or crystalline bodies in the spongy mesophyll cells (Plate 7b, Fig. A). The leaves in which typical mottling symptom was observed, the palisade cells were disrupted in contrast to the healthy leaf. Disintegration of chloroplasts can be seen in some cells of the parenchyma. Some of the cells of lower epidermis were found to be large and some of them were irregular in shape (Plate 7b, Fig. B). The leaf sections of puckered leaves showed hyperplasia of cells. Moreover, the palisade cells appeared irregular in outline (Plate 7c, Fig. A). In general, the palisade parenchyma of infected leaves was disorganized and showed an irregular shape against the regular columnar shape seen in healthy leaves. The palisade cells and the spongy mesophyll cells were deformed and it was difficult to distinguish.

4.5. BIOLOGICAL CHARACTERIZATION OF THE VIRUS

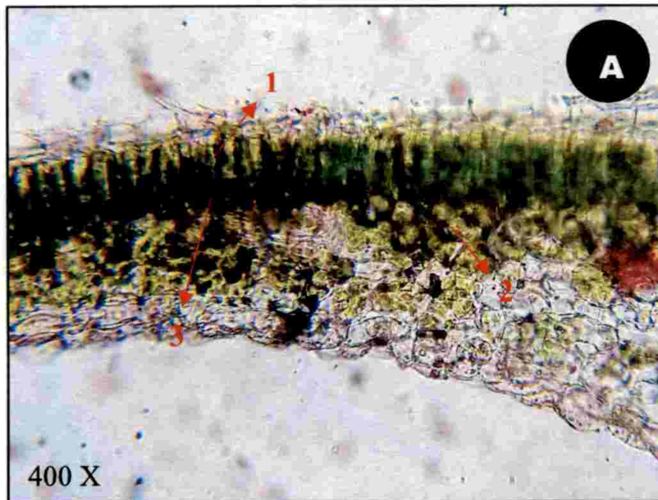
4.5.1. Transmission Studies

4.5.1.1. Mechanical Transmission

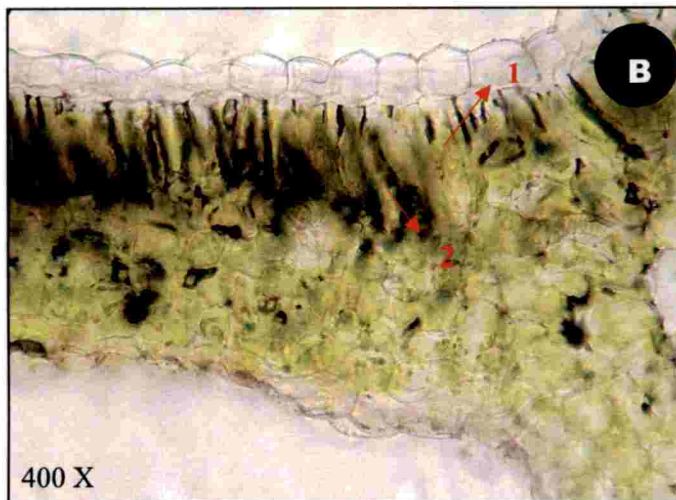
The experiment was conducted on seedlings of susceptible papaya variety Red Lady in primary leaf stage raised under insect proof net house conditions as mentioned in section 3.5.1.1. The results revealed that PRSV is readily transmitted to healthy plants through sap and 100 per cent transmission rate was recorded. The symptoms developed following mechanical inoculation included mild chlorosis, chlorotic spots, vein clearing, mottling, puckering, leaf malformation and shoestring symptoms on leaves. Oily spots on stem were also observed following artificial inoculation. The symptomatology under artificial conditions is already explained in detail under section 4.3.2. The ELISA test conducted on test plants confirmed the presence of PRSV. The details of the number of plants infected and transmission percentage are given in Table 11 and the symptoms developed after mechanical transmission are depicted in Plate 6a -6c.

Table 10: Histopathological changes in leaf due to virus infection

Character	Healthy	Chlorosis	Green island symptom	Puckering	Mottling
Upper epidermis	Thick cuticle Single layered parenchyma, tightly packed	Thick cuticle Single layered parenchyma, tightly packed	Thick cuticle Single layered parenchyma, tightly packed	Thick cuticle, single layered parenchyma, tightly packed	Thick cuticle Single layered parenchyma, tightly packed
Mesophyll-Palisade tissue	Single layered, tightly packed, long axis parallel, plenty of intact chloroplast, regular columnar shaped cells	Single layered, disintegrated mass of chloroplast in cytoplasm, disorganized parenchyma	Single layered, tightly packed, long axis parallel, plenty of chloroplast suspended in peripheral cytoplasm	Single layered parenchyma, disruption of palisade cells	1-2 layered, tightly packed, disruption of palisade cells
Mesophyll-Spongy tissue	5-6 layered parenchyma, irregular shape, loosely arranged, starch grains present, intercellular air space present	4-5 layered parenchyma, irregular shape, loosely arranged, starch grains abundant, intercellular air space present	3-4 layered parenchyma, irregular shape, loosely arranged, plenty of chloroplast, crystalline bodies present	5-4 layered parenchyma, irregular shape, loosely arranged, more chloroplasts in some cells	3-4 layered parenchyma, irregular shape, disintegration of chloroplasts in some cells, intercellular air space present,
Lower epidermis	Single layered parenchyma, tubular shape, stomata with sunken guard cells	Single layered parenchyma, tubular shape, stomata with sunken guard cells	Single layered parenchyma, tubular shape, increased number of chloroplasts, stomata with sunken guard cells	Single layered parenchyma, tubular shape, chloroplast present in some cells, stomata with sunken guard cells	Deformed, single layered parenchyma, irregular shape, some cells are large, stomata with sunken guard cells

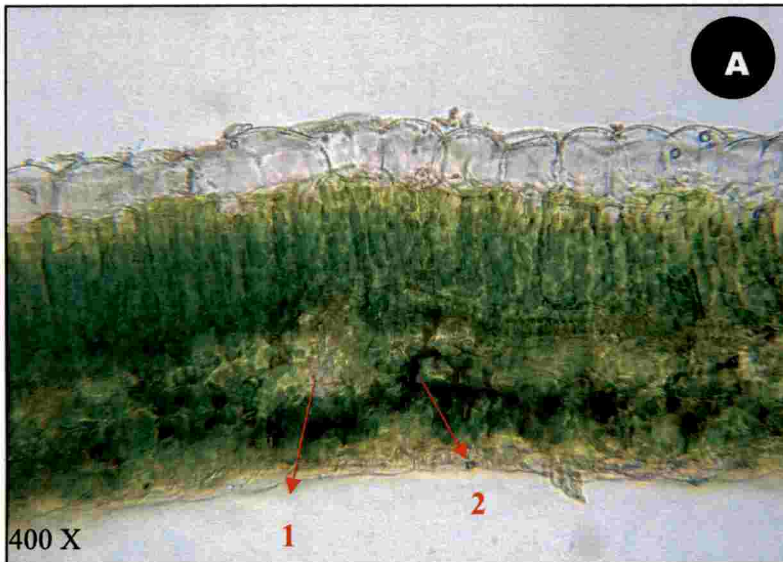


(A) Healthy leaf: 1) Well organized parenchyma with regular columnar shape 2) Spongy cells regular in outline 3) Numerous chloroplasts suspended in cytoplasm

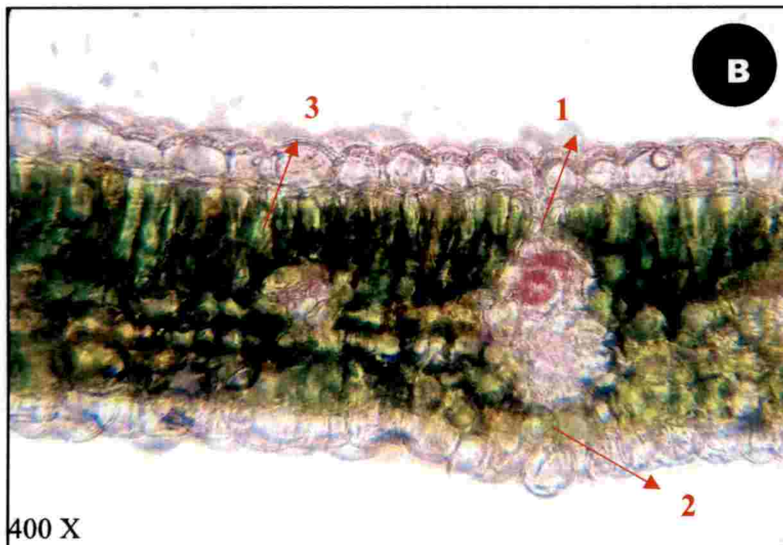


(B) Chlorosis symptom: 1) Malformed palisade parenchyma 2) Disintegrated mass of chloroplasts in cytoplasm

Plate 7a: Histopathological changes due to virus infection

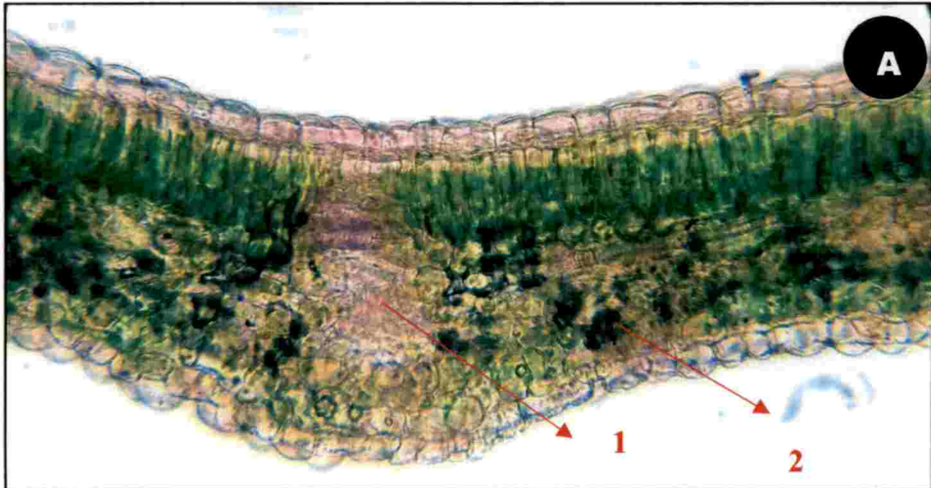


(A) Green island symptom: 1) Presence of crystalline bodies
2) Increased number of chloroplasts in some cells of lower epidermis



(B) Mottling symptom: 1) Disruption of palisade cells
2) Irregular shape of lower epidermal cells 3) Disintegration of chloroplasts

Plate 7b: Histopathological changes in leaf due to virus infection



(A) Puckering symptom: 1) Hyperplasia of cells

2) More number of chloroplasts concentrated in some cells

Plate 7c: Histopathological changes in leaf due to virus infection

4.4.1.2. Seed Transmission

The seeds of infected fruits were tested for the presence of PRSV. None of the seedlings raised from seeds of infected fruits expressed symptoms up to 60 DAS. The absence of the virus was confirmed using DAC- ELISA as described under section 3.6.1.The results of the study are presented in Table 12.

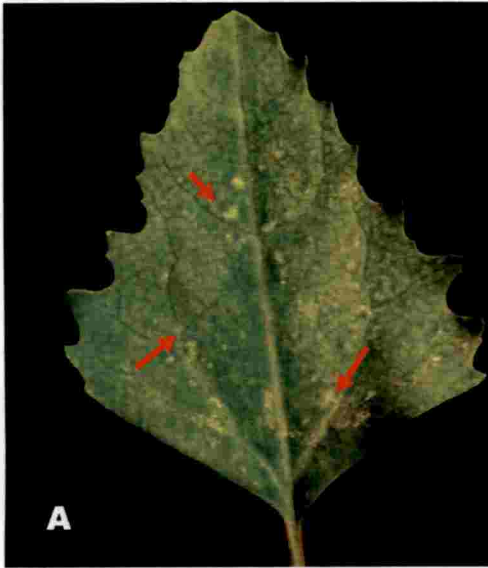
4.5.2. Host Range Studies

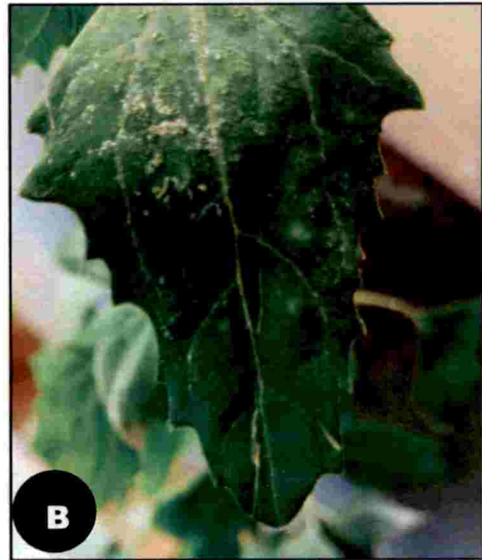
In the present investigation, 21 plants belonging to 6 families were tested for their susceptibility to PRSV through mechanical inoculation under insect proof net house conditions. Out of these, only five plant species expressed visible symptoms. The results of the host range experiment is detailed in Table 13. The infection was further confirmed through ELISA test of the symptomatic plants and also by back inoculation to indicator plant *Chenopodium amaranticolor*.

The results revealed that, other than Caricaceae, the host range of PRSV was limited to 2 families *viz*., Chenopodiaceae and Cucurbitaceae. When *C. amaranticolor* was inoculated with virus inoculum from infected papaya plants, the leaves exhibited local lesions in the form of mild chlorotic spots 8 to 10 DAI all over the leaf lamina. Pinpoint necrotic lesions were observed 12 to 14 DAI (Plate 8a, Fig. A). Very distinct chlorotic spots were observed on inoculated leaves 21 DAI (Plate 8a, Fig. B). No systemic symptoms were observed on these plants on further monitoring. The data revealed 40 per cent of transmission of PRSV to *C. amaranticolor*.

Four plant species belonging to Cucurbitaceae were studied *viz*., *Trichosanthes cucumerina* (snakegourd), *Momordica charantia* (bittergourd), *Cucurbita moschata* (pumpkin) and *Cucumis sativus* (cucumber).

Snakegourd produced local symptoms like necrotic local lesions on the inoculated leaves 12 to 14 DAI (Plate 8a, Fig. C). The size of the necrotic lesions





Symptoms on *Chenopodium amaranticolor* (A) Pinpoint necrotic lesions (B) Distinct chlorotic spots

Symptoms on snake gourd (C) Necrotic local lesions with chlorotic halo

Plate 8a: Host range of PRSV

developed ranged from 1 mm which were pinpoint in size to 8 mm. The lesions were surrounded by a chlorotic halo. The plants did not exhibit any kind of systemic symptom up to 60 DAI. Back inoculation of the leaves symptomatic leaves of snake gourd to *C. amaranticolor* failed to produce local lesions. The percent transmission of PRSV recorded in snakegourd was 60 per cent. DAC- ELISA conducted with symptomatic leaves of snakegourd failed to give positive reaction.

On mechanical inoculation of PRSV to bittergourd, systemic symptoms were produced 14 DAI. Initially, a discolouration was observed on the leaves presenting an uneven appearance of dark and light green areas (Plate 8b, Fig. A). In some plants, mild chlorotic spots were developed on the apex of the leaf lamina 14 DAI (Plate 8b, Fig. B). The apical leaves also expressed puckering 22 - 25 DAI (Plate 8b, Fig. C). Cent per cent transmission of PRSV was recorded in bittergourd. Back inoculation of symptomatic leaves to *C. amaranticolor* developed pinpoint necrotic lesions and mild chlorotic spots on the inoculated leaf 14 to 17 DAI (Plate 8b, Fig. D). The presence of virus was confirmed by positive reaction obtained by conducting DAC-ELISA of the test plants.

Systemic symptoms were also observed in case of cucumber inoculated with PRSV. The incubation period of the virus was 14 days. Initially, the leaves gave a pale green appearance indicating mild chlorosis 14 DAI (Plate 8c, Fig. A). Additionally, distinct chlorotic spots began to appear on the leaves 21 DAI (Plate 8c, Fig. B). In few inoculated cucumber plants, vein clearing was also observed 25 - 27 DAI (Plate 8c, Fig. C). The results revealed 100 per cent transmission of PRSV to cucumber plants. The infection was further confirmed through back inoculation of cucumber to *C. amaranticolor* which produced pinpoint necrotic lesions on the inoculated leaves 12 DAI (Plate 8c, Fig. D). The presence of PRSV was also confirmed through DAC-ELISA of the symptomatic cucumber leaves.

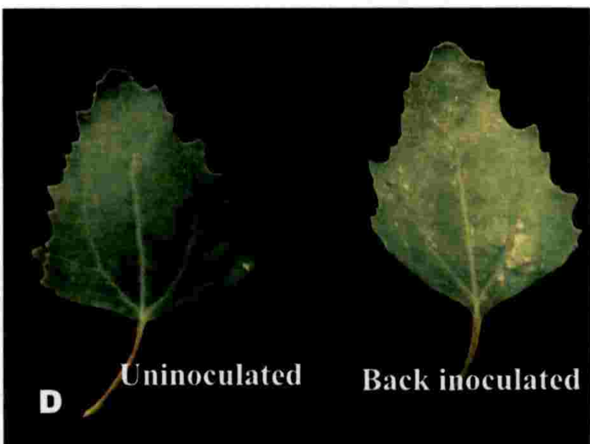
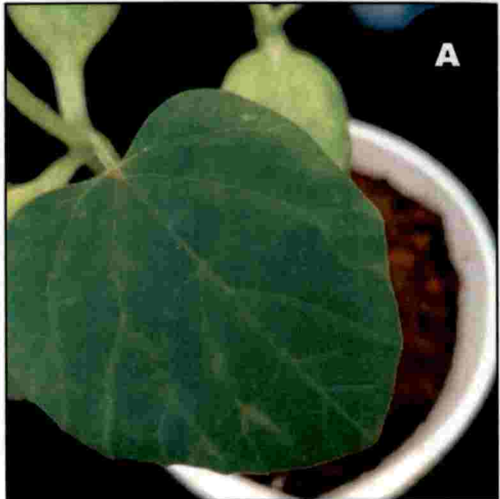
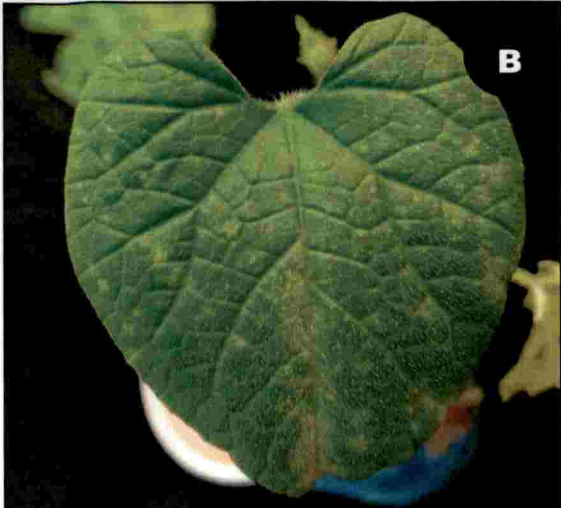
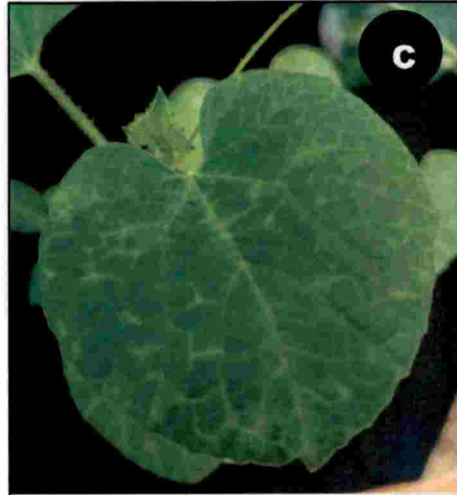


Plate 8b: Host range of PRSV: Symptoms on bitter gourd (A) Discolouration of leaves (B) Pale chlorotic spots on leaf apex (C) Puckering in leaves (D) Necrotic lesions developed on back inoculation to *C. amaranticolor*







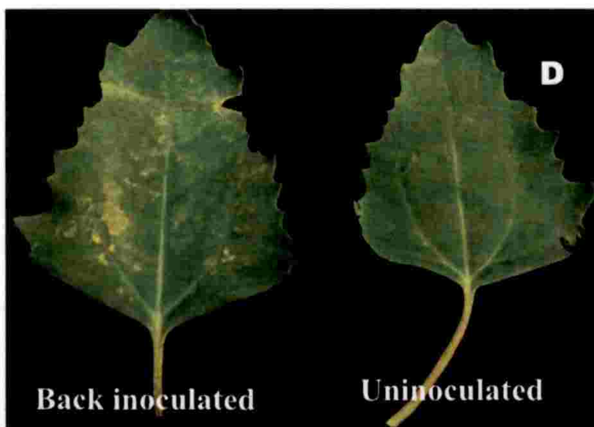


Plate 8c: Host range of PRSV: Symptoms on cucumber (A) Mild chlorosis of leaves (B) Chlorotic spots on leaves (C) Vein clearing (D) Necrotic lesions developed on back inoculation to *C. amaranticolor*

In the case of pumpkin, systemic symptoms were produced in the form of mild chlorosis on the leaves 14 DAI (Plate 8d, Fig. A). The leaves later exhibited mottling with irregular distribution of areas of dark and light green 25 to 27 DAI. Puckering was also seen on the leaves 27 DAI (Plate 8d, Fig. B). Back inoculation to *C. amaranticolor* produced mild chlorotic spots. Pinpoint necrotic lesions were also observed (Plate 8d, Fig. C). The transmission percentage recorded in case of pumpkin was 50 per cent.

Among the 21 species of plants tested for host range, only members of Chenopodiaceae and Cucurbitaceae were found to be the hosts of PRSV. Other 16 plant species belonging to Amaranthaceae, Fabaceae, Solanaceae, Malvaceae, Asteraceae, Musaceae and Cleomaceae which were tested for host range did not develop any visible symptoms.

4.5.3. Electron Microscopy

Electron microscopy was done by leaf dip method using JEOL CX- 100 electron microscope at Advanced Centre of Plant Virology, I.A.R.I., New Delhi. The leaf dip samples were prepared using negative stain, uranyl acetate (2%) (pH: 4.5) from infected putative papaya leaves. The electron micrographs revealed the presence of typical flexuous rod particles. The particles were of 807.74 nm in length and 12 nm in width (Plate 9).

Based on the size and shape of the particles, the virus responsible for the symptom development in papaya was identified and confirmed as *Papaya ringspot virus*, which possess (+) ssRNA belonging to genus *Potyvirus*, family *Potyviridae*.

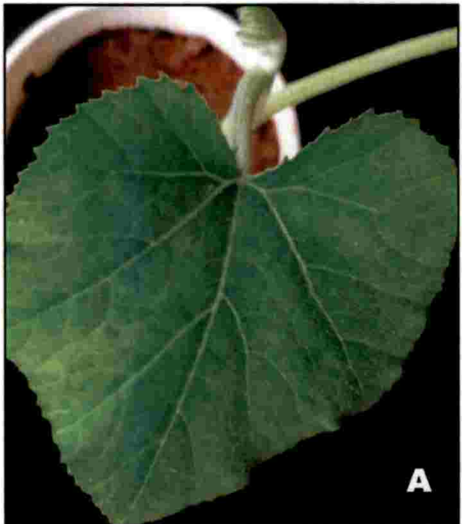


Plate 8d: Host range of PRSV: Symptoms on pumpkin (A) Mild chlorosis of leaves (B) Mottling and puckering (C) Necrotic lesions developed on back inoculation to *C. amaranticolor*

Table 11: Transmission of PRSV through mechanical inoculation

Test Plant	No. of plants		Types of symptoms*	Percent transmission	Incubation period of virus	Confirmatory test (ELISA)**
	Inoculated	Infected				
Papaya var. Red Lady	20	20	CS, CL, VC, Mot, P, SS, OS	100	14 - 15 days	+

*VC = Vein clearing, CL= Chlorosis, Mot= Mottling, CS= Chlorotic spots on leaves, P= Puckering, SS= Shoestring symptom, OS= Oily spots

** - '+' = positive reaction to ELISA

Table 12: Transmission of PRSV through seeds

Sl. No.	Variety	No. of seeds		Germination percentage (%)	No. of plants expressing symptoms	Per cent transmission	Confirmatory test (ELISA) *
		Sown	Germinated				
1.	Red Lady (Diseased)	25	20	80	0	0	--
2.	Red Lady (Apparently healthy)	25	19	76	0	0	--
3.	Local (Diseased)	25	22	88	0	0	--
4.	Local (Apparently healthy)	25	24	96	0	0	--

* '--': negative reaction to ELISA

Table 13: Host range of PRSV

Sl. No.	Host	Family	Types of symptoms*	No. of plants		Per cent transmission	Confirmation of infectivity	
				Inoculated	Infected		Back inoculation	ELISA
1.	*Amaranthus polygamous* (green)	Amaranthaceae	NS	10	0	0	-	-
2.	*Amaranthus polygamous* (red)		NS	10	0	0	-	-
3.	*Gomphrena globosa*		NS	10	0	0	-	-
4.	*Alternanthera sessilis*		NS	10	0	0	-	-
5.	*Chenopodium amaranticolor*	Chenopodiaceae	Chlorotic spots,necrotic lesions	5	2	40	-	-
6.	*Momordica charantia*		Leaf discolouration, chlorotic spots, puckering	10	10	100	Mild chlorotic spots, pinpoint necrotic lesions	+
7.	*Cucurbita moschata*	Cucurbitaceae	Chlorosis, mottling and puckering	10	5	50	Necrotic lesions	+
8.	*Cucumis sativus*		Chlorotic spots, vein clearing	10	10	100	Necrotic lesions	+
9.	*Trichosanthes cucumerina*		Necrotic local lesions with chlorotic halo	10	6	60	NS	--

Table 13- Contd…

10.	*Vigna unguiculata*	Fabaceae	NS	10	0	0	-	-
11.	*Nicotiana tabaccum*	Solanaceae	NS	10	0	0	-	-
12.	*Nicotiana glutinosa*		NS	10	0	0	-	-
13.	*Solanum lycopersicum*		NS	10	0	0	-	-
14.	*Solanum melongena*		NS	10	0	0	-	-
15.	*Capsicum annuum*		NS	10	0	0	-	-
16.	*Ageratum conyzoides*	Asteraceae	NS	5	0	0	-	-
17.	*Synedrella nodiflora*		NS	5	0	0	-	-
18.	*Sphaegneticola trilobata*		NS	10	0	0	-	-
19.	*Musa paradisica*		NS	10	0	0	-	-
20.	*Abelmoschus esculentus*	Malvaceae	NS	10	0	0	-	-
21.	*Cleome viscosa*	Cleomaceae	NS	5	0	0	-	-

*NS = no symptoms, '+' = positive reaction to ELISA '--' = negative reaction to ELISA

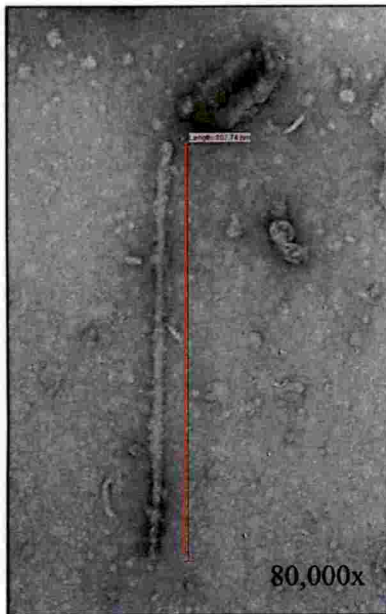
4.6. DETECTION OF PRSV BY PROTEIN - BASED METHOD

4.6.1. Direct Antigen Coating ELISA (DAC – ELISA)

The seriodiagnostic detection of PRSV was carried out at Advanced Research Centre for Plant Disease Diagnosis, College of Agriculture, Vellayani. The antiserum was purchased from DSMZ, Germany. Healthy and suspected symptomatic leaves of papaya plant were used to perform DAC- ELISA. The titre of antiserum used for the study was 1: 200 which was standardized by Krishnapriya (2015). In the present investigation, the serodiagnostic detection of PRSV was validated. The absorbance values were recorded at 405 nm in Microplate Reader, 680 (BIORAD) for healthy and suspected samples. The values recorded in infected samples were more than twice the value recorded in healthy samples. This clear distinction in the absorbance values indicated that the suspected sample is infected with PRSV and the PRSV specific primary antibody gave high reactivity (Plate 10, Table 14).

Table 14: DAC ELISA: Absorbance value at 405 nm

Sample	Sample 1	Average	Sample 2	Average
Blank	0.103	**0.129**	0.131	**0.164**
	0.148		0.188	
	0.136		0.173	
Healthy	0.196	**0.212**	0.258	**0.222**
	0.233		0.211	
	0.208		0.198	
Infected	1.217	**1.218**	1.131	**1.114**
	1.211		1.117	
	1.227		1.095	



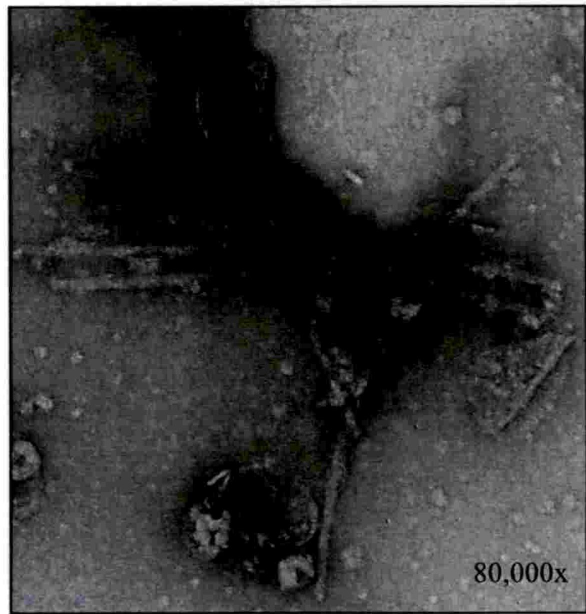


Plate 9: Electron micrographs depicting flexuous rod shaped particles (807.74 x 12nm)

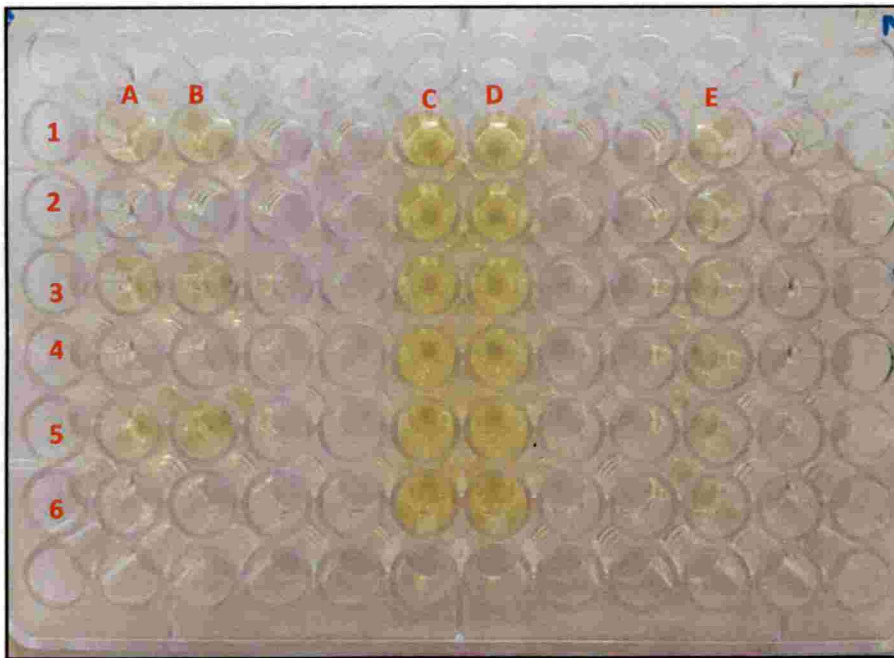


Plate 10: Detection of PRSV by DAC- ELISA. Healthy: A (1,3,5), B (1,3,5); Infected: C(1-6), D (1-6); Blank: E (1-6)

4.7. MOLECULAR CHARACTERIZATION OF PRSV

Molecular characterization studies of five isolates collected from Thrissur district was undertaken. Total RNA was isolated from the virus infected plant, followed by cDNA synthesis. Amplification of partial nuclear inclusion b (NIb) and coat protein (CP) gene was carried out using PRSV specific primers. The amplicons obtained in PCR was outsourced to Agri Genome, Kochi for sequencing. The sequences of the different isolates obtained were subjected to phylogenetic analysis. The isolates were named according to the location from which the leaf sample was collected.

4.7.1. Isolation of RNA

Electron microscopic studies revealed that the virus in the present study is a single stranded (ss) RNA virus belonging to *Potyviridae*. In order to carry out molecular detection, the isolation of total RNA was carried out from the leaf samples of infected and healthy papaya plants using TRIzol reagent method. The total RNA obtained was quantified using Nanodrop spectrophotometer. The concentration and the absorbance ratios of the RNA isolated are given in Table 15. The RNA with an absorbance value ($A_{260/280}$) greater than 1.8 was considered as good quality RNA. Gel documentation of the total RNA isolated was done under BIORAD Molecular Imager, (Gel Doc^{TM} XR+) and the gel image revealed three distinct rRNA bands with no apparent degradation of RNA (Plate 11).

4.7.2. Synthesis of First Strand Complementary DNA (cDNA)

The virus in present investigation was confirmed as ss RNA virus. Hence, for detection of the virus at molecular level through PCR, reverse transcription was performed first to convert RNA to cDNA. The conversion of RNA to cDNA using Revert Aid First Strand cDNA synthesis kit is described under section 3.7.2. The cDNA obtained was quantified and the ratio of the absorbance value ($A_{260/280}$) was recorded to assess the purity of cDNA (Table 16).

Table 15: Yield and absorbance ratio of total RNA isolated from leaf samples

Location	Isolate	RNA yield (ng/ μl)	Purity of RNA($A_{260/280}$)
Vellanikkara	VK -1	1735.1	1.97
Koratty	KR - 2	1651.8	1.98
Melur	ML -3	1429.7	2.06
Pazhayanur	PZ -4	1236.5	2.09
Puthur	PT -5	1699.9	1.95

Table 16: Quantity and quality of cDNA

Isolate	Concentration (ng/ μl)	Absorbanca ratio ($A_{260/280}$)
VK -1	1601.8	1.64
KR -2	1544.6	1.98
ML -3	1462.8	2.09
PZ -4	1588.3	1.89
PT -5	1332.5	1.96

4.7.3. Standardization of Reverse Transcriptase -Polymerase Chain Reaction (RT-PCR)

RT-PCR conditions such as dilution of template cDNA and annealing temperature were standardized simultaneously and the results are given below.

4.7.3.1. Standardization of Template cDNA Dilution

RT-PCR was carried out at different dilutions of the cDNA template. Good quality bands were obtained in 1:1 dilution of cDNA. No amplification was observed in other dilutions (Table 17). The concentration of cDNA at which amplification was obtained ranged from 44.40 - 54.40 ng/µl.

4.7.3.2. Standardization of Annealing Temperature

The annealing temperature was determined by performing gradient PCR. Amplification was observed at 45° C. There was no amplification at 46, 47, 48, 49 and 50° C (Table 18) (Plate 12).

Table 17: Effect of dilutions of template cDNA on RT-PCR amplification

Template cDNA dilutions	Nature of amplification
1.1	Amplified with good quality bands
1:0.5	No amplification
1: 0.25	No amplification
1: 0.1	No amplification

Table 18: Standardization of annealing temperature for RT-PCR

Temperature	Result
45° C	Amplified with good bands
46° C	No amplification
47° C	No amplification
48° C	No amplification
49° C	No amplification
50° C	No amplification

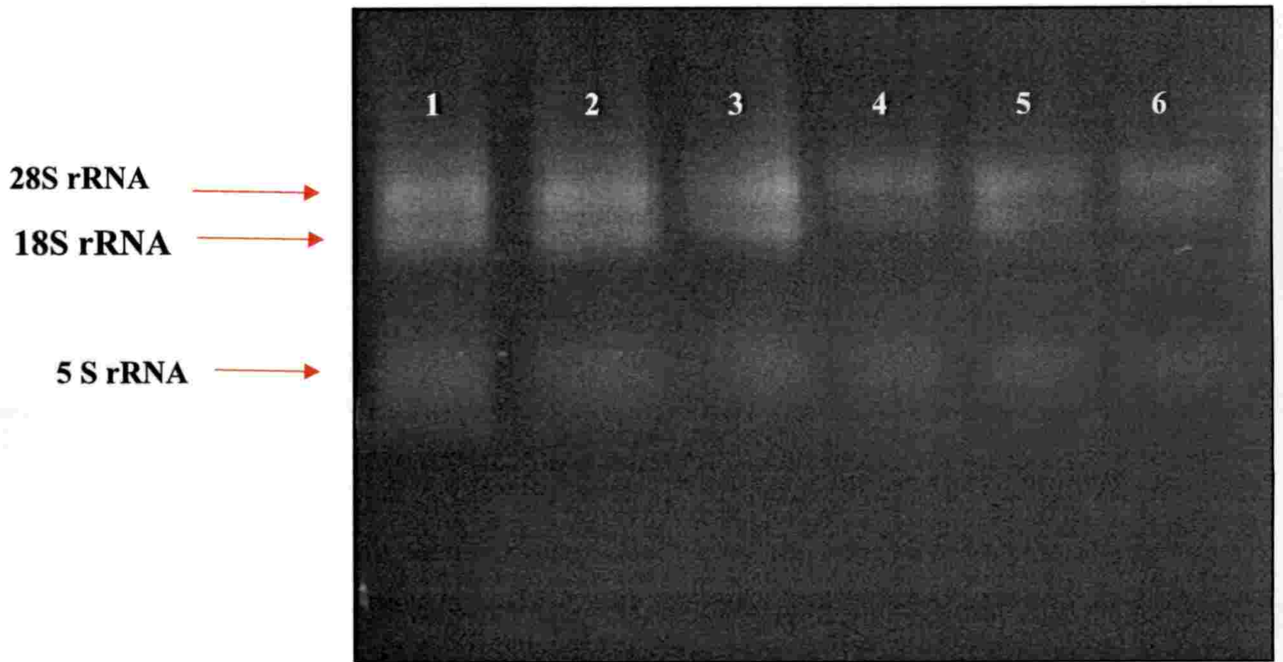


Plate 11: Total RNA isolated from leaf samples: Lane 1- 5: Infected isolates - VK1, KR-2, ML-3. PZ-4 and PT-5 Lane 6: Healthy control

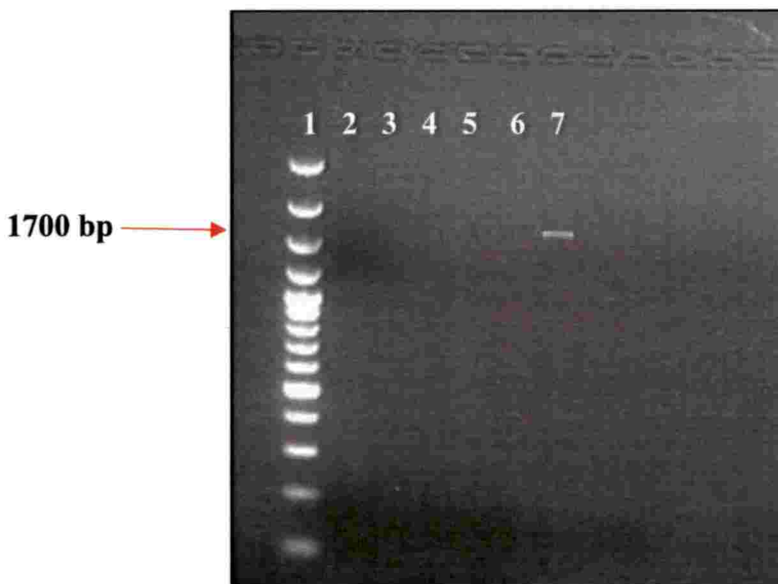


Plate 12: Standardization of annealing temperature: Lane 1: 100 bp plus Generuler DNA ladder; Lane 2: 50°C; Lane 3: 49°C; Lane 4: 48°C; Lane 5: 47°C; Lane 6: 46°C; Lane 7: 45°C

4.7.4. Detection of PRSV by RT-PCR

RT- PCR was carried out using reported primers (Srinivasulu and Sai Gopal, 2011), as mentioned under section 3.7.3. The mastermix composition described under section 3.7.3. was followed for carrying out the rection. The reaction yielded amplified products of expected size 1700 bp which was visualized after gel documentation. The gel electrophoresis was carried out using 1.2 per cent agarose in 1 X TAE buffer. Thick bands were visualized and documented in infected isolates which indicated the presence of virus. No band was present in case of healthy control (Plate 13).

4.7.5. Sequencing

The PCR products obtained were sent to Agri Genome, Kochi for purification and sequencing. The nucleotide sequences of the five isolates obtained are presented in Fig. 1a- 1e.

4.7.6. Sequence Analysis

The nucleotide sequences of five isolates obtained were analyzed using nucleotide BLAST and it showed significant sequence similarity to reported NIb and CP gene sequences of PRSV in NCBI data bank. BLAST analysis of nucleotide sequences confirmed the presence of virus.

The amino acid sequences were deduced from nucleic acid sequences using ExPASy translator tool and the amino acid sequences of five isolates varied from each other with respect to the number of amino acids present in each sequence (Fig. 2a -2e). Protein BLAST analysis revealed that amino acid sequences of present study showed similarity to sequences in NCBI data bank.

4.7.6.1. In silico Analysis of PRSV Isolate - VK-1

Nucleotide BLAST of the sequence of isolate VK-1 revealed maximum sequence similarity (90%) to PRSV isolate KE- Ca (DQ 666640.1) from Calicut,

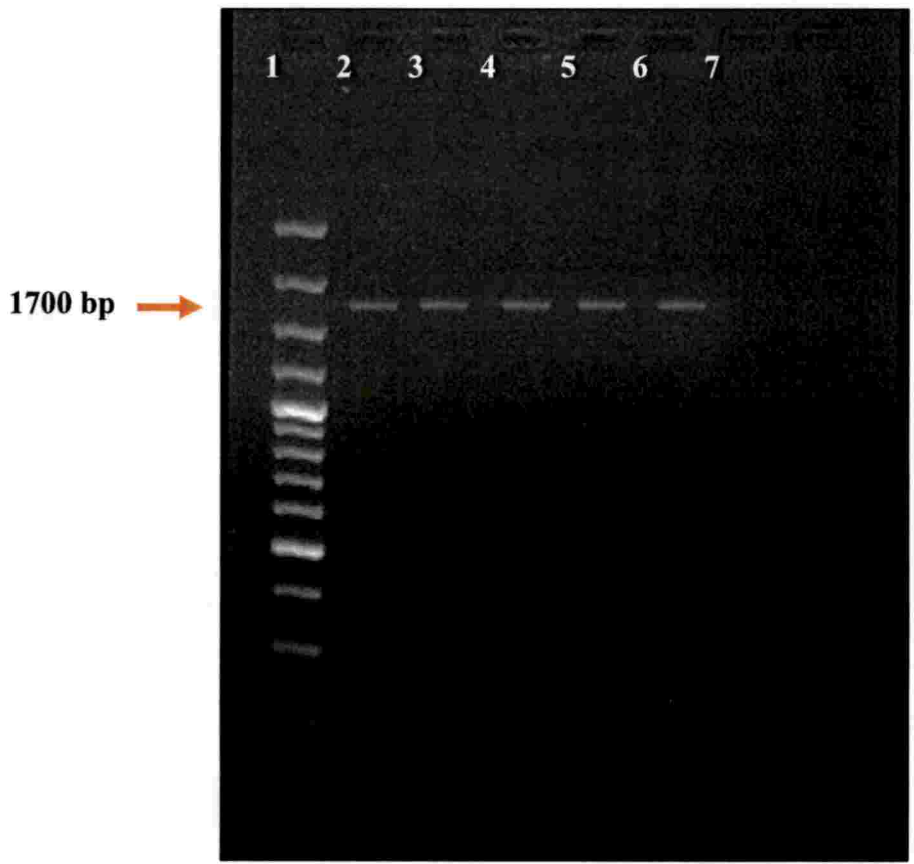


Plate 13: Gel profile of PCR products: Lane 1: 100 bp plus Generuler DNA ladder; Lane 2-6: PRSV isolates showing amplified product of 1700 bp; Lane 7: Healthy control

5’ACGCAACTTTTTCGGTCGTAGATTCAAGAAGAGATGTGTGTGTTTTATATCA
ATGGTGATGATCTCTGTATCGCCATTCACCCAGATCATGAGTATGTTCTTGAC
TCATTTTCTAGTTCTTTTGCTGAACTTGGGCTTAAGTATGATTTCACACAAAGA
CATAGGAATAAACAGGATTTGTGGTTTATGTCACATCGAGGTGTTCTTATCAA
TAACATTTACATTCCGAAACTTGAACCTGAGCGAATTGTTGCAATTCTTGAGT
GGGACAAGTCCAAGCTTCCGGAGCATCGTTTGGAGGCAATCACAGCAGCAAT
GATAGAATCATGGGGTTATGGTGAGCTAACACACCAAATTCGCAGATTCTAC
CAATGGGTTCTTGAACAAGCTCCATTCAATGAGTTGGCTAGGCAAGGGAGAG
CCCCTTATGTTTCGGAGGTTGGATTAAGAAGGCTGTATACCAGTGAGCGTGGT
TCGATGGATGAGTTGGAAGCTTACATCGACAAATACTTCGAACGTGAACGTG
GAGATTCTCCTGAGTTATTAGTGTACCATGAATCAAAAAGTCCGGGTGATCAT
CAGATTGTCGGTCACATGAATGAGCATGTTTATCATCAATCTAAGACTGAAA
GCGGTGGATGCAGGTCTCAATGATAAGCTGAAAGAGAAGGAAAAAGAAAAA
GATAAAGAAAAAGAAAAAGAAAAGAAAGACAAGAAGGATGCTAGTGACGG
AAAGTGATGTGTCAACTAGCAAAAGAATTGGAGAGAGAGATAGAGATGTCA
ATGCTGGAAACTAGTGTTACATTTGCAGTTCCAAGAACCAAGCCATTTACTGA
TAAATGATTTTGCCTAGAATTAGGGAAAAGTTGTTCTTAATTTAACCATCTTC
TTCAGTATAATCCACAGCAAATTGACATCTCGAACACTCGTGCCACACAATCA
CAGGTTTGAAGAAGTGGTATGAGGGAGTTGAAAATGACTATGGTCTTAATGG
ATAAATGAATTGCAAGTGAATGTTGAACGGACTTAATGTATGGTGTATCGGA
AATTGTAACATCACAAGGACTATTCTGGGTGCTGGGTCAATGATGGAATGGG
TGAACCTCCAGGTGACTTATCATTAAACCGGTAATTGGACATGCCACTCCCTT
CTCATTGAGCAAATATGGACTACACTTCCGAGTAACGCGCCGAAGCCTTCATC
GGCAAGCGTAAATGCCAACTGGTGAAGAGTCTACATGTCCCCGCG 3’

Fig. 1a: Nucleotide sequence of partial NIb and CP gene of PRSV isolate VK-1 (1249 bp)

5'GCGGCGATGCCAGAGATGAGAGCTCAGAGGAATGTGTGTGTTTTATAT
CAATGGTGATGATCTCTGTATTGCCATTCACCCGGATCATGAGTATGTTC
TTGACTCATTCTCTAGTTCATTTGCTGAACTTGGGCTTAAGTATGATTTC
ACACAAAGGCATAGAAATAAACAGGATTTGTGGTTCATGTCACATCGAG
GTGTTCTGATCGATGACATCTACATTCCGAAACTTGAACCTGAGCGAAT
TGTTGCAATTCTTGAGTGGGACAAATCCAAGCTTCCGGAGCATCGTTTG
GAGGCAATCACAGCAGCAATGATCGAATCATGGGGTTATGGTGAGCTA
ACACACCACATCCGCAGATTTTACCAATGGGTTCTTGAGCAAGCTCCAT
TCAATGAGTTGGCTAGGCAAGGGAGAGCCCCTTACGTCTCGGAAGTTGG
ATTAAGAAGGCTGTATACCAGTGAGCGTGGTTCGATGGATGAGTTGGAA
GCGTATATCGATAAATACTTCGAACGTGAGCGTGGAGATTCTCCTGAGC
TATTAGTGTACCATGAATCAAAAAGCTTGGATGATAATCAGATTATCTG
TCACATGAATGAGCATGTTTATCACCAATCTAAGACTGAAGCGGTGGAT
GCAGGTCTCAATGATAAGCTGAAAGAGAAGGAAAAAGAAAAAGATAA
AGAAAAAGAAAAGGAAAAGAAAGACAAGAAGGCTGCTAGTGACGAAA
GTGATGTGTCAACTAGCACAAAAACTAGGGAGAGAGATAGAGATGTCA
ATGCTGGAACTAGTGGTACATTCGCAGTTCCAAGAATCAAACCATTTAC
TGATAAATGATTTTGCCCAGAATTAAGGGAAAAGTTGTTCTTAATTTGA
ATCATCTTCTTCAGTATAATCCAACAGCAAATTGACATCTCGAACACCTC
GTGCCACACAATCACAGTTTGAGAAGTGTATGAGGGAGTGAGGAATGA
CTTATGGCCTTATGATAATGAAATGCAAGTGATGTTGAACGACTAATGG
CTTGGTGTATCGAATGGTACATCACCAGAATAATCTGTGTTTGGGTCAT
GATGATGTGAAACTCAAGTCGATTAATTCCAGTTAACGGTTAATGACCA
TGCAACTCGTCATAGCCAGTACATGCTCCATCAGATACGGACGATGCTA
TATGGCAGGCGTAATTGCACCTATGGGAGAGTG 3'

Fig. 1b: Nucleotide sequence of partial NIb and CP gene of PRSV isolate KR-2 (1204 bp)

5’AGGCCGTTTGCTGTGGGTACGTGGCTCAGAGAATGTGTGTGTTTTATAT
CAATGGTGATGATCTCTGTATCGCCATTCATCCGGATCATGAGTATGTTC
TTGACTCATTTTCTAGTTCATTTGCTGAACTTGGGCTTAAGTATGATTTCA
CACAAAGACATAGGAATAAACAGGATTTGTGGTTCATGTCACATCGAGG
TGTTCTGATCGATAACATTTACATTCCGAAACTTGAACCTGAGCGAATTG
TTGCAATTCTTGAGTGGGACAAATCCAAACTTCCGGAACATCGCTTGGAG
GCAATCACAGCAGCAATGATAGAATCATGGGGTTATGGTGAGCTAACAC
ACCAAATCCGCAGATTCTACCAATGGGTTCTTGAACAAGCTCCATTCAAT
GAGTTGGCTAGGCAAGGGAGAGCCCCTTATGTCTCGGAGGTTGGATTAA
GAAGGCTGTATACCAGTGAGCGTGGTTCAATGGATGAGTTGGAAGCATA
CATTGACAAATACTTCGAACGTGAGCGTGGAGATTCTCCCGAGTTATTAG
TGTATCATGAATCAGAAAGTTCGGATGGTCATCAGATTATCGATCACATG
AATGAGCACGTTTATCATCAATCTAAGACTGAAGCGGTGGATGCAGGTCT
CAATGATAAGCTGAAAGAGAAGGAAAAAGAAAAAGATAAAGAAAAAGA
AAAAGAAAAGAGAGACAAGAAGGATGCTAGTGACGGAGGTAGTGTGTC
AACTAGCACAAAAATTGGAGAGAGAGATAGAGATGTCAATGCTGGAACT
AGTGGTACATTTGCAGTTCCAAGAACCAAGCCATTTACTGATAAAATGAT
TTTGCCCAGAATTAAGGGAAAAGTTGTTCTTAATTTGAATCATCTTCTTCA
GTATAATCCACAGCAAATTGATATCTCGAACACTCGTGCACACAATCACA
GTTTGAAGAGTGTATGAGGAGTGAGGAGTGACTTATGGCCTTAATGATAT
GAATGCAAGTGATGTGAACGCCTTAATGTTGTGTATCGAAATGGTACATC
GCAGACAATCTGAGTTCTGGTCATGATGAATGGTGAACTCAGGTCGATTA
TCGATAAACTGTATGGCATGCACTCCTCATTAGCATCATGGTCACTTCAG
CTACGGCAGCTAGCTCGAAGGCATGCATCTTGAAGGTCATGCG 3’

Fig. 1c: Nucleotide sequence of partial NIb and CP gene of PRSV isolate ML-3 (1185 bp)

5’GGGTAAAAAATCCAAGAGATGAGAGATCAGAGGCATGTGTGTGTTTTATAT
CAATGGTGATGATCTCTGTATTGCCATTCACCCGGATCATGAGTATGTTCTTG
ACTCATTCTCTAGTTCATTTGCTGAACTTGGGCTTAAGTATGATTTCACACAA
AGGCATAGAAATAAACAGGATTTGTGGTTCATGTCACATCGAGGTGTTCTGA
TCGATGACATCTACATTCCGAAACTTGAACCTGAGCGAATTGTTGCAATTCTT
GAGTGGGACAAATCCAAGCTTCCGGAGCATCGTTTGGAGGCAATCACAGCAG
CAATGATCGAATCATGGGGTTATGGTGAGCTAACACACCACATCCGCAGATT
TTACCAATGGGTTCTTGAGCAAGCTCCATTCAATGAGTTGGCTAGGCAAGGG
AGAGCCCCTTACGTCTCGGAAGTTGGATTAAGAAGGCTGTATACCAGTGAGC
GTGGTTCGATGGATGAGTTGGAAGCGTATATCGATAAATACTTCGAACGTGA
GCGTGGAGATTCTCCTGAGCTATTAGTGTACCATGAATCAAAAAGCTTGGAT
GATAATCAGATTATCTGTCACATGAATGAGCATGTTTATCACCAATCTAAGAC
TGAAGCGGTGGATGCAGGTCTCAATGATAAGCTGAAAGAGAAGGAAAAAGA
AAAAGATAAAGAAAAAGAAAAGGAAAAGAAAGACAAGAAGGCTGCTAGTG
ACGAAAGTGATGTGTCAACTAGCACAAAAACTAGGGAGAGAGATAGAGATG
TCAATGCTGGAACTAGTGGTACATTCGCAGTTCCAAGAATCAACCATTTACTG
ATAAAATGATTTTGCCCAGAATTAAGGGAAAAGTTGTTCTTAATTTGAATCAT
CTCTTCAGTATAATCCACAGCAAATTGACATCTCGAACACTCGTGCCACACAA
TCACAGTTTGAGAAGTGGTATGAGGGAGTGAGGGATGACTATGGCCTTAATG
ATAATGGAAATGCAGTGATGTTGAACGCTTAATGGTTTGGTGTATCGAAATTG
TTACATCACCAGACAATATCTGGTGTTGGTCATGATGGATGTGAACTCAGGTC
GAATTATCCATTAAACCGTTATTGAGCATGCACTTCGCATTTAGGCAATCATG
CTCATCCAGTAACGCGGCGAGCGTATTGCCTGGAATTGCCCTGGAGAGTATA
CATTGCTG3’

Fig. 1d: Nucleotide sequence of partial NIb and CP gene of PRSV isolate PZ-4 (1209 bp)

5’GTGTGTGTTTATATCAATGGTGATGATCTCTGTATCGCCATTCATCCGGAT
CATGAGTATGTTCTTGACTCATTTTCTAGTTCATTTGCTGAACTTGGGCTTAA
GTATGATTTCACACAAAGACATAGGAATAAACAGGATTTGTGGTTCATGTCA
CATCGAGGTGTTCTGATCGATAACATTTACATTCCGAAACTTGAACCTGAGC
GAATTGTTGCAATTCTTGAGTGGGACAAATCCAAACTTCCGGAACATCGCTT
GGAGGCAATCACAGCAGCAATGATAGAATCATGGGGTTATGGTGAGCTAAC
ACACCAAATCCGCAGATTCTACCAATGGGTTCTTGAACAAGCTCCATTCAAT
GAGTTGGCTAGGCAAGGGAGAGCCCCTTATGTCTCGGAGGTTGGATTAAGAA
GGCTGTATACCAGTGAGCGTGGTTCAATGGATGAGTTGGAAGCATACATTGA
CAAATACTTCGAACGTGAGCGTGGAGATTCTCCCGAGTTATTAGTGTATCAT
GAATCAGAAAGTTCGGATGGTCATCAGATTATCGATCACATGAATGAGCACG
TTTATCATCAATCTAAGACTGAAGCGGTGGATGCAGGTCTCAATGATAAGCT
GAAAGAGAAGGAAAAAGAAAAAGATAAAGAAAAAGAAAAAGAAAAGAGA
GACAAGAAGGATGCTAGTGACGGAGGTAGTGTGTCAACTAGCACAAAAATT
GGAGAGAGAGATAGAGATGTCAATGCTGGAACTAGTGGTACATTTGCAGTTC
CAAGAACCAAGCCATTTACTGATAAAATGATTTTGCCCAGAATT 3’

Fig. 1e: Nucleotide sequence of partial NIb and CP gene of PRSV isolate PT-5 (820 bp)

MCVFYINGDDLCIAIHPDHEYVLDSFSSSFAELGLKYDFTQRHRNKQDLWFMSHRGVLIN
NIYIPKLEPERIVAILEWDKSKLPEHRLEAITAAMIESWGYGELTHQIRRFYQWVLEQAP
FNELARQGRAPYVSEVGLRRLYTSERGSMDELEAYIDKYFERERGDSPELLVYHESKSPG
DHQIVGHMNEHVYHQSKTESGGCRSQ

Fig. 2a: Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate VK-1

MCVFYINGDDLCIAIHPDHEYVLDSFSSSFAELGLKYDFTQRHRNKQDLWFMSHRGVLID
DIYIPKLEPERIVAILEWDKSKLPEHRLEAITAAMIESWGYGELTHHIRRFYQWVLEQAP
FNELARQGRAPYVSEVGLRRLYTSERGSMDELEAYIDKYFERERGDSPELLVYHESKSLD
DNQIICHMNEHVYHQSKTEAVDAGLNDKLKEKEKEKDKEKEKEKKDKKAASDESDVST
STKTRERDRDVNAGTSGTFAVPRIKPFTDK

Fig. 2b: Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate KR-2

MCVFYINGDDLCIAIHPDHEYVLDSFSSSFAELGLKYDFTQRHRNKQDLWFMSHRGVLID
NIYIPKLEPERIVAILEWDKSKLPEHRLEAITAAMIESWGYGELTHQIRRFYQWVLEQAP
FNELARQGRAPYVSEVGLRRLYTSERGSMDELEAYIDKYFERERGDSPELLVYHESESSD
GHQIIDHMNEHVYHQSKTEAVDAGLNDKLKEKEKEKDKEKEKEKRDKKDASDGGSVST
STKIGERDRDVNAGTSGTFAVPRTKPFTDKMILPRIKGKVVLNLNHLLQYNPQQIDISNTR
AHNHSLKSV

Fig. 2c: Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate ML-3

MCVFYINGDDLCIAIHPDHEYVLDSFSSSFAELGLKYDFTQRHRNKQDLWFMSHRGVLID
DIYIPKLEPERIVAILEWDKSKLPEHRLEAITAAMIESWGYGELTHHIRRFYQWVLEQAP
FNELARQGRAPYVSEVGLRRLYTSERGSMDELEAYIDKYFERERGDSPELLVYHESKSLD
DNQIICHMNEHVYHQSKTEAVDAGLNDKLKEKEKEKDKEKEKEKKDKKAASDESDVST
STKTRERDRDVNAGTSGTFAVPRINHLLIK

Fig. 2d: Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate PZ-4

MSHRGVLIDNIYIPKLEPERIVAILEWDKSKLPEHRLEAITAAMIESWGYGELTHQIRRFYQ
WVLEQAPFNELARQGRAPYVSEVGLRRLYTSERGSMDELEAYIDKYFERERGDSPELLV
YHESESSDGHQIIDHMNEHVYHQSKTEAVDAGLNDKLKEKEKEKDKEKEKEKRDKKDA
SDGGSVSTSTKIGERDRDVNAGTSGTFAVPRTKPFTDKMILPRI

Fig. 2e: Deduced amino acid sequence of partial NIb and CP gene of PRSV isolate PT-5

Kerala. It also showed 88 per cent similarity to four other accessions namely, KA- Gu from Gulbarga, Karnataka (DQ666639.1), TA- Ti from Tiruvallur, Tamil Nadu (DQ 666641.1), AP- Ko, Kovvur isolate (DQ 666638.1) and HYD, Hyderabad isolate (KP743981.1) from Andhra Pradesh with minimum E value. There were 100 Blast hits against the sequence of isolate VK-1 with 96 per cent query coverage (Fig. 3a and 3b). On translating the nucleotide sequence using ExPASy translator tool, the longest frame *i.e.* 5'3' Frame 3 was used to deduce the amino acid sequence from the six possible open reading frames (ORFs) obtained. It comprised of 206 amino acids. However, protein BLAST analysis of this deduced amino acid sequence showed highest similarity to the amino acid sequence of Hospet isolate from Karnataka (AAX07289.1).

4.7.6.2. In silico Analysis of PRSV Isolate - KR-2

BLASTn analysis of the nucleotide sequence of isolate KR-2 showed maximum sequence similarity (93%) to PRSV isolate KE- Ca (DQ666640.1) from Calicut, Kerala. The BLAST results also revealed significant similarity to other south Indian isolates with an identity percentage ranging from 89 to 92 per cent. Isolate KA-Gu (DQ666639.1) is found to be the most similar (92%) among the south Indian isolates other than those from Kerala. It also showed 91 per cent similarity to four accessions namely isolate HYD (KP743981.1) and AP-Ko (DQ 666638.1) from Andhra Pradesh, TA-Ti from Tamil Nadu (DQ666641.1 and KA-Ho from Karnataka (AY839865.1). It was found to be less similar to Andhra Pradesh isolates *viz*., AP-Te (AY839864.1) from Hyderabad and AP-Ra (AY839863.1) from Rly Kodur with an identity of 90 and 89 per cent respectively. There were 100 Blast hits against the sequence of isolate KR-2 with 92 per cent query coverage (Fig. 4a, 4b). On translating the nucleotide sequence using ExPASy translator tool, the longest frame, 5'3' Frame 2 was used to deduce the amino acid sequence from the six possible ORFs obtained. It comprised of 268 amino acids. Protein BLAST analysis of the deduced amino acid sequence of isolate KR-2 exhibited 95 per cent identity to amino acid sequence of isolate KE-Ca from Calicut (ABG72803.1).

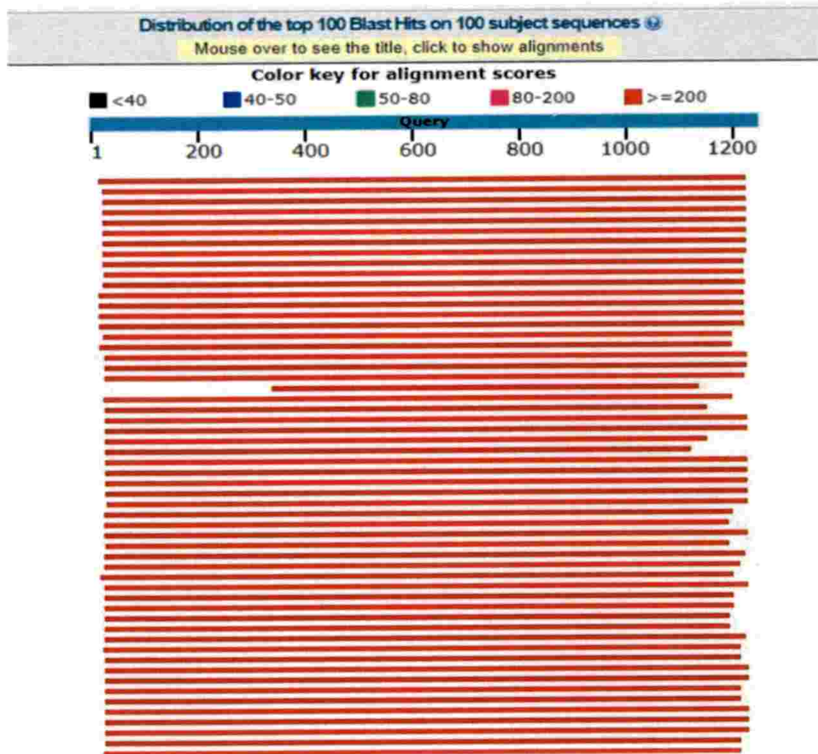


Fig. 3a: Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate VK-1

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Papaya ringspot virus isolate PRSV-KE Ca polyprotein gene, partial cds	1526	1526	96%	0.0	90%	DQ666640.1
Papaya ringspot virus isolate PRSV-KA Gu polyprotein gene, partial cds	1391	1391	96%	0.0	88%	DQ666639.1
Papaya ringspot virus isolate PRSV-TA Ti polyprotein gene, partial cds	1386	1386	96%	0.0	88%	DQ666641.1
Papaya ringspot virus isolate PRSV-AP Ko polyprotein gene, partial cds	1386	1386	96%	0.0	88%	DQ666638.1
Papaya ringspot virus isolate HYD, complete genome	1380	1380	96%	0.0	88%	KP743981.1
Papaya ringspot virus isolate PRSV-AP Te polyprotein gene, partial cds	1325	1325	96%	0.0	87%	AY839864.1
Papaya ringspot virus isolate PRSV-KA Ho polyprotein gene, partial cds	1295	1295	96%	0.0	87%	AY839865.1
Papaya ringspot virus isolate PRSV-AP Ra polyprotein gene, partial cds	1230	1230	96%	0.0	86%	AY839863.1
Papaya ringspot virus isolate E2, complete genome	1013	1013	95%	0.0	82%	KC345609.1
Papaya ringspot virus W from India, complete genome	1011	1011	95%	0.0	82%	EU475877.1
Papaya ringspot virus isolate PRSV-W-1, complete genome	1005	1005	95%	0.0	82%	DQ374153.1
Papaya ringspot virus isolate PG, complete genome	1003	1003	96%	0.0	82%	EU126128.1
Papaya ringspot Virus complete genomic RNA	1003	1003	96%	0.0	82%	X67673.1
Papaya ringspot virus (strain P) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	1003	1003	96%	0.0	82%	D00595.1
Papaya ringspot Virus genomic RNA for nuclear inclusion protein b and coat protein	1003	1003	96%	0.0	82%	X67672.1
Papaya ringspot virus Mex-VrPO, complete genome	1002	1002	93%	0.0	83%	AY231130.1
Papaya ringspot virus (strain W) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	998	998	94%	0.0	82%	D00594.1

Fig. 3b: Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate VK-1

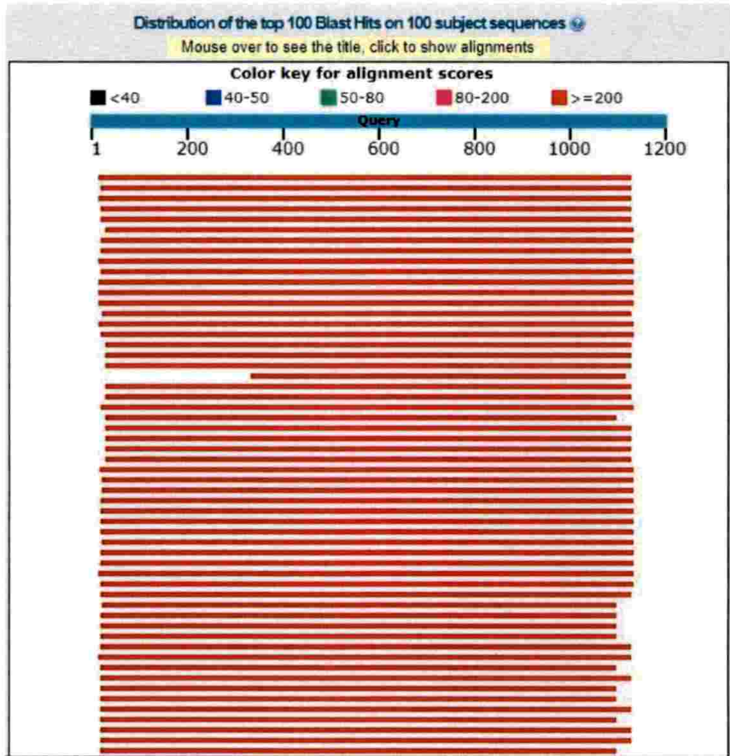


Fig. 4a: Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate KR-2

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Papaya ringspot virus isolate PRSV-KE Ca polyprotein gene, partial cds	1639	1639	92%	0.0	93%	DQ666640.1
Papaya ringspot virus isolate PRSV-KA Gu polyprotein gene, partial cds	1541	1541	92%	0.0	92%	DQ666639.1
Papaya ringspot virus isolate HYD, complete genome	1526	1526	92%	0.0	91%	KP743981.1
Papaya ringspot virus isolate PRSV-TA Ti polyprotein gene, partial cds	1507	1507	92%	0.0	91%	DQ666641.1
Papaya ringspot virus isolate PRSV-AP Ko polyprotein gene, partial cds	1474	1474	92%	0.0	91%	DQ666638.1
Papaya ringspot virus isolate PRSV-KA Ho polyprotein gene, partial cds	1439	1439	91%	0.0	91%	AY839865.1
Papaya ringspot virus isolate PRSV-AP Te polyprotein gene, partial cds	1415	1415	92%	0.0	90%	AY839864.1
Papaya ringspot virus isolate PRSV-AP Ra polyprotein gene, partial cds	1336	1336	92%	0.0	89%	AY839863.1
Papaya ringspot virus isolate PG, complete genome	1147	1147	92%	0.0	85%	EU126128.1
Papaya ringspot virus isolate E2, complete genome	1144	1144	92%	0.0	85%	KC345609.1
Papaya ringspot Virus complete genomic RNA	1142	1142	92%	0.0	85%	X67673.1
Papaya ringspot virus (strain P) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	1142	1142	92%	0.0	85%	D00595.1
Papaya ringspot Virus genomic RNA for nuclear inclusion protein b and coat protein	1142	1142	92%	0.0	85%	X67672.1
Papaya ringspot virus W isolate B9, complete genome	1125	1125	91%	0.0	85%	KX655867.1
Papaya ringspot virus (strain W) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	1125	1125	92%	0.0	85%	D00594.1
Papaya ringspot virus Mex-VrPO, complete genome	1105	1105	92%	0.0	85%	AY231130.1
Papaya ringspot virus W isolate 38NT, complete genome	1094	1094	91%	0.0	85%	KX655872.1
Papaya ringspot virus W isolate 24NT, complete genome	1094	1094	91%	0.0	85%	KX655871.1

Fig. 4b: Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate KR-2

4.7.6.3. In silico Analysis of PRSV Isolate - ML-3

BLASTn analysis of nucleotide sequence of isolate ML-3 exhibited 92 per cent identity to PRSV isolate KE- Ca (DQ666640.1) from Calicut, Kerala. It also showed 90 per cent sequence similarity to two isolates *viz*., HYD (KP743981.1) from Hyderabad and KA-Gu (DQ666639.1) from Gulbarga. However, other isolates from south India namely, TA-Ti from Tiruvallur, Tamil Nadu (DQ666641.1), AP-Ko (DQ 666638.1) from Kovvur, Andhra Pradesh and KA-Ho (AY839865.1) from Hospet, Karnataka showed an identity percentage of only 89 per cent with minimum E value (Fig. 5a, 5b). On translating the nucleotide sequence using ExPASy translator tool, the longest frame 5'3' Frame 1 was used to deduce the amino acid sequence from the six possible ORFs obtained. It comprised of 308 amino acids. Protein BLAST of the deduced amino acid sequence exhibited 96 per cent identity to isolate KE- Ca (ABG72803.1) from Calicut.

4.7.6.4.. In silico Analysis of PRSV Isolate - PZ-4

Nucleotide BLAST analysis carried out for sequence of isolate PZ-4 revealed that this isolate also showed maximum similarity to isolate KE- Ca (DQ666640.1) from Calicut, Kerala with 94 per cent identity. The sequence had also shown similarity (92%) to three other accessions *viz*., isolate KA- Gu (DQ666639.1) from Gulbarga, Karnataka, isolate HYD (KP743981.1) from Hyderabad and isolate TA- Ti (DQ666641.1) from Tamil Nadu with minimum E value. An identity of 91 per cent was seen in case of isolates AP-Ko (DQ 666638.1) and KA-Ho (AY839865.1) from Andhra Pradesh and Karnataka respectively (Fig. 6a, 6b). The nucleotide sequence was translated to six possible ORFs and the longest frame 5'3' Frame 2 was used to deduce the amino acid sequence. It consisted of 268 amino acids. Protein BLAST of the deduced amino acid sequence exhibited 95 per cent similarity to isolate KE- Ca (ABG72803.1).

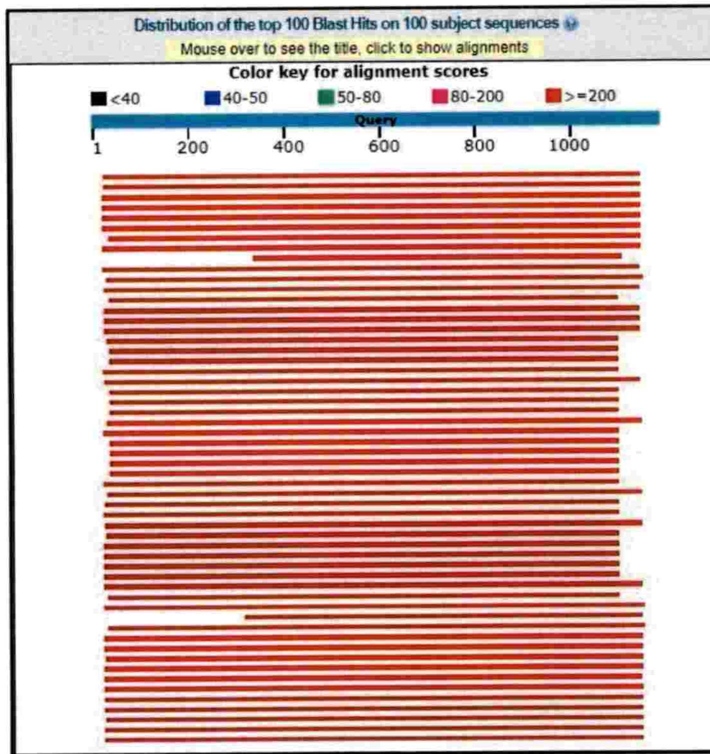


Fig. 5a: Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate ML-3

Sequences producing significant alignments:

Select All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Papaya ringspot virus isolate PRSV-KE Ca polyprotein gene, partial cds	1576	1576	94%	0.0	92%	DQ666640.1
Papaya ringspot virus isolate HYD, complete genome	1434	1434	94%	0.0	90%	KP743981.1
Papaya ringspot virus isolate PRSV-KA Gu polyprotein gene, partial cds	1430	1430	94%	0.0	90%	DQ666639.1
Papaya ringspot virus isolate PRSV-TA Ti polyprotein gene, partial cds	1424	1424	94%	0.0	89%	DQ666641.1
Papaya ringspot virus isolate PRSV-AP Ka polyprotein gene, partial cds	1408	1408	94%	0.0	89%	DQ666638.1
Papaya ringspot virus isolate PRSV-AP Te polyprotein gene, partial cds	1369	1369	94%	0.0	89%	AY839864.1
Papaya ringspot virus isolate PRSV-KA Ho polyprotein gene, partial cds	1362	1362	93%	0.0	89%	AY839865.1
Papaya ringspot virus isolate PRSV-AP Ra polyprotein gene, partial cds	1297	1297	94%	0.0	88%	AY839863.1
Papaya ringspot virus isolate PRSV-AP polyprotein gene, partial cds	1029	1029	64%	0.0	91%	AF329637.1
Papaya ringspot virus isolate E2, complete genome	1018	1018	94%	0.0	83%	KC345609.1
Papaya ringspot virus P isolate DEL, complete genome	1018	1018	94%	0.0	83%	EF017707.1
Papaya ringspot virus isolate PG, complete genome	1014	1014	94%	0.0	83%	EU126128.1
Papaya ringspot virus W isolate 39NT, complete genome	1011	1011	89%	0.0	84%	KX655872.1
Papaya ringspot Virus complete genomic RNA	1009	1009	94%	0.0	83%	X67673.1
Papaya ringspot virus (strain P) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	1009	1009	94%	0.0	83%	D00595.1
Papaya ringspot Virus genomic RNA for nuclear inclusion protein b and coat protein	1009	1009	94%	0.0	83%	X67672.1
Papaya ringspot virus W isolate B9, complete genome	1007	1007	89%	0.0	84%	KX655867.1
Papaya ringspot virus W isolate 24NT, complete genome	1005	1005	89%	0.0	84%	KX655871.1
Papaya ringspot virus W isolate 4NT, complete genome	1005	1005	89%	0.0	84%	KX655860.1
Papaya ringspot virus Mex-WPO, complete genome	1000	1000	90%	0.0	84%	AY231130.1
Papaya ringspot virus (strain W) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	998	998	94%	0.0	83%	D00594.1
Papaya ringspot virus W isolate 45C, complete genome	994	994	89%	[illegible]	[illegible]	[illegible]

Activate Windows
Go to Settings to activate Windows

Fig. 5b: Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate ML-3

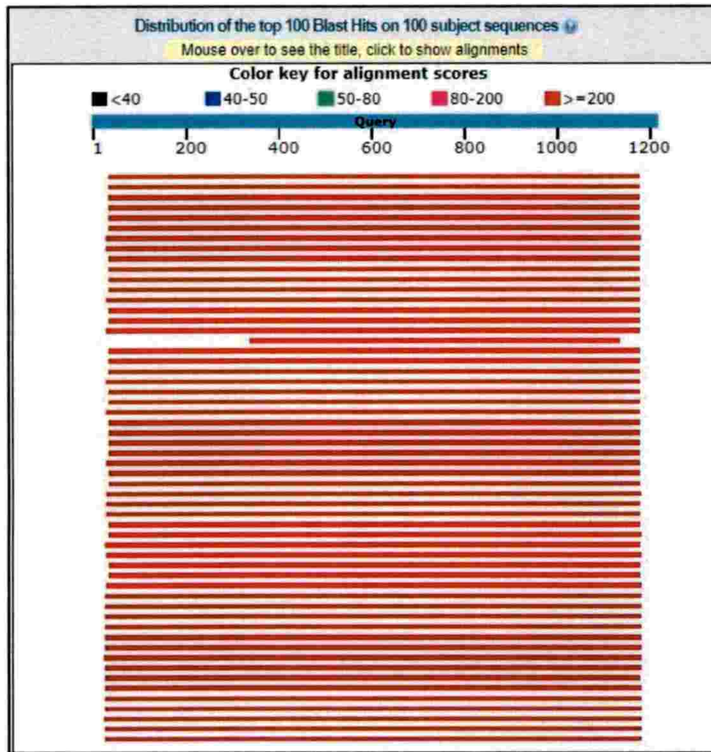


Fig. 6a: Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate PZ-4

Sequences producing significant alignments:

Select All None Selected:0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Papaya ringspot virus isolate PRSV-KE Ca polyprotein gene, partial cds	1722	1722	93%	0.0	94%	DQ666640.1
Papaya ringspot virus isolate PRSV-KA Gu polyprotein gene, partial cds	1600	1600	93%	0.0	92%	DQ666639.1
Papaya ringspot virus isolate HYD, complete genome	1589	1589	93%	0.0	92%	KP743981.1
Papaya ringspot virus isolate PRSV-TA Ti polyprotein gene, partial cds	1578	1578	93%	0.0	92%	DQ666641.1
Papaya ringspot virus isolate PRSV-AP Ko polyprotein gene, partial cds	1533	1533	93%	0.0	91%	DQ666638.1
Papaya ringspot virus isolate PRSV-KA Ho polyprotein gene, partial cds	1507	1507	93%	0.0	91%	AY839865.1
Papaya ringspot virus isolate PRSV-AP Te polyprotein gene, partial cds	1491	1491	94%	0.0	90%	AY839864.1
Papaya ringspot virus isolate PRSV-AP Ra polyprotein gene, partial cds	1399	1399	94%	0.0	89%	AY839863.1
Papaya ringspot virus isolate PG, complete genome	1181	1181	93%	0.0	86%	EU126128.1
Papaya ringspot Virus complete genomic RNA	1181	1181	93%	0.0	86%	X67673.1
Papaya ringspot virus (strain P) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	1181	1181	93%	0.0	86%	D00595.1
Papaya ringspot Virus genomic RNA for nuclear inclusion protein b and coat protein	1181	1181	93%	0.0	86%	X67672.1
Papaya ringspot virus isolate E2, complete genome	1166	1166	94%	0.0	85%	KC345609.1
Papaya ringspot virus (strain W) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	1164	1164	93%	0.0	85%	D00594.1
Papaya ringspot virus W isolate B9, complete genome	1158	1158	93%	0.0	85%	KX655867.1
Papaya ringspot virus Mex-WPO, complete genome	1133	1133	94%	0.0	85%	AY231130.1
Papaya ringspot virus isolate PRSV-AP polyprotein gene, partial cds	1133	1133	85%	0.0	93%	AF323637.1
Papaya ringspot virus W isolate 38NT, complete genome	1129	1129	93%	0.0	85%	KX655872.1
Papaya ringspot virus W isolate 24NT, complete genome	1129	1129	93%	0.0	85%	KX655871.1
Papaya ringspot virus W isolate 4NT, complete genome	1129	1129	93%	0.0	85%	KX655869.1
Papaya ringspot virus isolate PRSV-W-1, complete genome	1127	1127	94%	0.0	85%	DQ374153.1

Fig. 6b: Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate PZ-4

4.7.6.5. In silico Analysis of PRSV Isolate - PT-5

The nucleotide sequence of isolate PT- 5 under the present study was also subjected to nucleotide BLAST. The analysis showed the highest sequence similarity (95%) to isolate KE-Ca (DQ666640.1) from Calicut, Kerala. It also showed 92 per cent similarity to other south Indian isolates *viz.*, HYD (KP743981.1) and AP-Ko (DQ 666638.1) from Andhra Pradesh, KA-Gu (DQ666639.1) from Gulbarga, Karnataka, TA- Ti (DQ666641.1) from Tiruvallur, Tamil Nadu. Isolate PT- 5 showed a similarity of only 91 per cent to isolate KA-Ho (AY839865.1) from Hospet, Karnataka. Isolate AP- Ra (AY839863.1) from Rly Kodur, Andhra Pradesh was found to be divergent compared to other reported isolates of PRSV isolates from south India with an identity of only 89 per cent (Fig. 7a, 7b). Translation of nucleotide sequence using ExPASy translator tool gave six possible ORFs and the longest frame 5'3'Frame 2 was used to deduce the amino acid sequence. It consisted of 223 amino acids. Protein BLAST of the deduced amino acid sequence revealed that it was most similar to isolate KE-Ca (ABG72803.1) with an identity of 95 per cent.

4.7.6.6. Phylogenetic Analysis

A neighbor joining phylogenetic tree was constructed upon alignment of nucleotide sequences of five isolates generated from the study and 21 other sequences coding for partial NIb and CP region of PRSV reported from India and elsewhere available in the Genbank database given in Table 7. The phylogenetic tree illustrating the relationship of PRSV isolates from different geographical locations is shown in Fig. 8.

The analysis on the basis of partial NIb and CP gene of nucleotide sequences generated in this study and other isolates from distinct geographical locations resulted in multiple subgroups. The tree produced two major and distinctly branched groups *i.e* Group 1 and Group 2 which was an outgroup. Group 1 further branched into two subgroups *i.e* subgroup 1 A and subgroup 1B. Subgroup 1A consisted of mainly the

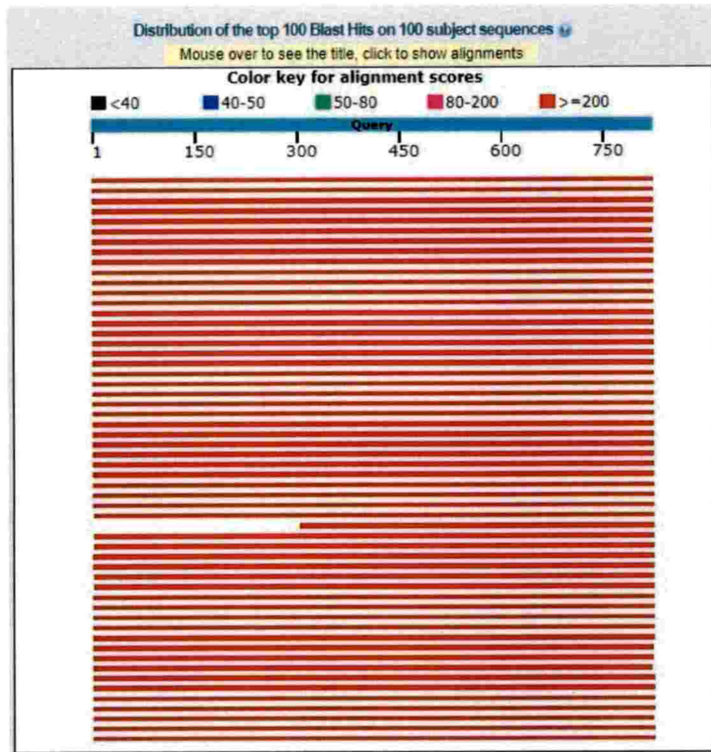


Fig. 7a: Blastn graphical output of nucleotide sequence of partial NIb and CP region of PRSV isolate PT-5

Sequences producing significant alignments:

Select: All None Selected:0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Papaya ringspot virus isolate PRSV-KE Co polyprotein gene, partial cds	1277	1277	100%	0.0	95%	DQ666640.1
Papaya ringspot virus isolate HYD, complete genome	1149	1149	100%	0.0	92%	KP743981.1
Papaya ringspot virus isolate PRSV-KA Gu polyprotein gene, partial cds	1144	1144	100%	0.0	92%	DQ666639.1
Papaya ringspot virus isolate PRSV-TA Ti polyprotein gene, partial cds	1138	1138	100%	0.0	92%	DQ666641.1
Papaya ringspot virus isolate PRSV-AP Ko polyprotein gene, partial cds	1138	1138	100%	0.0	92%	DQ666638.1
Papaya ringspot virus isolate PRSV-AP Te polyprotein gene, partial cds	1081	1081	99%	0.0	90%	AY839864.1
Papaya ringspot virus isolate PRSV-KA Hu polyprotein gene, partial cds	1074	1074	100%	0.0	91%	AY839865.1
Papaya ringspot virus isolate PRSV-AP Ra polyprotein gene, partial cds	1011	1011	100%	0.0	89%	AY839863.1
Papaya ringspot virus W isolate 38NT, complete genome	819	819	100%	0.0	85%	KX655872.1
Papaya ringspot virus W isolate 24NT, complete genome	808	808	100%	0.0	85%	KX655871.1
Papaya ringspot virus W isolate 4NT, complete genome	808	808	100%	0.0	85%	KX655860.1
Papaya ringspot virus isolate E2, complete genome	808	808	100%	0.0	85%	KC345609.1
Papaya ringspot virus P isolate DEL, complete genome	808	808	100%	0.0	85%	EF017707.1
Papaya ringspot Virus complete genomic RNA	808	808	100%	0.0	85%	X67673.1
Papaya ringspot virus (strain P) gene for polyprotein precursor, partial cds (nuclear inclusion protein and coat protein region)	808	808	100%	0.0	85%	D00595.1
Papaya ringspot Virus genomic RNA for nuclear inclusion protein b and coat protein	808	808	100%	0.0	85%	X67672.1
Papaya ringspot virus isolate PRSV_CH, complete genome	802	802	100%	0.0	84%	KT275936.1
Papaya ringspot virus isolate PRSV_VR, complete genome	802	802	100%	0.0	84%	KT275937.1
Papaya ringspot virus isolate PG, complete genome	802	802	100%	0.0	84%	EU126128.1
Papaya ringspot virus Mei-VrPO, complete genome	802	802	100%	0.0	84%	AY231130.1
Papaya ringspot virus W isolate 45C, complete genome	797	797	100%	0.0	84%	KX655870.1

Fig. 7b: Blastn text output of nucleotide sequence of partial NIb and CP region of PRSV isolate PT-5

south Indian isolates. The isolates under the present study grouped into two clusters although showing very little diversity among them. Isolate KR- 2 and PZ - 4 are closely related to each other than remaining isolates in the present study. Bootstrap value of 100 indicateds the maximum probability of obtaining the same clustering pattern. Isolates VK1, ML-3 and PT-5 fall in a common cluster. However, the isolates ML-3 and PT- 5 split again into two branches from a common node indicating that these two isolates are more closely related to each other than isolate VK-1. These five isolates showed maximum similarity to isolate KE- Ca (DQ666640.1) from Calicut, Kerala which is well supported by a bootstrap value of 97. The isolates from Calicut and those under the present study are also related to isolate PRSV-AP (AF323627.1) from Andhra Pradesh possessing the same lineage and this is supported by a bootstrap value of 87. It can also be noted from the tree that all south Indian isolates namely, KA- Gu (DQ666639.1), TA-Ti (DQ666641.1), AP-Te (AY839864.1), AP-Ra (AY839863.1), KA-Ho (AY839865.1), AP- Ko (DQ 666638.1) arise from a common node as that of the isolates from the present study, grouped together in a single subgroup 1A and a bootstrap value of 100 indicates the maximum probability of obtaining the same clustering pattern on reconstruction of the tree. The isolates from Tamil Nadu reported in cucurbits *viz.*,TN TDV SG1 (KP161501.1) and TN MET PUM1 (KP161500.1) showed slight divergence from the PRSV isolates under the present study coming under a separate subgroup i.e. subgroup 1B.

The isolate PRSV-UP (AF323628.1) from north India, exhibited divergence from the south Indian isolates and clustered with the PRSV isolates reported from Pakistan *viz.*, isolate PK- Dar 3 (JX661505.1) and TJ 11 (JX024999.1) falling into a different subgroup *i.e.* subgroup 2A and this clustering pattern is supported by a bootstrap value of 100. The subgroup 2B comprised of PRSV isolates reported in cucurbits and in *Clitoria ternatea* except isolate PRSV-HA (X67672.1) reported in papaya from China. Interestingly, the south Indian isolates reported in cucurbits from Tamil Nadu *viz.*, TN NGK PUM1 (KP161494.1), TN NGK PUM2 (KP161495.1) and

126

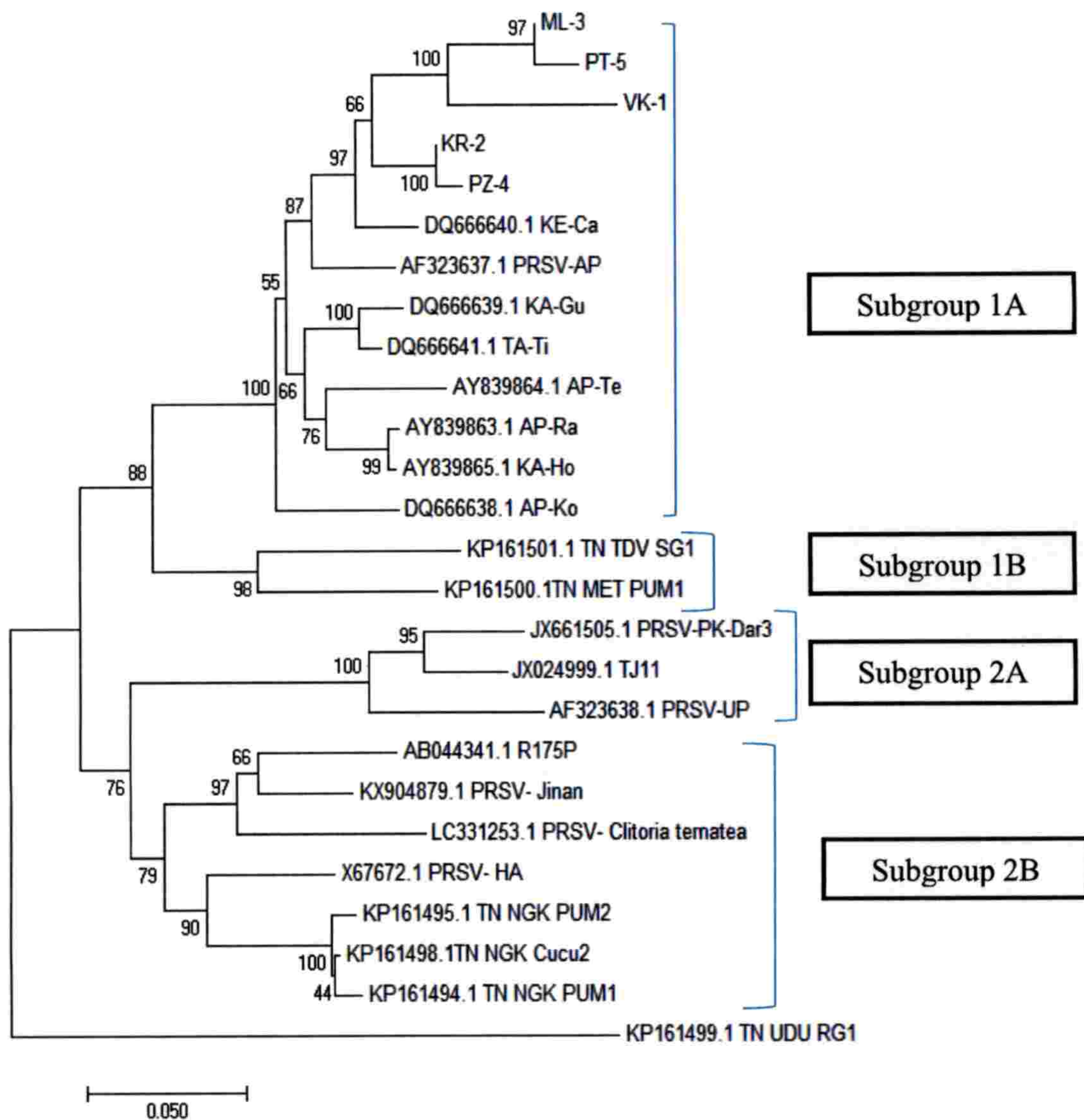


Fig. 8: Neighbor-joining tree representing phylogenetic relationships of PRSV partial polyprotein sequences from present study and other reported sequences. Numbers are percentage support of branching based on bootstrap analysis (1000 replications). The scale bar represents nucleotide substitutions per site.

TN NGK Cucu2 (KP161498.1) were grouped in a separate cluster in subgroup 2B and showed similarity to isolate PRSV- HA from China. Among all the sequences subjected to phylogenetic analysis, isolate TN UDU RG1 (KP161498.1) reported from Udumalpet, Tamil Nadu in ridge gourd exhibited maximum divergence from the isolates under present study and formed an outgroup.

4.8. MANAGEMENT OF PAPAYA RINGSPOT DISEASE

The experiment on evaluation of different chemicals, plant extracts, micronutrients and microbial formulations for the management of PRSD was undertaken in pot culture and insect proof conditions and the observations were recorded at weekly intervals. The results of the experiment are presented in Table 19. The experiment included chemicals which are reported to induce systemic resistance in plants following application *viz*., salicylic acid, acetyl salicylic acid (aspirin), humic acid and potassium silicate, microbial formulations *viz*., *Pseudomonas fluorescens*, PGPR mix II and mycopesticide *Lecanicillium lecanii,* two plant extracts *viz.* leaf extracts of *Bougainvillea spectabilis* and *Mirabilis jalapa*, a commercial viricide Perfekt, micronutrient formulations like Sampoorna and Solubor. A pre-inoculation application was first given to the test plants followed by challenge inoculation with PRSV one week after first application. After that, post - inoculation treatments were given at fortnightly intervals as described in detail under section 3.8.

The symptoms appeared in the inoculated plants about 14 to 15 days after inoculation (DAI). The vulnerability index indicating the severity of the disease was assessed every week from the first appearance of symptoms using 0-5 scale developed by Bos (1982) as described under section 3.1.

The treatments were found to be significantly different following second application *i.e.* 3 weeks after inoculation (WAI). 10 per cent leaf extract of *Bougainvillea spectabilis* (T7) and Solubor (0.1%) (T13) showed comparatively lower disease severity *viz*., 20 and 24.44 per cent respectively. The treatments were found to

be on par with each other. The maximum disease severity of 60 per cent was recorded in *Lecanicillium lecanii* (T5) and untreated control plants (T14). Sampoorna (T9) also recorded a considerably high disease severity of 53.33 per cent after the second application. The analysis revealed that these three treatments *viz*., T5, T9 and T14 were statistically on par. *P. fluorescens* (T3), PGPR mix II (T4), 10 per cent *Mirabilis jalapa* leaf extract (T6) and Solubor (0.1%) (T13) were also found to be on par after the second application.

A variation in the data was observed after the third application, *i.e.* 5 WAI, where, *P. fluorescens* (T3), *Bougainvillea* leaf extract (T7) and Solubor (0.1%) (T13) were found to be statistically on par and the disease severity recorded was 15.55, 11.11 and 15.55 respectively. *L. lecanii* (T5) did not show any reduction following third application and the severity recorded was 77.77 per cent which was found to be on par with untreated control plants (T14). Sampoorna (T9) and acetyl salicylic acid (T2) showed better results compared to T5 after third application and the two were found to be on par with each other. A disease severity ranging between 22.22 to 26.67 per cent was recorded in the other treatments which were found to be statistically on par.

After the fourth application, 7 WAI, *Bougainvillea* leaf extract (T7) exhibited the least disease severity and showed statistically significant difference from other treatments and was superior to all of them. *P. fluorescens* (T3) and Solubor (0.1%) (T13) were on par showing a disease severity of 13.33 and 15.55 per cent respectively. The same trend was observed in case of *L. lecanii* (T5) which did not show any reduction in the disease severity and was on par with control. Statistical analysis also revealed that 10 per cent leaf extract of *Mirabilis jalapa* (T6) and 0.3 per cent potassium silicate (T12) were on par.

Finally, after the fifth application, *i.e* 9 WAI , *Bougainvillea* treated plants gave the least PDS of 6.67 per cent as against 97.77 per cent in untreated control plants. This was followed by *P. fluorescens* (T3) which gave a disease severity of 11.11 per cent

over control after final treatment application. Both these treatments were found to be significantly different and superior to the remaining treatments.

To summerise, T7, *Bougainvillea* leaf extract (10%) was found to be the best treatment which gave consistently low values of PDS throughout the experiment. Initially, the PDS recorded was 20 per cent which decreased to 6.67 per cent after final application among all the treatments after final application. Hence, *Bougainvillea* treated plants gave a per cent reduction of 93.17 per cent in disease severity over untreated control plants. This was followed by *P. fluorescens* (T3) which gave a per cent reduction of 88.63 per cent and Solubor (0.1%) (T13) which gave a per cent reduction of 84.10 per cent over untreated control plants (Table 20, Fig. 9).

In order to deduce the conclusion, the data was further analysed statistically based on a ranking method proposed by Arunachalam and Bandyopadhyay (1984) where a score was allotted to each observation taken at weekly intervals for every treatment. The scores allotted for observations at weekly intervals were summed up and the treatment with the maximum value was considered as the best treatment. Based on the final score, it was confirmed that the best treatment was 10 per cent leaf extract of *Bougainvillea spectabilis* (T7) followed by 0.1 per cent Solubor (T13) and 2 per cent *P. fluorescens* (T3). The least effective treatments were found to be *L. lecanii* (T5) and 1 per cent Sampoorna micronutrient formulation (T9) (Table 21).

The biometric characters of the test plants *viz.* plant height and mean girth of stem were recorded for each treatment two weeks after every application and is presented in detail in Table 22. After the first application, the plants did not differ significantly in terms of plant height. Significant difference was observed between the treatments in the following applications. After the final application of treatments, the maximum plant height was recorded in *P. fluorescens* (T3) *i.e.* 71 cm, followed by 70 cm in plants sprayed with *Bougainvillea* leaf extract (T7). Both the treatments differed significantly from other treatments with respect to plant height. In plants treated with

Solubor (0.1%) (T13), the height recorded after final application was 60.66 cm and 61.10 cm in the treatment T6, *M. jalapa* leaf extract (10%). However, both T6 and T 13 were statistically on par. In case of untreated control plants (T14), the height recorded was only 35.60 cm.

A mean stem girth of 2.90 cm was recorded in plants treated with *P.fluorescens* (T3) and this was the highest value of girth of stem recorded. T3 differed significantly from other treatments. The second best treatment in terms of girth of the stem was *Bougainvillea* leaf extract (T7) *viz.*, 2.50 cm. Micronutrient formulation Sampoorna - 1 per cent (T9) was found to be the least effective treatment and was on par with untreated control plants. The effect of treatments on plant height and mean girth after final application is depicted in Fig. 10.

In addition to this, the influence of each treatment on the virus titre was studied by performing DAC- ELISA on plants under study. The results of ELISA are presented in Table 23. The plants applied with leaf extract of *B. spectabilis* (10%) (T7) gave the least absorbance value of 0.373 over 1.474 in control plants. The plants treated with *P. fluorescens* (2%) (T3) recorded an absorbance value of 0.509. However, these two treatments were found to be on par in terms of virus concentration. The virus titre recorded in plants treated with *L. lecanii* (T5) was 1.253 as against 1.474 in untreated control plants.

The chlorophyll content in the test plants was also recorded after the final application of the treatments using a SPAD meter. The results are presented in detail in Table 24 and depicted in Fig. 11. The maximum chlorophyll content of 44.67 SPAD units was recorded in plants treated with *Bougainvillea* leaf extract (T7). This was followed by *P. fluorescens* (T3) where the chlorophyll content recorded was 42.67 SPAD units. The next best result in terms of chlorophyll content was given by Solubor (0.1%) (T13) *viz.* 40.55 SPAD units. The value recorded in untreated control plants which expressed maximum disease severity and symptoms was only 17.66 SPAD units.

The test plants treated with 10 per cent *Bougainvillea* leaf extract (T7) and 2 per cent *P. fluorescnes* (T3) showing improved performance are depicted in Plate 14.

The per cent disease severity of untreated control plants increased gradually from 57.55 to 97.77 per cent. The symptom expression on these plants was very prominent when the temperature was low (Appendix VI).

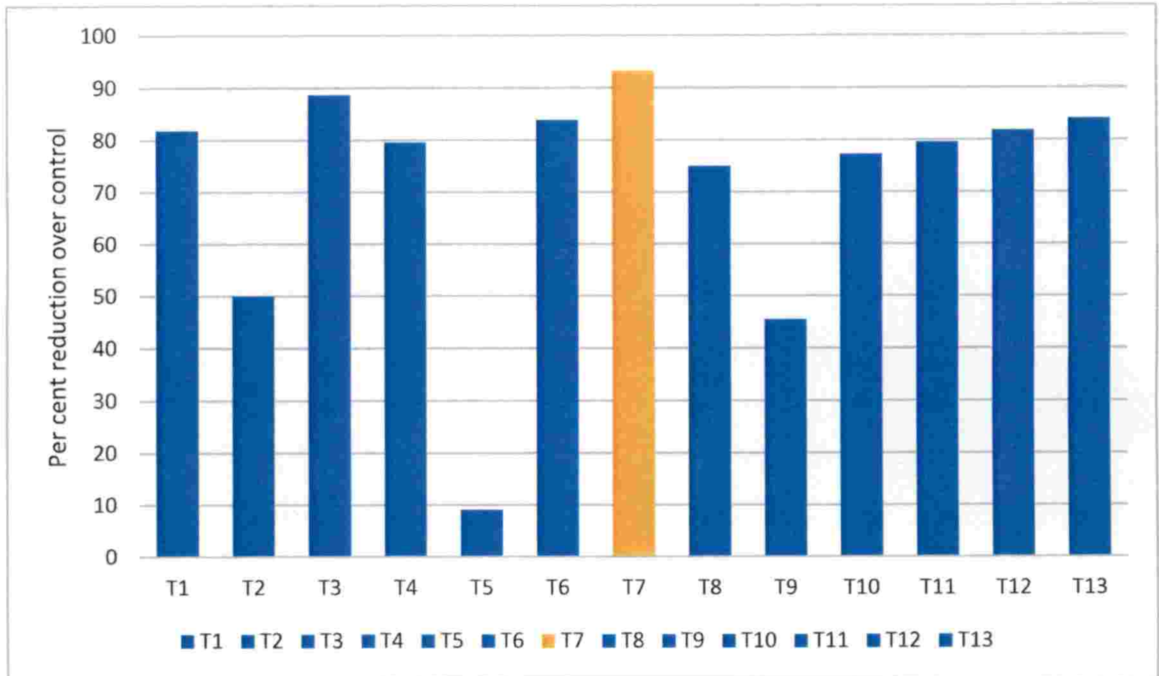
Table 19: Per cent disease severity at weekly intervals

Treatment	Per cent disease severity (PDS)							
	2WAI	3WAI	4 WAI	5WAI	6WAI	7WAI	8WAI	9WAI
Salicylic acid - 0.15 g L^{-1} (T1)	35.55 (5.93)^{cd}	31.11 (5.16)^{cd}	28.89 (5.36)^{c}	22.22 (4.70)^{c}	22.22 (4.70)^{d}	22.22 (4.70)^{d}	17.77 (4.19)^{ef}	17.77 (4.25)^{de}
Acetyl SA - 0.15 g L^{-1} (T2)	28.89 (5.36)^{de}	35.55 (5.99)^{bc}	48.88 (6.97)^{b}	60.00 (7.73)^{b}	55.55 (7.45)^{c}	51.11 (7.14)^{c}	48.89 (6.98)^{c}	48.89 (7.02)^{b}
P. fluorescens - 2% (T3)	35.55 (5.95)^{cd}	28.89 (5.41)^{de}	17.77 (4.19)^{d}	15.55 (3.92)^{d}	13.33 (3.65)^{e}	13.33 (3.65)^{f}	11.11 (3.29)^{g}	11.11 (3.37)^{f}
PGPR mix II - 2% (T4)	42.22 (6.49)^{bc}	28.89 (5.41)^{de}	26.67 (5.16)^{c}	24.44 (4.93)^{c}	22.22 (4.70)^{d}	22.22 (4.70)^{d}	22.22 (4.70)^{de}	20.00 (4.52)^{cde}
L. lecanii - 2% (T5)	57.77 (7.59)^{a}	60.00 (7.77)^{a}	73.33 (8.55)^{a}	77.77 (8.81)^{a}	75.55 (8.69)^{b}	77.77 (8.81)^{b}	84.44 (9.18)^{b}	88.89 (9.45)^{a}
Mirabilis jalapa leaf extract - 10% (T6)	28.89 (5.36)^{de}	28.89 (5.41)^{de}	24.44 (8.55)^{c}	22.22 (4.70)^{c}	22.22 (4.70)^{d}	20.00 (4.47)^{de}	20.00 (4.47)^{def}	17.77 (4.25)^{de}
B. spectabilis leaf extract-10% (T7)	35.55 (5.95)^{cd}	20.00 (4.52)^{f}	11.11 (4.93)^{e}	11.11 (3.29)^{d}	8.89 (2.93)^{f}	8.89 (2.93)^{g}	6.67 (2.58)^{h}	6.67 (2.67)^{g}
Perfekt - 0.1% (T8)	35.55 (5.95)^{cd}	31.11 (5.61)^{cd}	28.89 (5.36)^{c}	26.67 (5.16)^{c}	26.67 (5.16)^{d}	24.44 (4.93)^{d}	24.44 (4.93)^{d}	24.44 (4.98)^{c}
Sampoorna - 1% (T9)	55.55 (7.45)^{a}	53.33 (7.33)^{a}	71.11 (8.43)^{a}	62.22 (7.88)^{b}	62.22 (7.88)^{c}	55.55 (7.45)^{c}	55.55 (7.45)^{c}	53.33 (7.33)^{b}
Solubor -1% (T10)	43.33 (6.57)^{bc}	37.77 (6.18)^{b}	24.44 (4.93)^{c}	24.44 (4.93)^{c}	22.22 (4.70)^{d}	22.22 (4.70)^{d}	22.22 (4.70)^{de}	22.22 (4.75)^{cd}
Humic acid - 0.2% (T11)	44.44 (6.65)^{b}	35.55 (5.99)^{bc}	31.11 (5.57)^{c}	24.44 (4.93)^{c}	24.44 (4.93)^{d}	22.22 (4.70)^{d}	22.22 (4.70)^{de}	20.00 (4.75)^{cd}
Pot. silicate - 0.3%(T12)	44.44 (6.65)^{b}	35.55 (5.99)^{bc}	28.89 (5.36)^{c}	22.22 (4.70)^{c}	22.22 (4.70)^{d}	20.00 (4.47)^{de}	17.77 (4.19)^{ef}	17.77 (4.25)^{de}
Solubor -0.1% (T13)	24.44 (4.93)^{e}	24.44 (4.98)^{ef}	15.55 (3.92)^{de}	15.55 (3.92)^{d}	15.55 (3.92)^{e}	15.55 (3.92)^{ef}	15.55 (3.29)^{f}	15.55 (3.98)^{e}
Untreated control (T14)	57.77 (7.59)^{a}	60.00 (7.77)^{a}	77.77 (8.81)^{a}	84.44 (9.18)^{a}	91.11 (9.54)^{a}	95.55 (9.77)^{a}	97.77 (9.88)^{a}	97.77 (9.91)^{a}
CD (0.05)	0.67	0.51	0.66	0.65	0.59	0.57	0.61	0.60

Values in parentheses: square root transformed, WAI: weeks after inoculation

Table 20: Per cent reduction of disease over control after final treatment application

Treatment	PDS after final treatment application	Per cent reduction over control
Salicylic acid - 0 15 g L^{-1} (T1)	17.77 $(4.25)^{de}$	81.82
Acetyl SA - 0.15 g L^{-1} (T2)	48.89 $(7.02)^{b}$	50.00
P. fluorescens - 2% (T3)	11.11 $(3.37)^{f}$	88.63
PGPR mix II - 2% (T4)	20.00 $(4.52)^{cde}$	79.54
L. lecanii - 2% (T5)	88.89 $(9.45)^{a}$	9.08
M. jalapa leaf extract - 10% (T6)	17.77 $(4.25)^{de}$	81.82
B. spectabilis leaf extract-10% (T7)	6.67 $(2.67)^{g}$	**93.17**
Perfekt - 0.1% (T8)	24.44 $(4.98)^{c}$	75.00
Sampoorna - 1% (T9)	53.33 $(7.33)^{b}$	45.45
Solubor -1% (T10)	22.22 $(4.75)^{cd}$	77.27
Humic acid -0.2% (T11)	20.00 $(4.75)^{cd}$	79.54
Pot. silicate -0.3%(T12)	17.77 $(4.25)^{de}$	81.82
Solubor -0.1% (T13)	15.55 $(3.98)^{e}$	84.10
Untreated control (T14)	97.77 $(9.91)^{a}$	



T1 - Salicylic acid (0.15 g L^{-1})

T2 - Acetyl salicylic acid (0.15 g L^{-1})

T3 - *P. fluorescens* (2 %)

T4 - PGPR mix II - (2 %)

T5 - *L. lecanii* (2 %)

T6 - *M. jalapa* leaf extract (10 %)

T7- *B. spectabilis* leaf extract (10%)

T8 - Perfekt (0.1%)

T9 - Sampoorna (1%)

T10 - Solubor - 1%

T11 - Humic acid (0.2 %)

T12 - Potassium silicate (0.3%)

T13 - Solubor (0.1%)

T14 - Untreated control

Fig. 9: Per cent reduction in disease severity over control after final application

Table 21: Ranking of different treatments (Arunachalam and Bandyopadhyay, 1984)

Trt. No.	Treatment details	Scores alloted								Total
		2WAI	3WAI	4WAI	5WAI	6WAI	7WAI	8WAI	9WAI	
T1	Salicylic acid - 0.15 g L^{-1}	0.7	0.58	0.6	0.75	0.67	0.57	0.69	0.64	5.20
T2	Acetyl salicylic acid - 0.15 g L^{-1}	0.9	0.41	0.4	0.5	0.5	0.42	0.375	0.29	3.80
T3	*P. fluorescens* - 2%	0.7	0.75	0.8	1	0.83	0.86	0.875	0.86	6.68
T4	PGPR mix II - 2%	0.5	0.75	0.6	0.75	0.67	0.57	0.56	0.57	4.97
T5	*L.lecanii* - 2%	0.2	0.17	0.2	0.25	0.33	0.29	0.25	0.14	1.83
T6	*M. jalapa* leaf extract - 10%	0.9	0.75	0.6	0.75	0.67	0.64	0.625	0.64	5.58
T7	*B.spectabilis* leaf extract- 10%	0.7	1	1	1	1	1	1	1	**7.70**
T8	Perfekt - 0.1%	0.7	0.58	0.6	0.75	0.67	0.57	0.5	0.43	4.80
T9	Sampoorna - 1%	0.2	0.17	0.2	0.5	0.5	0.42	0.375	0.29	2.66
T10	Solubor -1%	0.5	0.33	0.6	0.75	0.67	0.57	0.56	0.5	4.48
T11	Humic acid - 0.2%	0.4	0.41	0.6	0.75	0.67	0.57	0.56	0.5	4.46
T12	Potassium silicate -0.3%	0.4	0.41	0.6	0.75	0.67	0.64	0.69	0.64	4.80
T13	Solubor -0.1%	1	0.91	0.9	1	0.83	0.79	0.75	0.71	6.89
T14	Untreated control	0.2	0.17	0.2	0.25	0.17	0.14	0.125	0.14	1.40

Table 22: Effect of treatments on biometric parameters

Treatment	After 1st spray*		After 2nd spray*		After 3rd spray *		After 4th spray*		After 5th spray*	
	Plant height (cm)	Girth of stem (cm)	Plant height (cm)	Girth of stem (cm)	Plant height (cm)	Girth of stem (cm)	Plant height (cm)	Girth of stem (cm)	Plant height (cm)	Girth of stem (cm)
T1	15.51	1.56^{ab}	40.53^{f}	1.90^{b}	47.30^{e}	2.03^{b}	49.20^{f}	2.03^{b}	49.20^{f}	2.06^{c}
T2	15.5	1.53^{b}	31.33^{i}	1.70^{cd}	40.63^{h}	1.78^{c}	41.33^{i}	1.78^{d}	41.33^{i}	1.78^{de}
T3	15.22	1.70^{a}	57.63^{a}	2.33^{a}	65.43^{a}	2.46^{a}	71.00^{a}	2.48^{a}	71.00^{a}	2.91^{a}
T4	14.91	1.66^{ab}	49.10^{c}	1.76^{bc}	57.30^{c}	1.86^{c}	59.20^{d}	1.86^{cd}	59.2^{c}	1.86^{d}
T5	15.13	1.00^{fg}	32.5^{h}	1.46^{ef}	38.83^{i}	1.49^{e}	39.70^{j}	1.49^{f}	39.70^{j}	1.50^{g}
T6	16.73	1.06^{efg}	55.13^{b}	1.58^{de}	61.16^{b}	1.65^{d}	61.1^{c}	1.65^{e}	61.10^{b}	1.68^{f}
T7	14.3	1.23^{cd}	57.13^{a}	1.81^{bc}	66.30^{a}	1.80^{c}	69.03^{b}	1.9^{c}	70.00^{a}	2.50^{b}
T8	13.43	1.10^{def}	38.53^{g}	1.46^{ef}	42.80^{g}	1.53^{e}	43.80^{h}	1.53^{f}	43.80^{h}	1.53^{g}
T9	14.36	0.93^{gh}	42.03^{e}	1.30^{g}	42.27^{g}	1.36^{f}	47.00^{g}	1.36^{g}	47.00^{g}	1.36^{h}
T10	14.26	0.83^{h}	17.26^{l}	0.90^{h}	32.32^{j}	0.86^{h}	36.00^{k}	0.93^{i}	56.16^{d}	1.00^{i}
T11	13.8	1.13^{cdef}	38.02^{g}	1.76^{bc}	44.97^{f}	1.78^{c}	49.20^{f}	1.83^{cd}	49.20^{f}	1.83^{de}
T12	14.66	1.20^{cde}	47.13^{d}	1.70^{cd}	51.26^{d}	1.78^{c}	53.26^{e}	1.78^{d}	53.26^{e}	1.78^{def}
T13	15.57	1.26^{c}	30.60^{j}	1.35^{fg}	41.77^{gh}	1.43^{ef}	47.50^{g}	1.80^{cd}	60.66^{bc}	1.73^{g}
T14	10.03	0.93^{gh}	20.20^{k}	1.00^{h}	23.33^{k}	1.13^{g}	24.50^{l}	1.13^{h}	35.60^{k}	1.38^{h}
CD (.05)	NS**	0.135	0.510	0.152	1.680	0.111	1.499	0.111	1.487	0.102

*Values are mean of 3 replication

** NS= treatments are non - significant

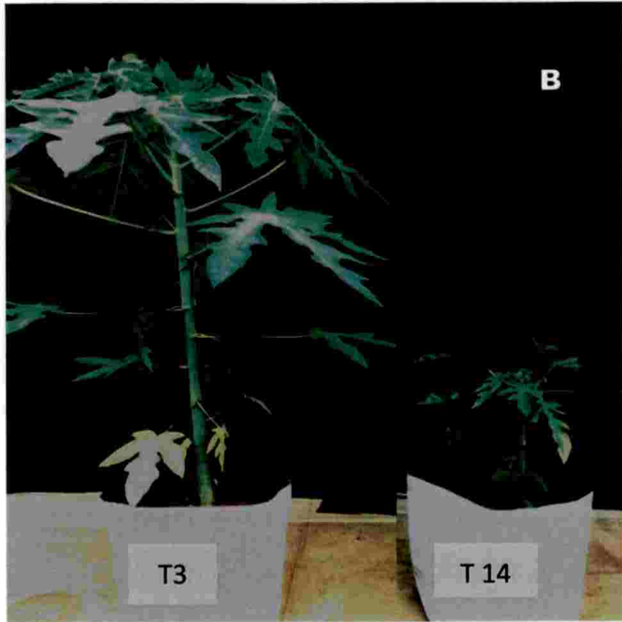
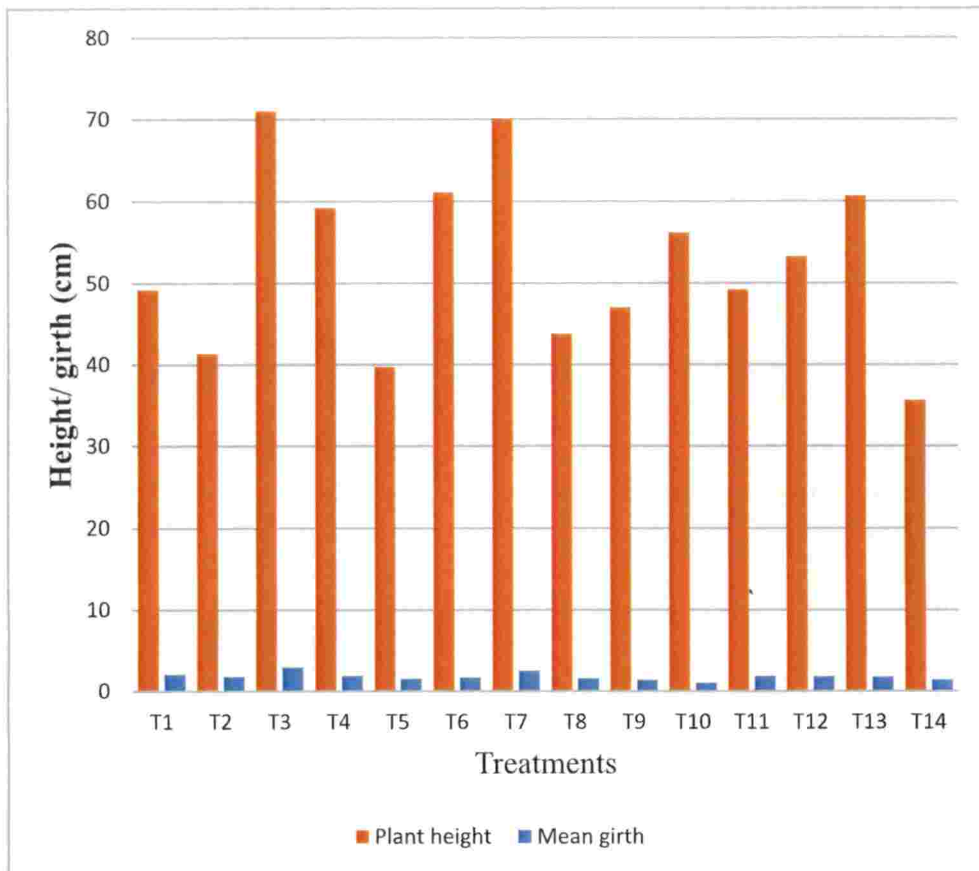


Plate 14: Papaya plants after final application (A) T7: Test plant treated with 10% leaf extract of *Bougainvillea spectabilis* T 14: Untreated control (B) T3: Test plant treated with 2% *Pseudomonas fluorescens* T 14: Untreated control



T1 - Salicylic acid (0.15 g L^{-1})

T2 - Acetyl salicylic acid (0.15 g L^{-1})

T3 - *P. fluorescens* (2 %)

T4 - PGPR mix II - (2 %)

T5 - *L. lecanii* (2 %)

T6 - *M. jalapa* leaf extract (10 %)

T7- *B. spectabilis* leaf extract (10%)

T8 - Perfekt (0.1%)

T9 - Sampoorna (1%)

T10 - Solubor - 1%

T11 - Humic acid (0.2 %)

T12 - Potassium silicate (0.3%)

T13 - Solubor (0.1%)

T14 - Untreated control

Fig. 10: Plant height and mean stem girth after final treatment application

Table 23: Influence of different treatments on virus titre of papaya plants

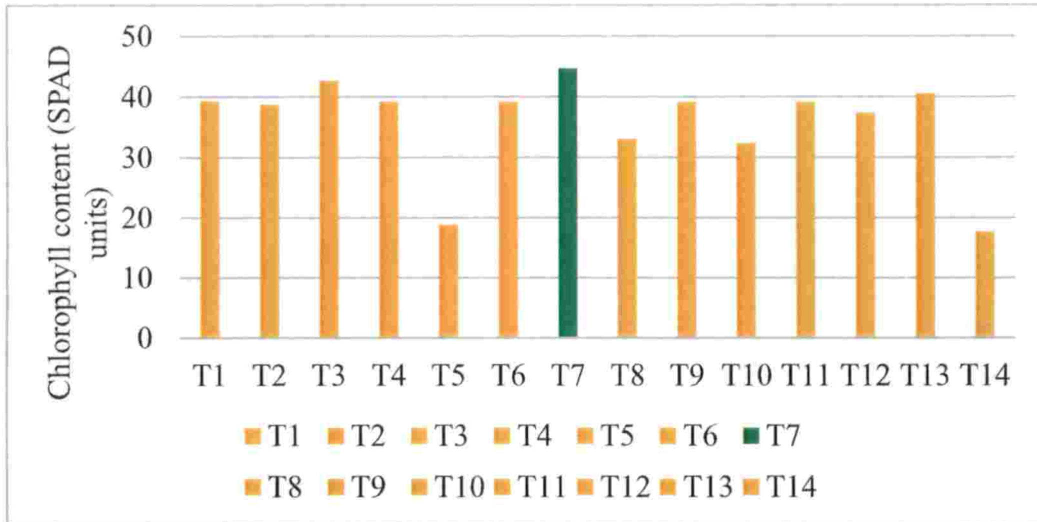
Treatment	Treatment details	Virus titre*
T1	Salicylic acid - 0.15 g L^{-1}	0.689[fg]
T2	Acetyl salicylic acid - 0.15 g L^{-1}	0.967[cd]
T3	*Pseudomonas fluorescens* - 2%	**0.509[hi]**
T4	PGPR mix II - 2%	0.714[efg]
T5	*Lecanicillium lecanii* - 2%	1.253[b]
T6	*Mirabilis jalapa* leaf extract - 10%	0.627[gh]
T7	*Bougainvillea spectabilis* leaf extract- 10%	**0.373[i]**
T8	Perfekt - 0.1%	0.834[def]
T9	Sampoorna - 1%	1.023[c]
T10	Solubor -1%	0.69[fg]
T11	Humic acid -0.2%	0.772[efg]
T12	Potassium silicate -0.3%	0.853[de]
T13	Solubor -0.1%	0.619[gh]
T14	Untreated control	1.474[a]

* Absorbance at 405 nm - mean of four replications

Table 24: Effect of treatments on chlorophyll content of leaves

Treatment	Treatment	Chlorophyll content (SPAD units)*
T1	Salicylic acid - 0.15 g L^{-1}	39.30
T2	Acetyl salicylic acid - 0.15 g L^{-1}	38.72
T3	*Pseudomonas fluorescens* - 2%	42.67
T4	PGPR mix II - 2%	39.20
T5	*Lecanicillium lecanii* - 2%	18.83
T6	*Mirabilis jalapa* leaf extract - 10%	39.10
T7	*Bougainvillea spectabilis* leaf extract- 10%	**44.67**
T8	Perfekt - 0.1%	33.075
T9	Sampoorna - 1%	39.10
T10	Solubor -1%	32.36
T11	Humic acid -0.2%	39.10
T12	Potassium silicate -0.3%	37.36
T13	Solubor -0.1%	40.55
T14	Untreated control	17.66

* Values are mean of three replications



T1 - Salicylic acid (0.15 g L^{-1})

T2 - Acetyl salicylic acid (0.15 g L^{-1})

T3 - *P. fluorescens* (2 %)

T4 - PGPR mix II - (2 %)

T5 - *L. lecanii* (2 %)

T6 - *M. jalapa* leaf extract (10 %)

T7- *B. spectabilis* leaf extract (10%)

T8 - Perfekt (0.1%)

T9 - Sampoorna (1%)

T10 - Solubor - 1%

T11 - Humic acid (0.2 %)

T12 - Potassium silicate (0.3%)

T13 - Solubor (0.1%)

T14 - Untreated control

Fig. 11: Chlorophyll content after final treatment application

Discussion

5. DISCUSSION

Papaya is an important fruit crop which is grown widely in the tropics and subtropics because of its great economic potential. In spite of its tree-like habit, papaya is considered as a herbaceous plant which could reach up to a height of 9 m and hence described as giant herbs (Malo and Campbell, 1986). Recently, considerable progress has been achieved regarding the biological activity and medicinal value of papaya and it is considered as a valuable nutraceutical fruit plant (Yogiraj *et al.*, 2014). Over the last few decades, the reports regarding the susceptibility of papaya to a wide range of viruses belonging to the genera *viz.*, Potyvirus, Rhabdovirus, Potexvirus, Geminivirus, Nepovirus, Ilarivirus, Tobravirus and Cucumovirus have been of major concern (Tennant *et al.*, 2007). These diseases caused by viruses are a major threat to papaya cultivation and production. Among the viral diseases, papaya ringspot disease caused by *Papaya ringspot virus* is reported to have a devastating effect on the economic yield of papaya fruits. The incidence of the disease has been reported from all over the world and in India, it was first reported in 1948 by Capoor and Varma from Pune. Since then, it has spread gradually to all the papaya growing states resulting in a negative impact on papaya production. This disease can cause up to 100 per cent yield loss (Tennant *et al.*, 2007) and render the crop uneconomical. Papaya plants of all ages are vulnerable to PRSV. The fruits of the infected plants become small and deformed making them unmarketable. In the recent years, the disease incidence has become very severe and this could be attributed to the change in climate which might have an influence on the secondary spread of virus through insect vectors.

The occurrence of this disease was first reported in Hawaii by Lindner (1945) and subsequently, it was reported from different papaya cultivating regions worldwide. In India, the disease was first reported in Pune by Capoor and Varma (1948) as already mentioned above and later reported from different states of the country *viz.*, Bihar (Mishra and Jha, 1955), Madhya Pradesh (Garga, 1963), Rajasthan (Surekha *et al.*, 1977) and in Maharashtra (Yemewar and Mali, 1980). In south India, it was reported

during 1995 from Karnataka (Byadgi *et al*., 1995). In the state of Kerala, it was first reported in 1999 by Philip and Nair (1999) from Vellayani, Thiruvananthapuram. Later, it was reported in Andhra Pradesh (Gourgopal and Jain, 2002) and Tamil Nadu (Sharma *et al*., 2005).

Considering the disease severity, spread and economic loss caused by PRSV, investigations were conducted on biological, immunological and molecular characterization of this devastating pathogen of papaya. The evaluation of microbial agents, plant products and various chemicals against the virus were also studied to develop possible recommendations for the management of the disease.

The project was initiated with a purposive sampling survey in selected papaya orchards in Thrissur district of Kerala namely Vellikulangara, Koratty, Venginissery, Muringoor, Pazhayannur, Puthur and Vellanikkara. The variety Red Lady was found to be widely cultivated in all the commercial orchards and was highly susceptible to the disease. The disease incidence varied from location to location. In Vellikulangara, the disease incidence was found to be as high as 99.60 followed by 98 per cent in both Koratty and Venginissery, 97.5 per cent in Puthur and 96 per cent in Pazhayannur. The percent disease severity recorded at Vellikulangara, Puthur and Pazhayannur were 48.67, 96.67 and 55 per cent respectively. In orchards of Koratty and Venginissery, the percent disease severity recorded was 65 per cent. Due to the increased demand of the variety Red Lady, large quantities of the fruits have been marketed in and around the district. Moreover, the fruits are also exported from Thrissur district to Middle - East countries. This has led to a significant increase in the area under papaya cultivation compared to last decade. The high incidence of disease in these areas might be due to large scale year- around cultivation of the crop which might have helped the virus to build up and endure in the papaya plants. Despite the high incidence of the disease in Vellikulangara, findings on percent disease severity revealed that the severity of the disease was comparatively less. This might be due to inborn tolerance of the papaya

plant which is trying to resist the infection. Even then, the fruits did not meet the quality standards for exporting due to the presence of ringspots on them.

High percent disease severity recorded in orchards surveyed in Puthur, Koratty and Venginissery suggests the severity of the disease was very high. The cultivation of a single variety *viz*. Red Lady in large areas might have helped in rapid spread of the disease once variety becomes susceptible after breakdown of resistance due to selection pressure of virus.

A wide variation was observed in the disease incidence recorded in three different plots of Muringoor *viz*., Plot 1 (85%), plot 2 (65.5 %) and plot 3 (25 %). High incidence in the first two plots may be attributed to the infestation of the orchard with aphids and mealybug and the weeds in between the rows where the insects would have colonized. Reports on insect transmission studies revealed that aphids and papaya mealy bug were the potential vectors of PRSV. A transmission percentage of 80 per cent was recorded in *Paracoccus marginatus* Williams and Garnara de Willink, followed by *Aphis gossypii* (40%) and *A. craccivora* (30%) (Krishnapriya, 2015). The lower incidence in plot 3 might be due to intercropping with banana which might have served as a barrier to viruliferous aphids. Consistent to this statement, earlier reports reveal that intercropped maize barriers have a reducing effect on the incidence and severity of PRSV in endemic areas (Prasad and Kudada, 2005; Mederos *et al*., 2013).

Purposive sampling survey was also conducted in papaya orchard at Melur. The disease incidence recorded at there was 39.28 per cent with a percent disease severity of 40 per cent. The farmer was spraying the orchard with micronutrient sprays at regular intervals and this might be the reason of reduced disease incidence. Use of micronutrients was one of the strategies outlined by Verghese *et al*. (2001) for the management of papaya ringspot disease. Manjunatha (2012) also studied the effects of micronutrients on the disease incidence of PRSV at different stages of the crop and observed a much reduced disease incidence compared to control plants.

A roving survey was also conducted at the main campus of Kerala Agricultural University, Vellanikkara and a PDI and PDS of 81.8 per cent and 51.8 percent were recorded.

The development of symptoms of the disease was investigated in detail under natural field conditions and artificial insect proof net house conditions. The most important symptoms on leaves included chlorosis, mosaic, mottling, chlorotic spots and vein clearing. In orchards where high severity was recorded, wavy leaf margins, puckering, vein thickening, formation of green islands, clustering of veins, leaf distortion and shoestring appearance were the symptoms that were observed throughout. Presence of oily streaks on the petiole and oily ringspots on the green regions of the stem were also observed as predominant symptoms. The fruits expressed typical symptom in the form of concentric or broken few to many oily ringspots depending on the degree of infection. The ringspots were observed in both young and mature fruits. The canopy of the infected plants showed a yellow discolouration from a distance and the leaves showed rosetting. Plants in advanced stages of infection failed to produce flowers and fruits. In cases where fruit was produced, they were found to be severely distorted and much reduced in size. In advanced stages of infection, there was complete defoliation and collapse of the plant leading to its death. Those which were affected at seedling stage, remain stunted and the newly developing leaves were completely malformed. Similar findings were described in the earlier reports on symptoms induced by PRSV (Torres and Giacometti,1966; Khurana and Bhargava,1970; Surekha *et al.*,1977; Almeida and Carvalho,1978; Yemewar and Mai,1980; Lana,1980; Kitajima *et al.*,1987; Lokhande and Moghe,1992; Thomas and Dodman,1993; Hussain and Varma,1994; Dahal *et al.*,1997; Gonsalves,1998).

The symptoms developed in papaya under artificial conditions when inoculated mechanically were documented and the plants developed initial symptoms 14 DAI.

Initially, leaves turned pale green in colour and exhibited mild chlorotic spots. Subsequently, the entire leaf produced mosaic symptoms. The incubation period and initial symptoms were in line with the findings of Kunkalikar (2003) and Krishnapriya (2015). In some cases, vein clearing was also observed in leaves after they turn pale green. Mottling was observed in leaves 25 - 30 DAI. Later, puckering was seen in leaves leading to complete distortion. By the end of 2 MAI, there was complete reduction in leaf lamina and the leaves presented a shoestring appearance.

Interestingly, there was masking of the symptoms in the artificially inoculated papaya plants when there was a rise in temperature during summer. The newly emerging leaves appeared healthy. Nevertheless, as soon as monsoon started and temperature began to fall, all the symptoms including those in the advanced stage also reappeared. Gonsalves and Ishii (1980) opined that the symptom expression is highly influenced by environmental conditions and were more severely expressed during cooler months. Findings by Kunkalikar (2003) and Singh and Shukla (2011) also suggest that the symptoms were masked in high temperatures leading to mild incidence of the disease.

The histological and cytological changes in the foliar tissue of papaya brought about by PRSV infection were studied and documented. The comparative observations revealed that the healthy tissues had intact palisade parenchyma, spongy mesophyll cells, definite lower and upper epidermis of the cell and stomata. However, in case of infected leaf, there was a disruption in the spatial arrangement of the cells. In general, the palisade parenchyma was malformed losing their regular columnar shape and the palisade and spongy cells were unable indistinguishable. Similar observations were made by Kunkalikar *et al.* (2007) during histological studies of infected leaves.

The chlorotic nature of the infected leaves was understood from the histological studies where complete disintegration of the chloroplasts was observed. In case of green island symptom, agglutination and increased concentration of chloroplasts was

observed all over the leaf tissue compared to the healthy tissue. It was also characterized by presence of dense bodies. Mottled leaves showed irregular distribution of chloroplasts and the chloroplasts were found to be aggregated in some regions and disintegrated at some regions of the leaf tissue. The leaves with puckering symptom exhibited hyperplasia of the cells. Similar ultrastructural and anatomical changes have been documented by Singh and Shukla (2012).

The present investigation revealed 100 per cent transmission of PRSV mechanically. The incubation period recorded was 14 days. The test plants also gave positive reaction to DAC- ELISA. The results are in accordance with previous reports (Reddy *et al.*, 2007, Krishnapriya, 2015). Even though other reports state that PRSV is mechanically transmitted, there is a difference in the incubation period and transmission percentage. Bayot *et al.* (1990) recorded a transmission of 60 per cent while 80 per cent was recorded by Kelaniyangoda and Madhubashini (2008). Earlier reports also reveal that the initial symptoms are expressed 18 to 22 DAI (Kunkalikar, 2003; Dhanam *et al.*, 2011). The variations might be due to the difference in the virus strain and environmental conditions prevailing at the time of inoculation.

Seed transmission studies were also conducted to check whether the virus is seed - borne. None of the seedlings germinated from seeds of infected fruits expressed any kind of symptom. Moreover, the seedlings gave negative reaction following testing through DAC- ELISA. The earlier reports on seed transmission are congruent with the present findings (Wang *et al.*, 1978; Prasad and Sarkar, 1989; Dahal *et al.*, 1997; Kunkalikar, 2003; Reddy *et al.,* 2007; Krishnapriya, 2015). Notwithstanding these reports, Bayot *et al.* (1990) reported a transmission of 0.15 per cent through seeds. This variety of the crop, virus strain and temperature are factors governing seed transmission (Gibbs and Harrison, 1976) which might be responsible for the variation in different reports.

In the present study, efforts were taken to ascertain the role of plants belonging to family other than Caricaceae in being the reservoir of the virus inoculum. The results of the experiment revealed that the host range of virus is limited to Caricaceae, Cucurbitaceae and Chenopodiaceae.

In case of *Chenopodium amaranticolor*, pin point necrotic lesions were observed on the inoculated leaves. Yeh and Gonsalves (1984) and Krishnapriya (2015) induced local lesions following artificial inoculation in *Chenopodium quinoa* and not *C. amaranticolor*. Moreover, in contrast to the results of the present study, several researchers failed to establish *Chenopodium* as the host of PRSV (Story and Halliwell, 1969; Opina, 1986; Ramos, 1987; Lakshminarayan Reddy, 2000). When Dhanam *et al.* (2011) conducted the experiment in *C. quinoa* and *C. amaranticolor*, the latter produced local lesions 20 DAI.

Momordica charantia and *Cucumis sativus* showed 100 per cent transmission on mechanical inoculation of PRSV. *Trichosanthes cucumerina* and *Cucurbita moschata* also presented symptoms with a transmission of 60 and 50 per cent respectively. All the cucurbits showed systemic symptoms like chlorosis, mottling and puckering except *T. cucumerina.* The symptom development in *T. cucumerina* was restricted to necrotic lesions with chlorotic halo. Back inoculation of the symptomatic leaves of cucurbits to indicator plant C. *amaranticolor* also developed local lesions except in case of *T. cucumerina*. The failure in development of systemic symptoms in this host might be due to the host resistance factors and the variety of the crop as all other cucurbits under study were inoculated at the same time with the same inoculum of virus and maintained under the same insect proof net house conditions. In congruence with the present findings in cucurbits other than *T.cucumerina*, Capoor and Varma (1958) proved the mechanical transmission of PRSV to seven cucubitaceous crops including *T. cucumerina* and *C. sativus*. CMI (1984) has reported that PRSV infecting papaya induces variable symptoms like vein clearing, mottling and malformed leaves in cucurbits. Similarly, other research workers reported the

transmission of PRSV from infected papaya to healthy cucurbits (Lima and Gomes,1975; Yemewar and Mali, 1980 and Yeh *et al*,1984, Singh *et al*., 2017). Chin and Ahamed (2007) reported that PRSV-P infect wild bittergourd which grow as weed plants. They also stated that mechanical transmission from the weed to cultivated bitter gourd plants produced vein clearing symptoms 2 weeks after inoculation. The cucurbitaceous plants under study except *T. cucumerina* were tested positive for DAC-ELISA. *T. cucumerina* did not give positive reaction in ELISA. Kunkalikar (2003) also proved successfully the transmission of PRSV to *M. charantia, T. cucumerina, C. sativus* and *C. moschata* which also gave positive reaction in DAC- ELISA.

In contrast to the above discussion, Kelaniyangoda and Madhubashini (2008) failed to establish cucurbits as hosts of PRSV. Similar experiment conducted by Dhanam *et al*. (2011) in different cucurbits revealed that zucchini alone expressed symptoms following artificial inoculation. Remaining test plants under study belonging to Amaranthaceae, Solanaceae, Fabaceae, Asteraceae, Musaceae, Malvaceae and Cleomaceae failed to produce symptoms following PRSV inoculum up to 60 DAI. The previous reports by Lakshminarayan Reddy (2000), Kunkalikar (2003) and Singh *et al*. (2017) are in harmony with these findings.

As a part of biological characterization, the morphology of the virus particles associated with the ringspot disease of papaya was also studied by taking electron photomicrographs of the putative samples and they revealed the presence of flexuous rod virus particles of size 807.74 nm x 12 nm. The length of the virus particles documented under the present study was slightly higher than the earlier reports on morphological studies where a size ranging from 750 - 800 x 12 nm was observed (Lana, 1980; Chen, 1984; Dahal *et al*., 1997, Kunkalikar, 2003, Tripathi *et al*., 2008, Dhanam *et al*., 2011).

ELISA is one of the sensitive tests for detection of virus. In the present study, DAC - ELISA was validated to detect PRSV in leaf samples. Absorbance value read

in ELISA reader were compared with that of healthy samples. In ELISA, generally the replicated samples having mean absorbance value which is at least double than that of the healthy sample were considered infected. Accordingly, the PRSV infection could be easily detected in 1:200 dilution of the antiserum which showed positive reaction with a high virus titre value. The absorbance value recorded in the suspected samples were five times more than the value recorded in healthy control. This striking difference in the absorbance values indicated that the suspected leaf samples are heavily infected with PRSV. Similarly, DAC - ELISA was used to detect different isolates of PRSV by many researchers (Bayot *et al.*, 1990, Kuan *et al.*, 1999, Kunkalikar, 2003, Krishnapriya, 2015).

An attempt was made to carry out molecular characterization of PRSV isolates from Thrissur. Molecular approaches have gained much importance in the detection and characterization of plant virus. The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) provides a reliable method to exponentially amplify virus specific cDNA *in vitro* using reported primers. Molecular or nucleic acid based techniques overcome the problems associated with serological detection which involve time consuming protocols.

Good quality RNA is essential for a reliable RT- PCR and PCR reaction. In the present study, RNA was isolated using TRIzol reagent. While standardizing the protocol for RNA isolation, it was observed that young leaves were more suitable than older leaves. On resolving the RNA isolated from older leaves, it was found to be degraded and contaminated with polysaccharides, phenols and proteins. This might be due to high amounts of tannins and phenolics in older leaves compared to younger ones. Isolation using TRIzol reagent gave good quantity of RNA in the present study ranging from 1236.5 to 1735.1 ng/µl as revealed by spectrophotometer analysis. The quality of RNA was assessed from the $A_{260/280}$ ratio obtained in the Nanodrop spectrophotometer which ranged from 1.95 to 2.09. It was further analyzed by resolving in formaldehyde agarose gel (1%). The RNA analyzed in the agarose gel

having three bands confirmed the purity of RNA without any DNA contamination. The extraction using TRIzol reagent was easy to perform and could be completed in a short period of time. Rio *et al.* (2010) reported that TRIzol is a monophasic solution which simultaneously solubilizes biological material and denatures protein. They also stated that the addition of chloroform following solubilization enables phase separation where RNA can be separated at the top layer in the aqueous phase without contamination of DNA and proteins which get resolved in the interface and organic phase respectively.

The total RNA isolated from the healthy and infected samples were converted to first strand cDNA using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit. The cDNA was further used in PCR for the characterization of PRSV. The quantity of cDNA synthesised from the different isolates ranged from 1332.5 to 1588.3 ng/µl as revealed by spectrophotometer analysis. The $A_{260/280}$ ratio ranged from 1.64 to 2.09. For carrying out PCR, the cDNA was further diluted so that it consisted of 44.40 to 54.40 ng/µl which gave good quality bands on amplification.

Reported primers (Srinivasulu and Sai Gopal, 2011) specific to partial NIb region, complete CP and 3' UTR region of PRSV were used for the detection of virus. Adams *et al.* (2005) reported that the CP coding region of members of *Potyviridae* had been mainly used to establish evolutionary relationships at both species and strain levels primarily because majority of potyvirus sequences in the database were derived from this region and it was multifunctional with major roles in virus life cycle and virus-vector interaction. Among the viruses belonging to *Potyviridae*, species identification was based on genetic information, host range, mode of transmission and antigenicity (Berger *et al.*, 2005). They stated that the genetic information mainly based on CP region such as, the amino acid sequence identity of the CP should be less than 80 per cent and nucleotide sequence identity should be less than 85 per cent over the whole genome. Moreover, the polyprotein cleavage site should be different from that of other species.

RT- PCR is considered the most reliable assay for the detection of virus in infected papaya plants even at low concentration. In the present study, leaf samples were also tested by RT-PCR to ensure that the plants with low virus load were not neglected. The reported primers which were used in the study *viz.*, PRSV-F: 5'-ATCACAATGTATTACGC- 3' and PRSV- R: 5'-CTCTCATTCTAAGAGGCTC-3' amplified the partial NIb region, complete CP region and 3' UTR yielded an expected product of size ~ 1700 bp in all positive samples. Previously, RT-PCR analysis with these primers had been successfully used for the detection of PRSV infecting papaya from Calicut, Gulbarga, Thiruvallur, Hospet and Hyderabad regions of south India. Similarly, Hema and Prasad (2004) used RT- PCR technique to amplify the CP region of a south Indian isolate which yielded a product of 900bp. Jain *et al.* (2004b) also carried out RT-PCR to amplify coat protein region of PRSV isolates from multiple locations in India. The product size of the amplicon varied from 840- 858 nucleotides, encoding protein of 280-286 amino acids.

The PCR products were sent to Agri Genome, Kochi for purification and sequencing. Nucleotide BLAST of the sequences of all the isolates showed maximum sequence identity to the Calicut isolate, KE-Ca (DQ666640.1) ranging from 90 to 95 per cent identity. The nucleotide sequences showed similarity to other south Indian isolates reported from Tamil Nadu, Andhra Pradesh and Karnataka. The amino acid sequences were also deduced from the nucleotide sequences using ExPASy translator tool and the sequences of isolates under present study varied with respect to size. On protein Blast, all the isolates except VK-1 showed maximum similarity to polyprotein sequence of isolate KE-Ca from Calicut (ABG72803.1). Protein blast of isolate VK-1 showed maximum similarity to polyprotein sequence of isolate KA- Ho (AAX07289.1). The variation observed in the isolates under present study might be due to the introduction of new isolates to the area, mutations or movement of the virus

through vectors and this might have caused change in the genetic composition of the virus.

The nucleotide sequences obtained were used for phylogenetic analysis which could be used to study genetic diversity. Availability of more sequences from Indian sub-continent will help in proper assessment of sequence divergence within PRSV population (Pushpa, 2014) . This would help to devise a long term management strategy to prevent the loss of resistance due to evolution of new strains of virus. Partial NIb and CP gene sequences in the present study were compared with 21 previously reported isolates from India and from other geographical locations available in the Genbank database. Further, a phylogenetic tree was constructed after aligning the sequences. It was revealed that the isolates in the present study showed maximum similarity to isolate KE- Ca from Calicut (DQ666640.1). They are hence more closely related to isolate KE- Ca. Isolates from Andhra Pradesh namely, PRSV-AP (AF323637.1), AP-Ko (DQ666638.1), AP-RA (AY839863.1) and AP-Te (AY839864.1), from Tamil Nadu namely, TA-Ti (DQ666641.1) and Karnataka isolates *viz.*, KA-Gu (DQ666639.1) and KA-Ho (AY839865.1) fall under the same subgroup as that of isolates under the present study and hence exhibit low degree of divergence. A similar clustering pattern of south Indian isolates was observed on phylogenetic analysis of isolates from different locations in India (Srinivasulu and Sai Gopal, 2011).

The south Indian isolates reported from Tamil Nadu in cucurbits *i.e.* isolate TN TDV SG1(KP161501.1) and TN MET PUM1 (KP161500.1) were more divergent than the above mentioned south Indian isolates from Andhra Pradesh, Tamil Nadu and Karnataka of PRSV in papaya. The divergence observed in these isolates might be because they are different strains of the same virus . Even then, the isolates are closely related to one another and exhibit low level of divergence compared to other known isolates from Indian subcontinent. This inference from the phylogenetic tree constructed is in accordance with the earlier reports by Pushpa (2014) who stated that the low level of divergence might be due to the recent occurrence of PRSV in this

region Another rationale behind this phenomenon might be the low selection pressure of virus as stated by Sharma *et al.*(2005). However, there are exceptions for south Indian isolate which do not fall into this category and which lie outside the group of interest such as isolates TN NGK PUM 1 (KP161494.1), TN NGK PUM2 (KP161494.1), TN NGK Cucu2 (KP161498.1). Moreover, isolate TN UDU RG1 (KP161499.1) showed maximum divergence to all the other south Indian isolates and formed an outgroup. Hema and Prasad (2004) reported that a recombination between PRSV-P isolates can occur which might significantly contribute to divergence. Interestingly, the north Indian isolate PRSV-UP (AF323638.1) was found to be very divergent from the south Indian isolates and showed similarity to PRSV isolates from Pakistan. The phylogenetic analysis conducted by Bateson *et al.* (2002) revealed that PRSV-P isolates from Indian subcontinent are more diverse which is consistent with the phylogenetic analysis in the present study. As opined in an earlier report (Jain *et al.*, 2004b), the higher sequence divergence within the PRSV population of the Indian subcontinent could be ascribed to the wide range of cropping systems and cultivation practices followed in different geographical regions.

Management of papaya ringspot disease under pot culture conditions was the next aspect of the study. For this, an experiment was conducted using a susceptible variety Red Lady. The efficacy of defense inducers, microbial formulations, micronutrient and plant extracts in reducing the disease severity was studied. Five applications of the treatments were given which included foliar spraying and soil drenching. In order to assess the percent disease severity, vulnerability index was calculated at weekly intervals after each spray. The first application of treatments out of all the five was done prior to challenge inoculation in order to assess their effects before inoculation if any.

Foliar spraying and drenching with 10 % leaf extract of *Bougainvillea spectabilis* (T7) gave the best result throughout the experiment. After the final spray, the least disease severity of 6.67 per cent was recorded in this treatment and it differed

156

significantly from all other treatments. This clearly indicates that *Bougainvillea* leaf extract is the most effective treatment for reducing disease severity. Prasad *et al.* (2007) studied the effects of different plant extracts including *Bougainvillea* leaf extract against BCMV infecting cowpea and found that it reduced the disease incidence up to 12 per cent compared to control plants under screenhouse conditions. When the same leaf extract was mixed with inoculum of BCMV and inoculated to young seedlings, a disease incidence of 42 per cent was recorded as against 80 per cent in control. A similar experiment was conducted by Rajanimala *et al.* (2009) where they evaluated the effect of defense inducers on expression of BGYMV, a potyvirus and reported that *Bougainvillea* leaf extract was the most superior treatment over others. A similar finding regarding the inhibition of virus activity by leaf extracts of *Bougainvillea* was reported by Reddy *et al.* (2006) where a 30 per cent reduction in expression of ULCV in urd bean plants was observed.

The performance of *Bougainvillea* leaf extract may be ascribed to the explanation by Verma and Dwivedi (1984), who isolated a virus inhibiting agent (VIA) developed in several plants *viz. Nicotiana* spp., *Lycopersicum esculentum, Cyamopsis tetragonaloba, Cucumis melo, Crotalaria juncea* and *Datura stramonium* following application of *Bougainvillea* leaf extract. The active molecule was identified as a proteinaceous compound which reached a maximum 24 hours after treatment with the leaf extract.

Solubor (0.1%) (T13), a micronutrient also gave considerable reduction over control plants. The percent disease severity recorded after final treatment application was 15.55 per cent. However, the severity recorded in case of Solubor (1%) (T10) was 22.22 per cent. Moreover, the application of higher dose of Solubor resulted in phytotoxicity following initial application in young leaves and the leaves became necrotic. There are numerous reports about the use of micronutrients, especially boron for management of papaya ringspot disease (Dar- Non- Wang and Ko, 1975; Lokhande and Moghe, 1992; Jahir Basha, 2002, Kunkalikar, 2003, Lokesh, 2014). However, the

results on percent disease severity in case of Sampoorna, micronutrient mix (T9), showed disparity from the previous reports. The per cent disease severity recorded was 53.33 per cent in plants applied with Sampoorna in contrast to only 15.55 per cent recorded in plants which were treated by 0.1 per cent Solubor.

Dordas (2008) described the role of micronutrients in controlling plant diseases. The efficieny of boron in reducing disease severity may be accredited to the fact that boron plays a major role in maintaining the cell wall structure and the cell membrane and most importantly, the metabolism of plants. Generally, the virus attack impair the cell and tissue structure which affects the translocation of nutrients. So, a foliar spray with boron may be actively absorbed through leaves and help in functioning of the cells bringing about some reduction in disease.

Pseudomonas fluorescens (2%) (T3) also gave results significantly different from other treatments. The percent disease severity recorded in this treatment after final application was 11.11 per cent *i.e.* a reduction of 86.66 per cent and it was found to be significantly different from other treatments after *Bougainvillea* leaf extract (T7). Earlier studies conducted on the evaluation of rhizobacteria in inhibition of diseases caused by plant viruses reveal that various strains of *Pseudomonas* play a major role. Van Loon *et al.* (1998) reported that different strains of *P. putida* and *P. fluorescens* confer resistance to *Cucumber mosaic virus* in cucumber and tomato; *Tobacco mosaic virus* and *Tobacco necrosis virus* in tobacco. Similarly, Kumar *et al.* (2005) reported that *P. fluorescens* was effective in reducing the disease incidence caused by BCMV, a potyvirus infecting french bean. Similarly, in a study conducted by Ashwini (2015) on the efficacy of *P. fluorescens* against BGYMV in bitter gourd, the highest yield was recorded in case of plants sprayed with 2 per cent *P. fluorescens*.

PGPR mix II (T4) which is also a microbial formulation consisting of a combination of *Pseudomonas* and *Bacillus*, developed at KAU, was also tested in the present study. After five applications, the percent disease severity recorded in this

treatment was 20 per cent. Ruwanthi *et al.* (2014) isolated *Bacillus* from the rhizosphere region of papaya and applied this to test plants as soil application prior to PRSV inoculation and spraying post inoculation. The results revealed a considerable reduction in symptoms and also the virus titre as against control plants. Earlier investigations also show that seed inoculation of watermelon seeds with *Bacillus polymyxa* and *B. megaterium* gave a disease reduction of 25.1 per cent and 35.5 per cent against viral disease caused by *Watermelon mosaic virus* -2 which is a potyvirus whose genome structure is closely related to PRSV. Similar results were reported by Abd El- Shafi and Yasser (2012) against ZYMV, a potyvirus in squash plants where two species of *Bacillus viz.*, *B. firmus* and *B. subtilis* were evaluated.

Kloeppen and Schroth (1978) described *P. fluorescens* as a biocontrol agent and as a plant growth- promoting microbe capable of reducing the concentration of virus. This explains the possibility of induction of certain defense molecules in the plant following the application.

Another botanical which was tested in the study was 10 per cent leaf extract of *Mirabilis jalapa* (T6) where a percent disease severity of 17.77 was recorded after final spray as against 97.77 per cent in control plants. A considerable reduction is observed in case of this treatment from the first spray where a disease severity of 28.89 per cent was recorded. Several research workers have published reports on the inhibitory effect of *Mirabilis jalapa* on viral diseases. Verma and Kumar (1980) reported that *Mirabilis* leaf extract causes a marked reduction in symptom suppression in several viral diseases namely, *Tomato yellow mosaic virus* and *Tomato yellow mottle virus* in tomato, *Cucumber mosaic virus* in cucumber. Reddy *et al.* (2006) tested the efficacy of various plant extracts on the inhibiton of ULCV in urdbean plants mechanically inoculated with the virus and it exhibited maximum reduction of 30 per cent in plants sprayed with *Mirabilis jalapa* leaf extract. Elsharkawy and El- Sawy (2015) also studied the effect of *Mirabilis jalapa* as a spray treatment to control BCMV, a potyvirus both in green house and field conditions. Earlier reports on the mode of action of *Mirabilis* suggest

that it contains an antiviral protein known as ribosome inactivating protein (RIP) which is effective against viruses that are mechanically transmitted (Kubo *et al.*, 1990; Takanami *et al.*, 1990; Vivanco *et al.,* 1999). The effect of Perfekt (T8), a commercial viricide was also evaluated in the study. The results reveal that it gave a reduction of 73.33 per cent with respect to control plants. Perfekt is a combination of eleven herbs as incidcated by the manufacturer and may follow the same principle as that of *Bougainvillea* and *Mirabilis* in disease reduction.

Two defense inducers namely salicylic acid (150ppm) (T1) and acetyl salicylic acid (150 ppm) (T2) was tested for inhibition of papaya ringspot disease. The disease severity recorded in case of salicylic acid was 17.77 per cent with 80 per cent reduction from control after the final spray and the result is on par with *Mirabilis jalapa* leaf extract (10%). The inhibition of several viral diseases by salicylic acid was reported earlier by different workers. Kuc (1982) reported that the exogenous application of salicylic acid induces SAR in plants. Qiu *et al.* (2003) described that root drenching of papaya seedlings with BTH, an analogue of SA increased the accumulation of PR1 gene in papaya which is used as a marker for SAR. Furthermore, Zhang *et al.* (2007) and Pushpa (2014) reported SA as an efficient defense molecule capable of reducing the incidence of PRSV. The mode of action of SA is explained by Radwan *et al.* (2007) stating that it activates resistance through inhibition of some antioxidant enzymes, catalase and peroxidase leading to accumulation of hydrogen peroxide in the cells.

In contrast to this, the severity recorded in case of acetyl SA (aspirin) was 48.89 per cent after the final spray and gave a reduction of only 48.88 per cent against control plants. Earlier reports suggest that aspirin is a potential antiviral chemical capable of activating the systemic resistance and cause reduction in severity of diseases caused by fungi, bacteria and viruses (Antoniw and White, 1979; Abd-el-Said *et al.*, 1996; Senaratna, 2000; El Mougy, 2004; Horotan and Apahidean, 2015). Despite this, Reddy *et al.* (2006) reported aspirin as the least effective among several defense inducers

174447

when tested against ULCV in urdbean which is consistent with the results of the present study.

Humic acid was another treatment which was tested in the present study. After the final application, the test palnts gave a reduction of 77.77 per cent against control plants. Earlier reports point out that humic acid is capable of activation of resistance in plants and suppression of diseases. The infectivity of TMV was reported to be considerably reduced following application of humic acid (Polak and Pospisil, 1995). Dmitrier *et al.* (2003) reported that application of humic acid enhanced the concentration of some antioxidants such as, β-carotene, superoxide dismutases, and ascorbic acid which played a major role in plant growth and development of disease resistance. In congruence with the results of the present study, Kshirsagar (2014) reported the delay in symptoms induced by PRSV on spraying seedlings with humic acid 72 hours prior to mechanical inoculation.

While reviewing the literature on effect of potassium silicate in disease suppression in plants, not much could be found regarding its effect on viral pathogens. In the present study, the disease severity recorded in case of potassium silicate (T12) is 17.77 per cent which is statistically on par with salicylic acid (T1) and 10 % leaf extract of *Mirabilis jalapa* (T6). Zellner *et al.* (2011) reported a delay in systemic symptom expression caused by *Tobacco ringspot virus* with high levels of silicate. In line with the present findings, Elsharkawy and Mousa (2015) found that silicon application to cucumber plants significantly reduced the severity of PRSV and its accumulation in leaves.

Lecanicillium lecanii (T5), an entomopathogenic fungus was also used in the present study. The treatment did not produce any beneficial results compared to other treatments. The percent disease severity recorded after 5 applications was 88.89 per

cent as against 97.77 per cent in control. Hence, this treatment, being an insect pathogen had no effect under insect proof net house conditions. However, in case of open field conditions, *L. lecanii* may be considered as a useful option as it is effective against aphids which are vectors of PRSV. Yeon *et al.* (2008) reported 72 to 97 per cent mortality in field evaluation of *Lecanicillium* against green peach aphid, *Myzus persicae* which is a potential vector of PRSV.

The results were further scrutinized on the basis on biometric characters. The plant height in case of *Bougainvillea* (T7) and *Pseudomonas* (T3) treated plants were significantly different and they were identified as the superior ones. In terms of girth of stem, *Pseudomonas fluorescens* (T3) exhibited a maximum value of 2.91 cm and they significantly differed from all other treatments. The least plant height and girth was recorded in untreated control plants. From this result, it is inferred that along with suppressing the disease, these treatments support the plant to carry out normal functioning by significantly reducing the virus inoculum in them.

In order to determine the influence of treatments on the virus titre, DAC-ELISA of the test plants was conducted. The data on virus titre of test plants revealed that 10 per cent *Bougainvillea* leaf extract (T7) was the most superior treatment with the least absorbance value of 0.373 as against 1.474 in untreated control plants. This clearly indicates that *Bougainvillea* is capable of activating the disease resistance mechanism of the plants and reducing the multiplication of virus in the host. *P. fluorescens* (2%) (T3) gave an absorbance value of 0.509 which is almost one- third of the value obtained in untreated control plants. Both T7 and T3 were statistically on par. The reduction in virus titre might be attributed to the presence of antiviral proteins reported in earlier publications already discussed in this chapter. The results of DAC-ELISA are in harmony with the PDS recorded in the experiment in the present study.

The data on chlorophyll content recorded after final application revealed that the leaves of plants treated with *Bougainvillea* leaf extract (10%) (T7) possessed the

highest chlorophyll content of 44.67 SPAD units. This was followed by *P. fluorescens* (2%) (T3) where a chlorophyll content recorded after final application was 42.67 SPAD units as against 17.66 SPAD units in untreated control plants. The high chlorophyll content recorded in these two treatments might be due the inhibition of viral particles that can allow normal growth and development of plants in comparison to the heavily infected plants.

The expression of symptoms on untreated control plants was very conspicuous throughout the experiment. The disease severity ranged from 57.55 to 97.77 per cent. The higher disease severity may be because of the low atmospheric temperature (Appendix VI). These observations are in line with earlier reports (Gonsalves and Ishii, 1980; Singh and Shukla, 2011).

To conclude, the present study revealed that Red Lady is the widely cultivated variety in Thrissur for commercial purposes and is highly susceptible to PRSV. The infected plants show a wide range of symptoms in the field which are reproduced in healthy papaya plants when artificially inoculated with the virus inoculum. Plants belonging to Cucurbitaceae also showed systemic symptoms following inoculation and this was confirmed through DAC- ELISA. The indicator host, *Chenopodium amaranticolor* also showed chlorotic local lesions after inoculation which confirms that it is also a host. The study on morphology of the virus particles revealed that they are typical rod shaped flexuous particles of size 807.74 x 12 nm confirming that they belong to Potyviridae. The study has also helped to elucidate the characterization of virus at the molecular level. As the coat protein gene is region specific, the availability of nucleotide and amino acid sequences may help to develop transgenic lines more suitable for that region in the future. Pot culture experiments revealed that different plant extracts, microbial formulations and micronutrients have an influence on reducing the virus titre. *Bougainvillea spectabilis* leaf extract and *Pseudomonas fluorescens* were superior to other treatments which is evident from the results of percent disease severity, virus titre in test plants and chlorophyll content.

Future Line of Work

In future, studies on the impact of the disease on the economic yield can be conducted. Simultaneously, the efficiency of treatments which performed well under pot culture conditions can be evaluated in field. A commercial product based of *Bougainvillea* which possess the anti-viral principles could be developed. Molecular clones of CP gene could be used for the production of antiserum through recombinant DNA technology and these could be used as disease diagnostic probes for more sensitive molecular techniques like Nucleic acid spot hybridization. Information obtained through characterization of CP gene could be used for development of region specific virus resistant transgenic papaya lines through coat protein mediated resistance

Summary

6. SUMMARY

Papaya is a fruit crop which is widely grown in both tropics and subtropics and is of great economic importance. However, the diseases especially those caused by viruses are a major hurdle for profitable cultivation. *Papaya ringspot virus* (PRSV) has devastating effects on the yield of the crop and 100 per cent yield loss has been reported from different locations across the globe.

The study entitled "Characterization, host range and management of *Papaya ringspot virus* (PRSV)" was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara, Kerala Agricultural University during 2016 - 2018 with the view to understand the symptomatology, mode of transmission, host range and morphological characters of the virus. Additionally, the present study was undertaken to characterize the coat protein gene of PRSV at molecular level. The present research was envisaged to evaluate the effects of different defense inducing chemicals, botanicals and microbial formulations on papaya ringspot disease.

Survey on incidence of papaya ringspot disease revealed the presence of PRSV from 10 locations surveyed and the disease incidence in surveyed locations ranged from 25.00 to 99.60 per cent. The maximum disease incidence of 99.6 per cent was recorded in Vellikulangara on papaya variety Red Lady followed by 98 per cent in Koratty and Venginissery on the same variety and 97.5 per cent in Puthur. The disease incidence was found to be comaparatively low in a plot surveyed at Muringoor (25 %) and Melur (39.28%). The disease was predominantly recorded on papaya variety Red Lady which has high susceptibility towards PRSV.

The symptoms of PRSV under natural conditions were documented during the survey in different locations. The most common symptoms noticed on leaves included chlororsis mosaic mottling and chlorotic spots. In fields recorded with high severity,

166

wavy leaf margins, puckering, thickening and clustering of veins and leaf distortion were recorded. In extreme cases, complete reduction in leaf lamina was observed making the leaf highly filiform and giving a shoestring appearance. Additionally, green islands were also observed as a typical symptom on leaves. On the petiole of affected leaves, elongated oily streaks were present which were discontinuous or broken and congregated at the base of the petiole. Similarly, oily ringspots were present on the green regions of the stem. On the fruits, typical ringspot symptom in the form of concentric or broken oily ringspots were present over the fruit surface in both young and mature fruits. The number of ringspots ranged from few to many depending upon the degree of infection. In advanced cases, the fruits were found to be malformed and highly reduced in size making them unmarketable.

Tapering was observed in the crown region of the infected plants and the newly emerging leaves presented a rosette appearance. In some cases, despite the infection, the plants produced flowers and fruits. However, the plants affected at seedling stage were found to be stunted and failed to yield fruits. Moreover, the girth of such plants were also found to be highly reduced. In terminal conditions, 100 per cent defoliation was observed finally leading to its death.

Typical foliar symptoms were reproduced on healthy seedlings of papaya when they were mechanically inoculated. The incubation period of the virus was found to be 14 days. Initially, the leaves expressed chlorotic spots which coalesced to give a mosaic mottled appearance. In some cases vein clearing was also seen as an initial symptom. Later, as the infection advanced, leaf distortion and filiformity was observed similar to that under natural conditions. Oily spots were observed on the stem region of the seedlings. The infected seedlings were found to be stunted.

Cent per cent transmission of virus through mechanical inoculation of infectious sap was recorded. However, the virus failed to induce the symptoms of the disease in seedlings emerged from seeds collected from infected fruits indicating that

it is not seed transmissible. As a part of biological characterization, host range studies conducted on 21 plant species belonging to 6 different families against PRSV indicated that the host range of PRSV is limited to Cucurbitaceae and Chenopodiaceae apart from Caricacae, the family to which papaya belongs. *Chenopodium amaranticolor* gave chlorotic local lesion symptoms on the leaves and did not show any systemic symptoms. *Cucumis sativus*, *Momordica charantia* and *Cucurbita moschata* exhibited systemic symptoms like chlororsis, vein clearing and mottling and the infection was confirmed through ELISA. In contrast, in *Trichosanthes cucumerina*, the symptoms were restricted to necrotic local lesions and failed to give systemic symptoms.

The morphological characters of the virus particles were studied through electron microscopy and electron micrographs revealed that the particles were typical flexuous rods of size 807.74 nm x 12 nm. Hence, the etiology of the disease was conformed as *Papaya ringspot virus*, belonging to the genus *Potyvirus* and family *Potyviridae*.

DAC- ELISA, an immunodiagnostic assay was validated by using the antiserum specific to PRSV. The high absorbance value recorded in suspected samples against healthy samples revealed the presence of virus.

Molecular diagnosis was standardized for the detection of PRSV. RNA extraction was standardized using TRIzol reagent and cDNA from viral RNA was also synthesized. PCR product of approximately 1700 bp was amplified using reported priemrs specific to coat protein gene of PRSV which again proved the etiology of the disease as PRSV. *In silico* analysis of five PRSV isolates revealed 95 to 90 per cent nucleotide homology with PRSV strain KE- Ca reported from Calicut. The isolates under present study also showed significant homology of amino acid identity (96-95 %) to the same strain KE- Ca. However, isolate VK-1 showed maximum amino acid identity (94 %) to strain KA- Ho from Hospet. The phylogenetic analysis by the alignment of CP gene sequences of present study with 21 other isolates also revealed

that the isolates under present investigation were more similar to KE - Ca isolate (DQ666640.1) from Calicut.

A pot culture experiment was conducted to evaluate the efficiency of different defense inducers, botanicals, micronutrients and microbial formulations in reducing the severity of papaya rinspot disease. *Bougainvillea* leaf extract (T7) showed maximum reduction in the disease severity right from the first spray and significantly differed from all other treatments. After final spray, the severity recorded in this treatment was only 6.67 per cent as against 97.77 per cent in untreated control plants. *Pseudomonas fluorescens* (2%) (T3) was also found to be superior where a disease severity of 11.11 per cent was recorded after the final spray. Since 14 treatments were tested against PRSV, in order to deduce a conclusion, the results of percent disease severity was further scrutinized by the statistical method proposed by Arunachalam and Bandyopadhyay (1984) which again revealed that *Bougainvillea* leaf extract - 10 % (T7) was the best treatment. The experiment was terminated after five sprays and the apical leaves of the test plant were subjected to DAC- ELISA. The absorbance values recorded for the different treatments at 405 nm revealed that *Bougainvillea* leaf extract - 10 % (T7) and *Pseudomonas fluorescens* - 2 % (T3) were statistically on par and superior to other treatments.

The biometric characters like plant height and girth of the stem of the test plants were recorded at monthly intervals and a gradual increase in height and girth were recorded in all treatments when compared to untreated control plants. The maximum height of the plant (71 cm) and girth of the stem (2.91 cm) was recorded in *Pseudomonas fluorescens* - 2% (T3) and was found to be significantly different from all other treatments. This was followed by *Bougainvillea* leaf extract treated plants (T7) which showed the next best result in terms of plant height and girth of stem *viz*., 70cm and 2.5 cm respectively as against 35.60 cm and 1.38 cm in untreated control plants. The chlorophyll content of the test plants was also assessed using SPAD meter and the results revealed that the maximum value was recorded in *Bougainvillea* leaf extract

(T7) treated plants (44.67 SPAD units) followed by plants treated with 2 % *Pseudomonas fluorescens* (T3) where a chlorophyll content of 42.67 SPAD units was recorded.

174 447

References

7. REFERENCES

Abdalla, O.A., Bibi, S., and Zhang, S. 2017. Application of plant growth- promoting rhizobacteria to control *Papaya ringspot virus* and *Tomato chlorotic spot virus*. *Arch. Phytopathol. Pl. Prot.* 50(11/12): 584-597.

Abd-el- Said, W.M., Abd-el- Ghafar, N.Y., and Shehata, S.A.M. 1996. Application of salicylic acid and aspirin for induction of resistance to tomato plants against bacterial wilt and its effect on endogenous hormones. *Annu. Agric. Sci.* 41(2): 1007- 1020.

Abd-el-Shafi, S. and Yasser, H. 2012. Biological control of zucchini yellow mosaic potyvirus (ZYMV) by *Bacillus firmus* and *Bacillus subtilis* in squash plants. *Egyptian J. Botany.*s.n.

Acosta, J. C. M., 1969, Consideracions sorbe insectos vectores aphididae y la "deformacion foliar'' (distortion ring spot virus) de *Carica papaya* en cl Limon Estado Aragua, Venezuela. *Revista Facultad de Agronomia,* 5: 77-108.

Adams, M.J., Antoniv, J.F., and Fauquet, C.M. 2005. Molecular criteria for genus and species discrimination within the family Potyviridae. *Arch. Virol.* 150: 459-479.

Ali,A. Natsauki,T., and Okuda, S. 2004. Identification and molecular characterization of viruses infecting cucurbits in Pakistan. *J. Phytopathol.* 152(11/12) :677-682.

Almeida, A. M. R. and Carvalho, S. L. C. DE. 1978. Occurrence of papaw distortion ringspot virus in the state of Parana. *Fitopatologia B'rasileira,* 3: 65 - 69.

Alvizo, H.F. and Rojkind, C. 1987. Resistencia al virus mancha anular del papaya en Carica cauliflora. *Revista Mexicana De Fitopatologia*.5: 61-62.

Antoniw, J.F. and White, R.F. 1979. The effects of aspirin and polyacrylic acid on soluble leaf proteins and resistance to virus infection in five cultivars of tobacco. *Phytopathol. Z.* 98: 331-341.

Arunachalam, V. and Bandyopadhyay, A. 1984. A method to make decisions jointly on a number of dependent characters. *Indian J. Genet.* 44(3): 419- 424.

Ashwini, K.N. 2015. Management of bitter gourd mosaic by enhancing host resistance. M Sc (Ag.) Thesis, Kerala Agricultural University, Thrissur, 63p.

Bag, S., Agarwal, Surekha and Jain, R. K.2007.Sequence diversity in the coat proteins of Papaya ringspot virus isolates originating from different locations in India. *Indian Phytopathol.* 60: 244-250.

Barakat, O.S., Goda, H.A., Mahmoud, S.M., and Emara, Kh. S.2012.Induction of systemic acquired resistance in watermelon against watermelon mosaic virus-2. *Arab. J. Biotechnol.* 15(2): 23-44.

Barbosa, F. R. and Paguio, O. R., 1982. Incidence and effect on yield of papaya *(Carica papaya* L.). *Fitopatologia Brasiliera,* 7: 365 - 373.

Bateson, M. F., Lines, R. E., Revill, P., Chaleeprom, W., Ha, C.V., Gibbs, A. J. and Dale, J. L. 2002.On the evolution and molecular epidemiology of the potyvirus Papaya ringspot virus. *J. Gen. Virol.*, 83: 2575–2585.

Bateson,M., Henderson,J.,Chaleeprom,W.,Gibbs,A., and Dale,J. 1994. Papaya ringspot potyvirus: isolate variability and origin of PRSV type P (Australia). *J. Gen. Virol.*75: 3547- 3553.

Bayot, R.G.,Villegas, V.N.,Magdalita, P.M.,Jovellana,M.D., Espino,T.M., and Exconde,S.B. 1990. Seed transmissibility of *Papaya ringspot virus*. *Philipp. J.Crop Sci.* 15(2):107-111.

Berger, P.H., Adams, M.J., Barnett, O.W., Brunt, A.A., Hammond, J., Hill, J.H., Jordan, R.L., Kashiwazaki, S., Rybicki, E., Spence, N, Stenger, D.C., Ohki, S.T., Uyeda, I., van Jayen, A., Valkonen, J., and Vetten, H.J. 2005. Potyviridae: In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., and Ball, L.A. (eds.), *Virus Taxonomy. VIIIth Report of the International Committee on Taxonomy of Viruses*. Elsevier/ Academic Press, London, pp. 819-841.

Bhargava, K. S. and Khurana, S. M. P.1969.Papaya mosaic control by oil sprays. *J. Phytopathol.,* 64: 338 - 343.

Bondok, A.M. and Thabet, M.S. 2016. Riboflavin and humic acid induced resistance in tomato against *Tobacco mosaic virus* (TMV). *Middle East J. Agric. Res.* 4(4): 868- 880.

Bos, L. 1982. Crop losses caused by viruses. *Crop Prot.* 1(3): 263- 282.

Byadgi, A.A., Anahosur,K.H., and Kulkarni, M.S.1995. Ringspot virus in papaya.118 (252): 28

Capoor, S. P. and Verma, P. M., 1948, A mosaic disease on *Carica papaya* L. in the Bombay province. *Curr. Sci.*, 17: 265 – 266.

Capoor, S.P. and Varma, P.M.1958. A mosaic disease of papaya in Bombay. *Indian J. Agric. Sci.* 28: 225-235.

Castillo, X.A.O., Fermin, G., Tabima, J., Rojas, Y., Tennant, P.F., Fuchs, M., Sierra, R., Bernal, A.J., and Restrepo, S. 2011. Phylogeography and molecular epidemiology of Papaya ringspot virus. *Virus Res.* 159: 132-140.

Chavan, M.V., Tomar, S.P.S., and Dhale, M.G. 2010. Management of Papaya ringspot virus (PRSV-P) of papaya under Pune conditions. *Acta Hortic.* 851:447-452.

Chen, M. J. 1984. Electron microscopic observations of Papaya ringspot virus in infected papaya leaves. *Plant Prot. Bull.,* 26:23- 31.

Cheng, D.J., Huang, X.D., Zhang, J.W., Tian, Y.P., Wang, X.Y. and Li, X.D. 2017. First report of *Papaya ringspot virus* associated with a ringspot disease of zucchini in northern China. *Plant Dis.*101 (5): 847.

Cherian, A.K. 1998. Management of *Tomato leaf curl virus* in tomato through the control of its whitefly vector, *Bemisia tabaci* Genn. by entomopathogenic fungi. Ph D thesis, University of Agricultural Sciences, Bangalore, 226p.

Chin, M. and Ahamed, M.H. 2007. *Momordica charantia* is a weed host reservoir for Papaya ringspot virus type P in Jamaica. *Plant Dis.* 91: 1518.

CMI (Common Mycological Institute), 1984. Papaya ringspot virus: Descriptions of Plant Viruses. Kew UK, 360p.

Conover, R. A., 1962, Virus diseases of Florida. *Phytopathology,*52 : 6.

Costa, A.S., Carvalho, A.M., and Kamada, S. 1969. Constatado o mosaic do mamoeiro em Sao Paulo. O Agronomico. 21:38-43.

Cruz, F.C.S., Tanada, J.M., Elvira, P.R., Dolores, L.M., Magdalita, P.M., Hautea, D.M., and Hautea, R.A. 2009. Detection of mixed virus infection with PRSV in papaya grown in Philippines. *Philipp. J. Crop. Sci.* 34(1): 62- 74.

Dahal, C., Lecoq, H. and Albrechtsen, S. E., 1997, Occurrence of Papaya ring spot potyvirus and cucurbit viruses in Nepal. *An. Appl. Biol.* 130 : 491 – 502.

Dar-Non-Wang and Ko, W.H., 1975. Relation between deformed fruit disease of papaya and boron deficiency. *Phytopathology,* 65(4):445-447.

Davis, R.I., Mu, L., Maireroa N., Wigmore, W.J., Grisoni, M., Bateson, M.F., and Thomas, J.E. 2005. *Aust. Plant Pathol.* 34: 125-126.

Davis, R.I., Thomas, J.E., Mc Michael, L.A., Dietzgen, R.G., Callaghan, B., James, A.P., Gunua, T.G., and Rahamma, S. 2002. Plant virus surveys on the island of New guinea and adjacent regions of northern Australia. *Aust. Plant Pathol.* 31(4): 385-390.

Dennis, J.J.C. and Guest, D.I. 1995.Acetyl salicylic acid and β- ionone decrease the susceptibility of tobacco to tobacco nercrosis virus and *Phytophthora parasitica* var. *nicotianae. Australasian Plant Pathol.* 24(1): 57-64.

Dhanam, S., Saveetha, K.,Sankaralingam, K., Kannan, R., and Pant, R.P.2011. Biological and molecular characterization of Coimbatore isolate of Papaya ringspot virus. *Arch. Phytopathol. Plant Prot.* 44(10): 925-932.

Diallo, H.A. Monger, W., Kouassi, N., Yoro, D.T., and Jones, P.2007. First report of Papaya ringspot virus infecting papaya in Cote d'Ivoire. *Plant Pathol.* 56(4):718.

Dmitrier, A., Tena, M., and Jorrin,J. 2003. Systemic acquired resistance in sunflower (*Helianthus annuus* L.). *Tsitologiya-1-Genetika*, 37(3): 9-15.

Dordas, C. 2008. Role of nutrients in controlling plant diseases in sustainable agriculture. A review. *Agron. Sustain. Dev.* 28: 33.

El Mougy, N.S., 2004. Preliminary evaluation of salicylic acid and acetylsalicylic acid efficacy for controlling root rot disease of lupin under greenhouse conditions. *Egypt. J. Phytopathol.*, 32: 11-21.

Elsharkawy M.M. and Mousa K.M. 2015. Induction of systemic resistance against *Papaya ring spot virus* (PRSV) and its vector *Myzus persicae* by *Penicillium simplicissimum* GP17-2 and silica (SiO2) nanopowder. *Int. J. Pest Manage.* 61 (4): 353–358.

Elsharkawy, M.M. and El- Sawy, M.M.2015. Control of Bean common mosaic virus by plant extracts in bean plants. *Int. J. Pest Manage.* 61(1): 54-59.

FAOSTAT.2014. Production of Papayas [online]. Available:http://faostat.fao.org/site/535.[07 Dec 2016].

Fermin, G. and Gonsalves, D. 2003. Papaya: Engineering against Papaya ringspot virus by native, chimeric and synthetic transgenes. In: Loebenstein, G. and Thottapilly, G. (eds.). *Virus and Virus Like Disease Of Major Crops In*

177

Developing Countries. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 497- 518.

Gaffney, T., Friedrich, L., and Vernooij, B. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science,* 262: 754–756.

Garga, R. P., 1963, Studies on virus diseases of plants in Madhya Pradesh - A serious virus disease of papaya. *Indian Phytopathol.*, 16: 31–33.

Gibbs, A. and Harrison, B. 1976. Transmission by vectors and in other natural ways. In: John Wiley & Sons, N.Y. (eds.), *Plant Virology, The Principles*. Butler and Tanner Ltd., Prome, Great Britain, pp. 187- 191.

Gomez, K.A. and Gomez, A.A. 1984. *Statistical Procedures for Agricultural Research*, John Wiley & Sons, USA. 657pp.

Gonsalves, D. and Ishii, I., 1980. Purification and serology of papaya ringspot virus. *Phytopathology,* 70: 1028 -1032.

Gonsalves, D., Tripathi, S., Carr, J.B. and Suzuki, J.Y., 2010. *Papaya Ringspot virus. The Plant Health Instructor. doi: 10.1094*. PHI-I-2010-1004-01.

Gonsalves, D.1998.Control of Papaya ringspot virus in papaya: A case study. *Annu. Rev.Phytopathol..* 36: 415 - 437.

Gourgopal, R. and Jain, R.K.2002. Comparison of the coat protein genes of two papaya ringspot virus isolates wither other isolates from different geographic lcoations. *Indian Phytopathol.* 55(3): 335- 337.

1786

Ha,C., Coombs,S., Revil, P.A., Harding, R.M., Vu, M., and Dale, J.L. 2008. Design and application of two novel degenerate primer pairs for the detection and complete genomic characterization of potyviruses. *Arch. Virol.*153: 25-36.

Hema, M.V. and Prasad, D.T. 2004. Comparison of the coat protein of a south Indian strain of PRSV with other strains from different geographical location. *J. Plant Pathol.* 86(1): 35-42.

Hemavati, R.2005. Detection, diagnosis and management of PRSV on cucurbits.M. Sc. (Agri) Thesis, University of Agricultural Sciences, Dharwad. 109p.

Henson, J.M. and French, R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annu. Rev. Phytopathol.* 31:81-109.

Holz S., Zeise I., Bartoszewski G., Kneipp J., Kube M., Buttner C. 2014. Insights into molecular impact of silica on virus infected cucumber cultures. p. 88. In: Proceedings of the 6th International Conference on Silicon in Agriculture, Stockholm, Sweden, 26–30 August 2014, 205 pp.

Horkness, R. N. 1967. Papaya growing in Florida. Gainsville, University of Florida. *Circular.* pp: 1 -1 5.

Horotan, R.A. and Apahidean, A.S. 2015. Effects of fungicide and acetylsalicylic acid treatments on qualitative and quantitative tomato production. *Bull. UASVM Hortic.* 72(1): 88-94.

Huguenot, C., Furneaux, M.T., Thottapilly, G., Rossel., and Hamilton, R.I. 1993. Evidence that Cowpea aphid- borne mosaic virus and Blackeye cowpea mosaic virus are two different Potyviruses. *J.Gen. Virol.*74: 335-340.

Hussain,S. and Varma, A.A. 1994. Occurrence of Papaya ringspot virus from Amritsar (Punjab). *Indian J. Phytopathol.* 7: 77-78.

Ibaba, J.D., Laing, M.D., and Gubba, A. 2015. First Report of a Novel *Potyvirus* from the *Papaya ringspot virus* Cluster Infecting Zucchini (*Cucurbita pepo*) in KwaZulu-Natal, Republic of South Africa. 2015. *Plant Dis*. 99: 1289.

Jackson, C.W., Heale, J.B., and Hall, R.A. 1985. Traits associated with virulence to the aphid *Macrosiphoniella sanborni* in eighteen isolates of *Verticillium lecanii. Ann. Appl. Biol.* 106: 39-48.

Jahir Basha, C.R., 2002, Transmission approaches for management of papaya ring spot virus disease. M.Sc. (Ag.) Thesis, University of Agricultural Sciences, Bangalore, pp. (total pages).

Jain, R.K., Nasiruddin, K.M., Sharma, J., Pant, R.P., and Varma, A. 2004a. First report of occurrence of Papaya ringspot virus infecting papaya in Bangladesh. *Plant Disease*. 88 (2): 221.

Jain, R.K., Pappu, H.R., Pappu, S.S., Varma, A., and Ram, R.D.1998. Molecular characterization of papaya ringspot potyvirus isolates from India. *Ann. Appl. Biol.*132: 413- 425.

Jain, R.K., Sharma, J.,Siva Kumar, A.S., Sharma, P.K.,Byadgi, A.S., Verma, A.K., and Varma, A.2004b. Variability in the coat protein gene of *Papaya ringspot virus* isolates from multiple locations in India. *Arch. Virol.*149: 2435-2442.

Jensen, D.D. 1949. Papaya virus disease with special reference to Papaya ringspot. *Phytopathology* 39: 191-210.[Cross Ref.]

Kalleshwaraswamy, C.K., Verghese, A., Ranganath, H.R., and Krishna Kumar, N.K.2007.Role of transient aphid vectors on the temporal spread of papaya ringspot virus in south India.proceesdings on 1st international symposium on papaya. Eds. Y.K. Chan and R.E.Paull. Acta. Hort. 740.ishs 2007.

Kelaniyangoda, D. and Madhubashini, L.2008. Indicator plants: Tools for detecting Papaya ringspot potyvirus and Cucumber mosaic cucumovirus. *J. Food Agric.* 1(2): 64-69.

Khurana, S.M.P.and Bhargava, K.S.1970.Induced apocarpy and double papaya fruit formation in papaya with distortion ringspot virus infection. *Plant Dis.Rptr.*54: 181-183.

Kiritani,K. and Su Hong, J. 1999. Papaya ringspot, banana bunchy top and citrus greening in the Asia and Pacific region: Occurrence and control strategy. *Jpn Agric. Res.* 33: 23-20.

Kitajima, E. W., Mattos, J. K. A., Parente, T. V., Marinho, V. L. A., and SA, P. B. DE.1987. Occurrence of Papaw ringspot virus in the federal district. *Fitopatologia Brasileira,* 12: 106 - 108.

Kloepper, J.W. and Schroth, M.N. 1978. Plant growth promoting rhizobacteria in Radish. Proceedings of the 4th International Conference of Plant Pathogenic Bacteria Gibert- Clarey, (ICPPBG'1978), France, pp: 879-882.

Krishnapriya,P.J. 2015. Immunomolecular detection and characterization of Potyviruses infecting cowpea and papaya. MSc (Ag.) thesis. Kerala Agricultural University, Thrissur, 197p.

Kshirsagar, H.R. 2014. Studies on induced resistance by chemicals against Papaya ringspot virus (PRSV) in papaya. M Sc (Ag) thesis. Mahatma Phule Krishi Vidyapeeth, Maharashtra, 104p.

Kuan, C.P., Chen, K. H. AND Su, H. J., 1999, Serological characterization of Papaya ringspot virus isolates in Taiwan. *Bot. Bull. Acad. Sin.,* 40: 231 - 236.

Kubo, S., Ikeda, T., Imaizumi, S., Takanami,Y., and Mikami, Y. 1990. A potent plant virus inhibitor found in *Mirabilis jalapa* L. *Ann. Phytopathol. Soc. Jpn.* 56:481-487.

Kuc, J. 1982. Induced immunity to plant disease. *Biosci*. 32:854-860

Kulkarni, H. Y.1970.Decline viruses of papaw (*Carica papaya* L.) in East Africa. *Ann. Appl. Biol.,* 66: 1 – 9.

Kumar, H.B., Udayshankar, A.C., Prakash, H.S., and Shetty, H.S. 2005. Effect of Sanosil and *Pseudomonas fluorescens* on *Bean common mosaic potyvirus* incidence in French bean. *Int. J. Bot.* 1(2):163-167.

Kunkalikar, S. 2003. Molecular characterization, cloning of coat protein gene, epidemiology and management of papaya ringspot virus. PhD thesis, University of Agricultural Sciences, Dharwad, 290p.

Kunkalikar, S., Byadgi, A.S., Kulkarni, V.R., Krishnareddy, M. and Prabhakar, A.S.N., 2007. Histopathology and histochemistry of papaya ringspot disease in papaya. *Indian J. Virol.* 18(1): .33-35.

Lai R., Zheng, M., and Zhang, X.2012.Control efficiency of inducers to papaya ringspot virus disease in *Carica papaya. Plant Prot.*, 2: 204-209.

Lakshminarayan Reddy, C.N. 2000. Studies of Papaya ringspot virus disease on papaya (*Carica papaya* L.). MSc (Ag) thesis, University of Agricultural Sciences, Bangalore, 105p.

Lana, A. F. 1980.Transmission and properties of viruses isolated from *Carica papaya* in Nigeria. *J. Hortic. Sci.* 55: 191-197.

Laney, A.G., Avanzato, M.V., and Tzanetakis, I.E. 2012. High incidence of seed transmission of *Papaya ringspot virus* and *Watermelon mosaic virus*, two viruses newly identifies in *Robinia pseudoacacia*.

Lewsey, M., Palukaitis, P. and Carr, J. P.2009. Plant–virus interactions: defence and counter-defence. In Parker, J. (ed.) *Molecular Aspects of Plant Disease Resistance*. Oxford, UK: Wiley-Blackwell. pp.134–176.

Lima, R. C. A., Souza, Jr. M.T., Pio-Ribeiro, G. and Lima, J.A.A.2002.Sequences of the coat protein gene from Brazilian isolates of Papaya ringspot virus. *Fitopatologia Brasileira*, 27:174-180.

Lima,. J. M. A. and Gomes, M. N. S.1975.Identification of Papaw ringspot virus in Fortaleza,ceara. *Fitossanidade,* 1: 56 – 69

Lindner, R.C.,Jensen,D.D., and Ikeda,W.1945. Ringspot. New papaya plunderer. Hawaii Farm Home.8:10-14. [Cross Ref.]

Lokesh, S. 2014. Response of zinc and boron sprays on growth, yield and quality of papaya (*Carica papaya* L.) cv. Red Lady. M Sc (Hort.) Thesis, Y.S.R. Agricultural University, Andhra Pradesh, 124pp.

Lokhande, N.M. and Moghe, P.G.1992. Influence of nutreints and hormones on fruit quality traits and their correlation with yield in PRSV infected papaya cv. Honeydew (Carica papaya L.). *The Punjab Hort.* J. 32(1): 102-106.

Maderos, C.D., Zotto, D.A., Galdeano, E., Portal, O., and Giolitti, F.2016. First report of Papaya ringspot virus infecting Carica papaya in Argentina. *J. Plant Pathol.* 98(3): 687.

Madhusudhan,K.N.,Vinayarani,G., Deepak,S.A., Niranjana,S.R., Prakash,H.S., Singh,G.P., Sinha, A.K., and Prasad, B.C. 2011. Antiviral activity of plant extracts and other inducers against tobamoviruses infection in bell pepper and tomato plants. *Int. J.Plant Pathol.*2: 35-42.

Mahdy, A.M.M., Fawzy, R.N., Hafez, M.A., Mohammed, H.A.N., and Shahwan, E.M.S. 2007. Inducing systemic resistance against *Bean yellow mosaic potyvirus* using botanical extracts. *Egyptian J. Virol.* 4: 223- 241.

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. 1990. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science,* 250: 1002–1004.

Malo, S.E. and Campbell, C.W., 1986, The papaya. *In: Cooperative Extension Service Fruits Crops Fact Sheet*. Univ. Florida, Gainesville. 11pp.

Manjunatha, S. 2012. Effect of micronutrients and silica on growth and yield of papaya cv. Red Lady.M Sc (Hort.). University of Horticultural Sciences, Bagalkot, 106pp.

Maoka, T., Kawano. S., and Usugi,T. 1995. Occurrence of P strain of papaya ringspot virus in Japan. *Annu. Phytopathol. Soc.* 61: 34-37.

Mederos, D. C., Hernandez, D. G., Gonzalez, J. G., Orelvis Portal, O. 2013.Epiphyte management of Papaya ringspot virus using Zea mays L. barriers in *Carica papaya* L. *Rev. Prot. Veg.* 28(2): 127-131.

Mishra and Jha,J. 1955. Mosaic of papaya (*Carica papaya* L.) in Bihar. In: *Proceedings of Bihar Academy of Agricultural Science*. 4: 102-103.

Nagendran, K., Pandey, K.K., Rai, A.B., and Singh, B. 2017. Viruses of Vegetable Crops: Symptomatology, Diagnostics and Management, IIVR Technical Bulletin No. 75, IIVR, Varanasi, pp 48.

Naseem, S, Roofi, A., Zafar, Y., and Hafeez, F.Y. 2013. Detection and phylogenetic relationship of *Papaya ringspot virus* -P in Pakistan. *J. Plant Pathol.* 95(3): 643- 645.

Omar, A.F., El- Kewey,s.A., Sidaros,S.A., and Shimaa, A.K.2011. Egyptian isolates of Papaya ringspot virus form a molecularly distinct clade. *J. Plant Pathol.* 93(3):569-576.

Opina,O.S. 1986 Studies on a new virus disease of papaya in Philippines. In: *Plant virus diseases of horticultural crops in the tropics and subtropics.* Food Fertilizer Technology Centre for the Asian and Pacific Region, 157-168.

Palukaitis, P. and Carr, J. P.2008. Plant resistance responses to viruses. *J. Plant Pathol.*90: 53–171.

Papayiannis, L.C., Ioannou, N., Boubourakas, I.N., Dovas, C.I., Katis, N.I., and Falk, B.W. 2005. Incidence of viruses infecting cucurbits in Cyprus. *J. Phytopathol.*153 (9): 530 -535.

Parameswari, B., Mangrauthia, S. K., Praveen, S. and Jain, R. K.2007.Complete genome sequence of an isolate of Papaya ringspot virus from India. *Arch. Virol.*, 152: 843–845.

Perera, W.G.S., Suestsugu, T., and Saito, N. 1998. Occurrence of papaya ringspot virus strain P in Sri Lanka. JICA/ NPQS Occasional Paper, No. 1: 14pp.

Philip, S. and Nair, K. 1999. Kerala Karshakan. 44(10): 7-8.

Polak, Z. and Pospisil, F. 1995. Alleviation of plant virus infection by humic acids. *Biol. Plant.* 2: 315- 317.

Prasad, H.P., Shankar, U.A., Kumar, B.H., Shetty, S.H. and Prakash, H.S., 2007. Management of Bean common mosaic virus strain blackeye cowpea mosaic (BCMV-BlCM) in cowpea using plant extracts. *Arch. Phytopathol. Plant Prot.* 40(2): 139-147.

Prasad, S. M. and Kudada, N., 2005. Effect of barrier and inter crops on natural incidence of papaya ringspot virus disease and fruit yield of papaya. *Indian J. Virol.*16 (1/2): 24-26.

Prasad, S. M. and Sarkar, D. P. 1989.Some ecological studies on Papaya ringspot virus in Ranchi. *Indian J. Virology,* 5: 118-122.

Prowidentii, R., 1996, A Taiwan strain of papaya ring spot virus causing prominent symptoms on cultivated cucurbits. Report-Cucurbit-Genetics Co-operative, 19: 83-84.

Pushpa, R.N. 2014. Molecular characterization, epidemiology and integrated management of Papaya ringspot virus (PRSV) in papaya (Carica papaya L.). Ph D. thesis, University of Agricultural Sciences, Bengaluru, 178p.

Qiu, X., Wang, M. L., Zhu, Y. J., Moore, P. H. and Albert, H. H.2003.Global expression profiling of pathogen-defense related genes from papaya. *Am. Soc. Plant Physiol.*792: 168.

Qualls, R.G. 2004. Biodgradability of humic substances and other fractions of decomposing leaf litter. *Soil Sci. Soc. Am. J.* 68: 1705-1712.

Quemada,H.,L'Hostis,B.,Gonsalves,D., Reardon, I.M., Heinrikson, R., Hiebert, E.L., Sieu, L.C., and Slightom,J.L. 1990. The nucleotide sequences of the 3' terminal regiosn of papaya ringspot virus strains w and p. *J. Gen. Virol.*71: 203-210.

Radwan, D.E.M, Fayez, K.A., Mahmoud, S.Y., Hamad, A., and Lu, G. 2007. Salicylic acid alleviates growth inhibition and oxidative stress caused by zucchini yellow mosaic virus infection in *Cucurbita pepo* leaves . *Physiol. Mol. Plant Pathol.*69: 172-181.

Rajinimala, N., Rabindran, R., and Ramaiah. M. 2009. Management of bitter gourd yellow mosaic virus (BGYMV) by using virus inhibiting chemical, biocontrol agents, antiviral principles (AVP) and insecticide. *Arch. Phytopathol. Plant Prot.* 42(8): 738–750

Ramos, C.S. 1987. Characterization of an unidentified virus attacking papaya (*Carica papaya* L.) in the Philippines. MSc (Ag.) thesis, University of Philippines at Los Banos College, Laguna, 57pp.[Cross Ref.]

Rao, G.P., Myrta, A. and Ling, K.S. 2008. Characterization, diagnosis and management of plant viruses: Horticultural Crops. Stadium Press, Houston, 144p.

Reddy, C.R., Tonapi, V.A., Varanavasiappan, S., Navi, S.S., and Jayarajan, R. 2006. Management of urd bean leaf crinkle virus in urd bean (*Vigna mungo* L. Hepper). *Int. J. Agric. Sci.* 2 (1): 22-28.

Reddy, L. Nagaraju, C.N., Kumar, M.P.K., and Venkataravanappa, V. 2007. Transmission and host range of papaya ringspot virus (PRSV*). J. Plant Dis. Sci.* 2(1): 9-13.

Reddy, P.V.K., Gowda, V.N., Rajesh, A.M., Yathindra, H.A., and Harshavardhan,M. 2011. Survey for the incidence and variability of symptoms of papaya ringspot virus in southern Karnataka, India. *Plant Archives*. 11 (2): 1147-1149.

Rio, D.C., Ares, M.Jr., Hannon, G.J., and Nilsen, T.W. 2010. Purification of RNA using TRIzol (TRI reagent). *Cold Spring Harb. Protoc.* doi:10.1101/pdb.prot5439.

Ruwanthi, K.H.D., Ranasinghe, C., and De Costa, D.M. 2014. In: Proceedings of the Peradeniya University,International Research Sessions, Sri Lanka, 18

Ryals, J., Uknes, S. and Ward, E. 1996. Systemic acquired resistance. *Plant Physiol.* 104: 1109 - 1112.

Saitou, N. and Nei, M. 1987. The neighbor- joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*4: 406-425.

Scheuerell, S.J. and Mahaffee, W.F. 2006. Variability associated with suppression of gray mold (Botrytis cineria) on Geranium by foliar applications of non-aerated and aerated compost teas. *Plant Dis*. 90: 1201-1208.

Senaratna, T., Touchell, D., Bunn, E., and Dixon, K. 2000. Acetyl salicylic acid (Aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. *Plant Grow. Reg.* 30: 157- 161.

Sharma,J. Jain, R.K., Ramiah, M., and Varma, A.2005. Natural spread of Papaya ringspot virus to new areas: occurrence in Coimbatore, Tamil Nadu. *Indian Phytopathol.* 58(2) : 245-249.

Shrestha, S.K. and Albrechsten, S.E. 1992. Outbreaks and new records – Identification of new virus diseases in Nepal. *FAO Plant Prot. Bull.* 40(4):167 -170.

Singh, R.K., Singh, D., and Singh, S.J.2003. Incidence, distribution and detection of a virus infecting papaya (*Carica papaya* L.) in eastern Uttar Pradesh. *Indian J. Plant Pathol.* 21(1-2): 51-56.

Singh, S., Awasthi, L.P., Kumar,P., and Jagre,A.2017. Diagnostic characteristics of papaya ringspot virus isolates infecting papaya (*Carica papaya* L.) in India. *Immunol. Virol.* 1(4):1-9.

Singh, V. and Shukla, K. 2009. Effect of PRSV infection on prigment content and assimilation of carbohydrate in *Carica papaya* L.*An. Plant Prot. Sci.* 17(1): 152-156.

Singh, V. and Shukla, S. 2012. Morphological and histopathological changes in papaya due to virus isolate from middle gangetic plains of India. *J. Cell. Tissue Res.* 12(3): 3387- 3393.

Singh,V. and Shukla, K. 2011. Influence of inoculation time on severity of virus disease caused by Papaya ringspot virus.*Annu. Pl. Prot. Sci.*19 (1): 142-146.

Srinivasulu, M. and Sai Gopal, D.V.R. 2011. Coat protein sequence comparison of south Indian isolates of Papaya ringspot virus with other Indian subcontinent isolates. *Phytopathol. Mediterr.* 50:359 -367.

Story, G. E. and Halliwell, R. S.1969.Identification of Distortion ringspot virus disease of papaya of the Dominican Republic. *Plant Disease Reporter.* 53: 757 - 760.

Sultana, S., Roy, B., and Sherpa, A.R. 2018. Natural occurrence of papaya ringspot virus in *Clitoria ternatea* in India. *J. Plant Pathol.* Available: https://doi.org/10.1007/s42161-018-0128-5. [30 Jun 2018]

Surekha, S.K., Mathur, K., and Shukla, D.O.1977. Virus diseases of papaya (*Carica papaya*) in Udaipur. *Indian J. Mycol. Pl. Pathol.*7:115-121.

Takanami, Y., Kuwata, S., Ikeda,T., and Kubo, S. 1990. Purification and characterization of the anti-plant viral protein from *Mirabilis jalapa* L. *Ann. Phytopathol. Soc.Jpn*. 56:488-494.

Talukdar, B., Saikia, A. K. and Nath, P. D. 2013. Detection of through Double antibody Sandwich Enzyme-linked Immunosorbent Assay (DAS-ELISA) in Assam. *J. Mycol. Pl. Pathol*., 43(4): 442-446.

Tennant, P. F., Fermin, G.A. and R. E. Roye.2007.Viruses infecting papaya (*Carica papaya* L.): etiology, pathogenesis and molecular biology. *Plant Viruses*, 1:178–188.

Thomas, J.E. and Dodman, R.L. 1993. The first record of Papaya ringspot virus type-P from Australia.*Aust. Plant Pathol*. 22: 2-7.

Torres, M.R. and Giacometti, D.C. 1966. Virosis de la papaya (*Carica papaya* L.) en el valle del Cauca. Agricultura Tropical (Colombia). 22:27-38.

Tripathi, S., Suzuki, J.Y., Ferreira, S.A., and Gonsalves, D.2008. *Papaya ringspot virus*-P: characteristics, pathogenicity, sequence variability and control. *Mol. Plant Pathol*. 9(3): 269-280.

Tuo, D., Shen, W., Yang, Y., Yan, P., Xiaoying, L., and Zhou, P. 2014. *Viruses*. 6(10): 3893-3906.

Usharani, T.R., Laxmi, V., Jalali,S., and Krishnareddy, M.2012. Duplex PCR to detect both *Papaya ringspot virus* and *Papaya leaf curl virus* simultaneously from naturally infected papaya (*Carica papaya* L.). *Indian J. Biotechnol*. 12(2): 269-272.

Van Loon, L. C. and Kammen, V. A. 1970. Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. 'Samsun and 'Samsun NN' II. Changes in protein constitution after infection with tobacco mosaic virus. *Virol.* 40:199-211.

Van Loon, L.C., Bakker, P.A.H.M., and Pieterse, C.M.J. 1998. Systemic resistance induced by rhizospheric bacteria. *Annu. Rev. Phytopathol.*36: 453- 483.

Verghese,A., Anil Kumar, H.R., and Kamala Jayanthi, P.D.2001. Staus and possible management of papaya ringspot virus with special reference to insect vectors. *Pest Manag. Hortic. Ecosystems.* 7: 99-112.

Verma, H.N. and Dwivedi. 1984. Properties of a virus inhibiting agent, isolated from plants which have been treated with leaf extracts from *Bougainvillea spectabilis*. *Physiol. Pl. Pathol.*25: 93-101.

Verma, H.N. and Kumar, V. 1980. Prevention of plant virus diseases by *Mirabilis jalapa* leaf extract. *New Bot.* 7: 87-91.

Verma,R., Ram, R.D.,and Tomer, S.P.S.2007.Survey and surveillance of Papaya ringspot virus disease in India. *J. Maharashtra Agric. Univ.* 32(2): 277-278.

Vimla S., Rao, G. P. and Shukla, K.2005.Response of commercially important papaya cultivars to papaya ringspot virus in eastern U.P. conditions. *Indian Phytopath.*, 58(2): 212-216.

Vivanco, J. M., Querci, M., and Salazar, L. F. 1999. Antiviral and antiviroid activity of MAP containing extracts from *Mirabilis jalapa* roots. *Plant Dis.* 83:1116-1121.

Wahab, N. 1991. Papaya ringspot virus disease (PRV) is here in Malaysia. MAPPS Newsletter. 15 (3): 36.

Wan, S.H. and Conover, R.A. 1983. Incidence and distribution of papaya virus in Southern Florida. *Plant Dis*. 67: 353 - 356.

Wang, C.H. and Yeh, S.D.1997. Divergence and conservation of the genomic RNAs of Taiwan and Hawaii strains of papaya ringspot potyvirus. *Arch. Virol.* 142:271- 285.

Wang, H. L., Wang, C. C., Chiu, R. J. and Sun, M. H.1978.A preliminary study of Papaya ringspot virus in Taiwan. *Plant Prot. Bull.,* 20**:** 133 - 140.

Wu, M., Song, M, Liu, M., Jiang, C., and Li, Z. 2016. Fungicidal activities of soil humic/ fulvic acids related to their chemical structures in greenhouse vegetable fields with cultivation chronosequence. *Sci. Rep*.328 -58.

Xiao, H.G., Tetsuo, M.K., and Luo, X.H.1997. Investigation and identification of papaya ringspot virus and papaya leaf- distortion mosaic virus in South China. *J. S. China Agric. Univ*. 18(4): 52-53 (Ch), 58 (En)

Yeh, S. D. Gonsalves, D. and Prowidenti, R. 1984. Comparative studies on host range and serology of Papaya ringspot and Watermelon mosaic virus. *Phytopathology,* 74: 1081 - 1085.

Yeh, S.D. and Gonsalves, D. 1984. Evaluation of induced mutants of papaya ringspot virus for control by cross protection. *Phytopathology*. 74: 1086-1091.

Yeh, S.D., Jan, F.J., Chiang, C.H., Doong, P.J., Chen, M.C., Chung, P.H., and Bau, H.J. 1992. Complete nucleotide sequence and genetic organization of papaya ringspot virus RNA.*J. Gen. Virol.*73: 2531-2541.

Yeh,S.D. and Gonsalves, D.1985. Translation of Papaya ringspot virus RNA in vitro: detection of a possible polyprotein that is processed for capsid protein, cylindrical-inclusion protein and amorphous inclusion protein. *Virology*.143: 260-271.

Yemewar, S.I. and Mali, V.R.1980.On the identity of a sap transmissible virus of papaya in Marathawada. *Indian J. Mycol. Plant Pathol.*10: 155-160.

Yeon, K.H., Lee, H.B., Kim, Y.C., and Kim, I.S. 2008. Laboratory and field evaluations of entomopathogenic *Lecanicillium attenuatum* CNU-23 for control of green peach aphid (*Myzus persicae*). *J. Microbiol. Biotechnol.* 18(12): 1915- 1918.

Yogiraj, V., Goyal, P.K , Chauhan, C.S., Goyal, A., and Vyas, B. 2014. *Carica papaya* Linn. An Overview. *Int. J. Herb. Med.* 2(5): 1-8.

Zellner W., Frantz J., Leisner S. 2011. Silicon delays *Tobacco ringspot virus* systemic symptoms in *Nicotiana tabacum*. *J. Plant Physiol.* 168 (15): 1866–1869.

Zettler, F. W., Edwardson, J. R. and Purcifull, D. E.1968.Ultramicroscopic differences in inclusion of papaya mosaic and Papaya ringspot virus correlated with differential aphid transmission. *Phytopathology,* 58: 332 - 335.

Zhang, G., Ma, L., Wang, P., Liu, Y., Zhang, G., Hu, Z., 2007, Effect of salicylic acid on papaya ringspot virus disease. *Chinese J. Tropical Agric.*, 6: 20-22.

Zhang, R.P., Huang, G.L., Guo, M.D., Xu, Z.L., Yu, X.B., 2009, Effects of several chemicals on preventing papaya ringspot virus and defensing enzyme activities in papaya leaves. *Plant Physiol. J.*, 45 (2): 129-132.

Zheng, L.,Rodoni, B.C., Gibbs, M.J., and Gibbs, A.J. 2010. A novel pair of universal primers for the detection of potyviruses. *Plant Pathol.* 59(2).

Appendices

APPENDIX I

Composition of Buffer for Mechanical Transmission

0.1 M Potassium phosphate buffer (pH: 7.0)

Prepare 1 M each of dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4)

Combine 61.5 ml of 1M K_2HPO_4 and 38.5 ml of KH_2PO_4. Dilute the combined 1 M stock solution to 1 litre with distilled water. Adjust the pH to 7.0 with pH meter.

APPENDIX II

Composition of reagents used for DAC- ELISA

1. Phosphate buffered saline (PBS - pH: 7.4)

Sodium chloride - 8.0 g

Potassium dihydrogen phosphate - 0.2 g

Disoudium hydrogen phosphate - 1.1 g

Potassium chloride - 0.2 g

Sodium azide - 0.2 g

Water - 1000 ml

2. Wash buffer (PBS- T)

Add Tween- 20 @ 0.5 ml / L to PBS

3. Coating buffer (pH: 9.6)

Sodium carbonate - 1.9g

Sodium bicarbonate - 2.93g

Sodium azide - 0.2g

Water - 1000 ml

4. Antibody diluent buffer (PBS- TPO)

Add 20g PVP and 2 g ovalbumin to 1l PBS-T

5. Enzyme conjugate diluent buffer (PBS-TPO)

Same buffer used as antibody diluent buffer

6. Substrate solution (pH: 9.8)

Diethanolamine - 97 ml

Sodium azide - 0.2g

Water- 800 ml

APPENDIX III

Composition of buffer and dyes used for gel electrophoresis

1. DiEthyl Pyro Carbonate (DEPC)Water (0.1%) - pre-treatment of micropipette tips, centrifuge tubes and glasswares

DEPC - 1 ml (Added in an amber bottle)

To this, add one litre of millipore water. Mix the contents using a magnetic stirrer overnight

followed by double autoclaving.

2. 10 X MOPS buffer (500 ml)- buffer for gel documentation

MOPS (20 mM) - 20.93g

Sodium acetate (50 mM) - 0.205 g

Sodium EDTA (10 mM) - 1.861 g

DEPC water - 1000 ml

3. Formaldehyde agarose gel (1%)

Boil 0.5 g agarose in 42.3 ml DEPC water. Add 5 ml 10 X MOPS buffer, 2.7 ml formaldehyde and 2 µl ethidium bromide.

The dye was prepared as a stock solution of ethidium bromide (stock 10 mg/ml; working concentration 0.5 µg/ml) and was stored at room temperature in a dark bottle.

APPENDIX IV

Gel electrophoresis of PCR products

1. Electrophoresis buffer (50 X TAE)

Tris base: 242.0 g

Glacial acetic acid: 57.1 ml

0.5M EDTA (pH 8.0): 100 ml

Distilled water: 1000 ml

The solution was prepared and stored at room temperature

2. Loading Dye (6X)

0.25% Bromophenol blue

0.25% Xylene cyanol

30% Glycerol in water

The dye was prepared and kept in fridge at 4° C.

APPENDIX V

List of laboratory equipments used for the studies

High speed refrigerated centrifuge: Kubota

Electrophoresis unit: BIORAD, Power Pac, HV, USA

PCR machine: Applied Biosystems

Gel documentation: BIORAD Molecular Imager (Gel Doc™ XR+)

ELISA plate washer: BIORAD, PW-40

ELISA plate reader: Microplate reader, 680, BIORAD

APPENDIX VI

Weekly atmospheric temperature data recorded during pot culture experiment

Month	Weeks after planting	Temperature	
		Maximum (°C)	Minimum (°C)
October, 2017	0	31.4	22.8
	1	31.9	22.8
	2	30.9	22.2
	3	32.0	21.5
	4	33.5	22.7
November, 2017	5	32.3	21.9
	6	32.8	20.8
	7	33.8	21.6
	8	32.0	22.5
December, 2017	9	32.8	21.0
	10	32.7	21.4
	11	32.3	21.5
	12	32.8	20.3
January, 2018	13	33.2	19.8
	14	32.7	21.8
	15	33.8	20.7
	16	34.1	21.4
	17	34.3	20.5

Characterization, host range and management of *Papaya ringspot virus* (PRSV)

By

ATHEENA HARISH

(2016-11-009)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT PATHOLOGY)

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF HORTICULTURE

VELLANIKKARA, THRISSUR - 680 656

KERALA, INDIA

2018

ABSTRACT

Papaya is an important fruit crop which is cultivated extensively both in tropics and subtropics. During the last decade, the area under papaya cultivation has dramatically increased due to the introduction of superior varieties. A major setback subsequent to the introduction of such new varieties has been the incidence of papaya ringspot disease caused by *Papaya ringspot virus* (PRSV) which leads to almost 100 per cent yield loss. Considering the importance of the disease, the project was undertaken with the objectives of characterization of the virus, molecular and immunodiagnosis along with disease management.

The research project was initiated with purposive sampling surveys conducted in different papaya orchards of Thrissur district in order to document the symptoms under natural conditions, to assess the incidence and severity of the disease and to collect infected samples for further studies. The maximum disease incidence and disease severity recorded were 99.6 and 96.67 per cent on papaya variety Red Lady from Vellikulangara and Puthur respectively. The development of symptoms was studied under artificial conditions also through mechanical inoculation of healthy papaya seedlings with virus inoculum maintained in insect proof net house conditions. The salient diagnostic symptoms of the disease observed on leaves were chlorotic spots, mottling, vein thickening, puckering, leaf distortion, shoestring symptom along with presence of oily streaks on the petiole. The fruits of infected plants presented typical oily concentric or broken ringspots on fruit surface along with malformation. Histopathological studies of infected leaves revealed disruption of the epidermis, disorganization of parenchyma, disintegration of chloroplasts and deposition of crystalline bodies.

The studies on virus transmission confirmed that it is transmitted through plant sap from infected to healthy papaya plants. Seed transmission studies revealed that PRSV is not seed -borne. Twenty one plant species including weeds seen in and around papaya orchards were tested for studying the host range of the virus and only five plant species *viz.*, *Cucumis sativus*, *Cucurbita moschata*, *Trichosanthes cucumerina*, *Momordica charantia* and *Chenopodium amaranticolor* developed symptoms after artificial inoculation of PRSV and thus proved to be the hosts of the virus. Morphological characterization done using electron microscopy showed the presence of typical flexuous rod particles of size 807.74 nm x 12 nm which indicated that the virus belongs to genus *Potyvirus* and the etiology of the disease was confirmed as *Papaya ringspot virus*.

Immunodiagnostic technique was validated using Direct Antigen Coating - Enzyme Linked Immuno Sorbent Assay (DAC-ELISA) and infected samples showed positive reaction to PRSV antiserum and could be detected at 1:200 dilution of primary antibody and 1:10,000 dilution of secondary antibody. Molecular characterization of PRSV was also carried out through Reverse Transcription Polymerase Chain Reaction (RT-PCR). The nuclear inclusion b (NIb) gene and the coat protein (CP) gene were amplified using reported primer pairs which yielded amplicons of approximate size of 1700 bp. The PCR products were outsourced for sequencing and *in silico* analysis of the sequences obtained revealed that the isolates of present study are more similar to Calicut isolate of PRSV, PRSV- Ca (DQ666640.1)

A pot culture experiment under insect proof conditions was also conducted to evaluate the effects of selected botanicals, chemicals and biocontrol agents for disease management. Among the fourteen treatments, *Bougainvillea* leaf extract, 10 per cent (T7) was the most effective with lowest disease severity (6.67%) followed by foliar spray and soil drenching with *Pseudomonas fluorescens* 2 per cent (T3) with a disease severity of 11.11 per cent as against 97.77 per cent recorded in untreated control plants. Plant height recorded after each treatment application revealed that both 10 per cent *Bougainvillea* leaf extract (T7) and 2 per cent *P. fluorescens* are equally superior to all other treatments. However, with respect to mean girth of stem, maximum value (2.91 cm) was recorded in *P. fluorescens* (T3) The data on virus titre of all treated plants assessed through DAC-ELISA revealed that the concentration of virus particles was minimum in plants treated with T7 and T3.

The outcome of this study would facilitate early detection and elimination of source of virus infection and thereby prevent the spread of disease in the field. The information generated on molecular characterization of PRSV isolates under could be applied in genetic engineering. The project also revealed the potential of botanical, *Bougainvillea spectabilis* leaf extract and biocontrol agent, *Pseudomonas fluorescens* for the ecofriendly management of papaya ringspot disease.

174447