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Research Article

MOLECULAR CHARACTERIZATION, EPIDEMIOLOGY AND MANAGEMENT OF THE *Papaya ringspot virus* (PRSV) IN PAPAYA UNDER SOUTHERN INDIAN CONDITIONS

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Abstract- Papaya cultivation is globally affected by *Papaya ringspot virus* (PRSV) disease. PRSV disease management using cross-protection and transgenic plants has been hindered due to variability observed in PRSV gene sequences. Therefore, the characterization of local PRSV isolates combined with the understanding of disease epidemiology could serve a crucial role in designing the region-specific management practices. In this perspective, the Coat protein gene sequences of three south Indian PRSV isolates were determined and compared with the sequences of other PRSV isolates from different geographical locations in the Indian states. The phylogenetic tree analysis reveals close clustering of PRSV isolates from the south Indian states. Monitoring of the vector population in the field revealed the occurrence of eight aphid species under south Indian field conditions. Three species namely, *Aphis gossypii*, *A. craccivora* and *Myzus persicae* were observed majorly throughout the year and were found as efficient transmitters of PRSV in fields. Among the integrated management practices examined, growing papaya as intercrop with African Tall maize (1:1) and Grand Naine banana (2:1) as live barriers was found effective, recording 60-90% disease control with a cost to benefit (C:B) ratio of 1:9.2 and 1:6.5 in Red lady and with 1:3 and 1:1 in Arka Surya respectively. Similarly, growing papaya with silver reflective mulch was recorded as profitable treatment with C:B ratio of 1:1.9 and1:6.2 by controlling 90 and 100% disease in Arka Surya and Red Lady respectively. Moreover, frequent foliar spray of red seaweed extract was found to delay PRSV symptom expression and reduce yield loss.

Keywords- *Papaya ringspot virus,* Coat Protein, Aphids, papaya management, Seaweed.

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Introduction

Papaya fruit is considered highly nutritious being rich in antioxidants such as carotenes, vitamins and trace elements. It is produced globally in over 60 countries with most of it being produced from the developing countries. Asia is the leading producer of papaya accounting for 56.3 percent of global production [1]. Global papaya production however faces a serious threat from the disease of *Papaya ring spot virus* (PRSV). PRSV, belongs to the genus *Potyvirus* of the family *Potyviridae*, is a positive strand RNA virus [2]. The flexuous filamentous PRSV particle typically measuring 780-800 nm X 12 nm in dimension with an RNA genome of about 10.3 Kb is surrounded by repeating units of a coat protein of molecular mass of about 36 KDa [3, 4]. The virus is transmitted non-persistently by numerous species of aphids [5]. Additionally, the virus shows significant variation across different geographical regions [6, 7]. Therefore, the molecular characterization of local PRSV isolates, combined with the understanding of vector epidemiology will have a crucial role in designing the region-specific management practices for efficient production of papaya.

The lack of sexually compatible PRSV resistant papaya cultivars has limited the breeder's efforts in developing PRSV resistant varieties [8]. However, the characterization of genome sequences of PRSV isolates from different geographical regions has facilitated the development of PRSV-resistant papaya via pathogen-derived resistance technology. The development of transgenic papaya lines based on coat protein gene or replicase gene mediated post-transcriptional gene silencing has been by far the most promising technology for PRSV disease management [9-11]. The success of such gene technologies depends on the relatedness of a transgene with the gene sequence of the challenging virus. Therefore, the sequence knowledge of local isolates would be an essential requirement for achieving effective PRSV resistance [12]. Since, the first report of PRSV in India [13], CP sequences of eleven PRSV isolates originating from different locations in India have been characterized [7].These virus isolates from India showed considerable heterogeneity in both the sequence and the length of CP gene. Comparative sequence analyses suggest the PRSV isolates originated from India were divergent up to 15% at amino acid level [14]. Similarly, considerable heterogeneity in CP gene length has been noticed, with maximum heterogeneity was observed in southern isolates (up to 23 %), followed by central (up to 11 %), eastern and northern (up to 10 %) and western (up to 7 %) isolates [15]. In this context, the CP gene sequences determined in this study are likely to provide valuable insights towards understanding the complexity of PRSV population in the country.

In addition to developing transgenic PRSV resistant papaya other major method of PRSV management currently being followed is via vector control. PRSV spreads rapidly in the field and it is transmitted mechanically through sap by many species of aphid vectors in a non-persistent manner [5]. The main factors influencing

PRSV disease spread involve the amount of initial virus inoculum in the hosts, the status of aphids as transient vectors, variations in their life cycles, behavior, ability to transmit the virus and effect of environmental factors on aphid population dynamics [16]. Previous studies suggest that the spread of aphid borne non-persistent viruses follows the seasonality of vectors, especially the dominant species [17,18]. *A. gossypii*, *A. craccivora* (Koch) and *A. citricola* (Van der Goot) have been the major vectors of PRSV in India [19,20]. In addition to this knowledge, a detailed understanding of the seasonal dynamics and the species composition of aphids involved in the field spread of PRSV under South Indian conditions would be useful in strategizing region-specific vector control practices. Therefore, herein, we have explored a systematic understanding of PRSV-aphid vector relationship and the associated epidemiological information in the context of the southern Indian field conditions.

PRSV spreads through the field very rapidly causing crop losses of up to 85-90% in severely infected fields [21]. In certain countries wherein, transgenic varieties are not in practice, conventional integrated management practices have been the only option in maintaining papaya orchards PRSV free. Growing barrier crops such as Jowar or Maize, either as intercrop or along the border have been shown to be effective in reducing virus transmission by blocking aphids from reaching the target plants [22, 23]. Intercropped maize barriers have shown reducing effect on both the incidence and final severity of PRSV in endemic areas [24, 25]. In addition to these practices, foliar sprays of biological solutions such as Milk, Neem oil and other plant seed extracts have been reported to be effective to some extent in containing the virus spread [26, 27]. In this context, examining the roles of novel biocontrol agents such as seaweed extracts would provide additional alternate options for farmers in managing PRSV dissemination. In fact, seaweeds have been previously shown to produce a variety of compounds with various pharmacological activities including antimicrobial, antifungal, antiviral etc. [28,29]. The Carrageenans, sulfated linear polysaccharides, from red seaweeds have been reported to induce various plant defense pathways against viruses, fungus, bacteria and insects [30].

Overall, the containment of PRSV in papaya fields has become a major challenge to papaya growers and scientists worldwide. The versatile and destructive nature of PRSV demands the characterization of sequence variation of local PRSV isolates, understanding the vector diversity and development of innovative management practices. Herein, we present a case study of south Indian PRSV isolates with respect to coat protein gene characterization and identifying the aphid vector composition in the field. Additionally, novel management practices have been discussed including, growing African Tall maize and Grand Naine banana as live barriers along with frequent foliar applications of seaweed extract for the effective management of PRSV.

Material and Methods

RNA Isolation: All the plastic and the glass wares were washed and treated with 0.1 per cent Diethyl pyrocarbonate (DEPC) for 24 hours before sterilization. Isolation of total RNA from healthy and PRSV infected papaya samples was carried out using Trizol reagent [31]. Briefly, the PRSV infected papaya leaf samples were flash frozen in liquid nitrogen and ground to fine powder. About 100 mG of powdered leaf material was added with 1 mL of Trizol reagent. The tubes were centrifuged at 9,000 rpm for 10 minutes, supernatant was collected into fresh tubes and processed further [31].

Reverse transcription: Total RNA from healthy and PRSV infected papaya samples were used for reverse transcription. A 20 µL reverse transcription (RT) mixture was prepared by following the protocol of TaKaRaPrimeScript reverse transcription kit. 2.5 µG of viral RNA was used in these reactions while sterile water was used in no template control. The RT mixture was reverse transcribed at 39 °C for 60 minutes and then at 94 °C for 5 minutes. The cDNA thus obtained was used for performing further PCR reactions.

PCR amplification of Coat protein genes: The cDNA thus obtained was subjected to PCR amplification using 5' AGAAGCGTGGGTCAATGGA 3' and 5' CTCTCCAGTTTTTGTGCTAGTTG 3' as forward primer and reverse primers respectively. The reactions were carried out in an Eppendorf thermo-cycler in 15.0 µL reaction volume. A typical PCR reaction contained 2.5 µL of 10x PCR buffer, 0.5 µL of 25 mM MgCl2, 2.0 µL of cDNA, 2.0 µL of 10 µM primer, 2.0 µL of dNTPs mix (2.5 mM each) and 1 unit of Taq DNA polymerase and the total volume was adjusted to 15 µL with DEPC treated sterile water. The mixture was subjected to one cycle of initial denaturation at 94 °C for 4 minutes followed by 35 cycles of denaturation at 94 °C for 60 seconds, annealing at primer specific temperature for 45 seconds, extension at 72 °C for 90 seconds and a final extension at 72 °C for 10 minutes. After completion of the PCR reaction the products were analysed on a 0.8% agarose gel.

Sequencing of amplified PCR product: the amplified PCR product was directly sequenced using ABI 3730Xl DNA analyser available at Scigenome Labs Pvt. Ltd., Cochin-Kerala, India. Sequencing was done in both directions using forward and reverse primers.

Construction of phylogenetic tree; The sequence homology was analysed using BLAST (www.ncbi.nih.gov /BLAST). The Neighbour joining phylogenetic tree was generated using MEGA 6.06 software tool. To calculate the confidence limits placed in construction of phylogenetic tree, bootstrapping analysis was carried out using 1000 replicates resulting in a boot strapped Neighbour joining tree.

Monitoring of major aphid vectors and their identification: A field experiment was conducted at Main Research Station (MRS), UAS, Hebbal, Bangalore, to study the abundance of aphid species and their influence on PRSV incidence in relation to weather parameters *viz*., temperature, rainfall, relative humidity, direction and wind speed. Three papaya varieties *viz*., Red Lady, Sunrise Solo, Arka Surya were raised inside an insect proof glasshouse, ten seedlings of each variety were transplanted in the main field during *Kharif*2013-2014and the crop was maintained with recommended package of practices except for plant protection measures. Aphid abundance in the field was assessed by using yellow sticky traps 2' X 2.5' that were installed in all four directions at different heights *viz*., 2, 4, 6 & at 8 feet. Aphids trapped on yellow sticky traps in different directions and at different heights were collected in Eppendorf tubes containing alcohol at 3 days intervals [Fig-2]. The aphid species characterization was carried out with the help of taxonomists at the National Bureau of Agriculturally Important Resources (NBAIR), Bangalore. To record fresh PRSV infection, observations on characteristic symptoms were recorded once in 7 days on each plant from time of transplanting.

Correlation between incidence of aphid vectors and PRSV: Data were subjected to Pearson's correlation analysis to determine the extent and nature of association between number of aphids and per cent PRSV infection. Based on trap catch data, predominant species of aphids which contributed for the successful transmission and spread of PRSV in field namely, *Aphis gossypii* (Glover), *A. craccivora* (Koch), *A. nerii (*Boyer de Fonscolombe), *Astegopteryx bambusae (*Buckton), *Myzus persicae* Sulzer, *Hyperomyzus carduellinus (*Borner), *Brevicornia brassicae* (Linnaeus) and *Pentalonia nigronervosa (*Coquerel) were considered as independent variables and fresh incidence of PRSV as dependent variable. The influence of each species of aphids on the spread of PRSV was analysed by correlation analysis using 'IBM SPSS Statistics version-20' statistical software package. The contribution of different species of aphids on fresh PRSV infection was calculated.

Integrated management methods: To combat PRSV disease in field, an integrated management approach was laid out in farmer field at Mayaganahalli, Ramanagar District, Karnataka, Southern India, during 2015-16. The seedlings of two popular varieties of papaya, 'Arka Surya' and 'Red Lady' were raised in 6" X 4" polyethylene covers and maintained in an insect proof nylon mesh of 40X gauge. 60 days old seedlings were then transplanted into main field with a spacing of 6' X 6'. The recommended package of practices was followed till the end of experiment.

Different treatments were evaluated by using a simple CRD design with ten replications. The treatments imposed were:T-1: Growing African tall maize as live barrier (two months before transplanting the papaya seedlings, 'African tall maize' was grown densely all around the treatment plot and also in between the rows of papaya as live a barrier in the ratio of 1:1); T-2: Growing Grand Naine banana as live barrier **(**two months before transplanting papaya seedlings, 'Grand Naine banana' was grown all around the treatment plot and in between the rows of the papaya as a live barrier in the ratio of 1:2); T-3: Growing papaya plants with silver reflective row mulch (60 days old papaya seedlings of both 'Red Lady' and 'Arka Surya' were grown in row covered with silver reflective mulch which were set above each papaya row); T-4: Untreated control (The 60 days old papaya seedlings of both 'Red lady' and 'Arka Surya' were planted in the main field. These plants were maintained without imposing any of the above treatments and the normal agronomic practices were followed as in the earlier treatments). All the treatments were replicated ten times. The observations on number of plants infected based on DAS-ELISA, per cent PRSV incidence, growth and yield characters of papaya including plant height, number of fruits per plant and yield (kg) per plant.

During termination of the experiment, apical leaves of all treated and untreated papaya plants were tested serologically using DAS-ELISA technique for the presence of PRSV. The test wells positive for PRSV were recorded as infected and were compared with healthy and buffer control. Based on the ELISA absorbance values and yield per plant, the per cent disease incidence, per cent disease control and percent yield increase over control were calculated by using the following formulae:

$$\text{Per cent Disease incidence} = \frac{\text{No. of plants infected}}{\text{Total No. of plants}} \times 100$$

$$\text{Per cent Disease control over untreated} = \frac{C - T}{C} \times 100$$

T = No. of plants infected in treated plot
C = No. of plants infected in untreated plot

$$\text{Per cent yield increase over untreated} = \frac{\text{Yield of treated plant - Yield of untreated plant}}{\text{Yield of treated plant}} \times 100$$

Preparation of seaweed extract: The *Kappaphycus* species of seaweed biomass was cultivated in the tropical waters of southeast coastline of the Indian states. The seaweed biomass thus obtained was crushed to separate solid and liquid fractions. These fractions were further processed using the patented methods of extraction by Sea6 Energy Pvt Ltd (WO2016/ 181411, 17th Nov 2016).

Serological detection of PRSV by DAS-ELISA: PRSV was detected in papaya samples by using double antibody sandwich technique with anti-PRSV capture antibody and ALP labelled anti-PRSV detection antibody (Agdia, Inc., USA).

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Results and Discussion

I. Molecular characterization of Coat protein genes of three South Indian PRSV isolates

a. PCR amplification and sequencing of Coat protein gene

The leaf samples of three-month-old "red lady" variety of papaya seedlings expressing symptoms typical of PRSV infection were confirmed by DAS-ELISA using Anti-PRSV polyclonal antibodies. Upon confirming the PRSV infection, total RNA was isolated from the leaf samples that were flash frozen in liquid Nitrogen and the cDNA was synthesized by using reverse transcription. The 5' AGAAGCGTGGGTCAATGGA 3' and 5' CTCTCCAGTTTTTGTGCTAGTTG 3' were used as the forward and the reverse primers for amplifying part of the coat protein gene. The size of amplified product (500 base pairs) was confirmed on an agarose gel [Fig-1]. The PCR reactions of Bangalore (PRSV-BLR), Coimbator (PRSV-CBE) and Ernakulam (PRSV-EKM) isolates resulted a product of about 500 base pairs while the Tirupati isolate (PRSV-TPT) did not result in a product. This could possibly due to variation in the nucleotide sequences at the oligo annealing region of PRSV-TPT. The amplified PCR products were sequenced in both directions using forward and reverse primers. Comparison of CP nucleotide sequence of PRSV-BLR reveals 91% identity with PRSV-EKM isolate and 88% identity with PRSV-CBE isolate, while the CP sequences of PRSV-EKM and PRSV-CBE isolates share an identity of 87%. It is intriguing to observe a diversity of about 12-13% in the CP nucleotide sequences of neighbouring Indian states.

A

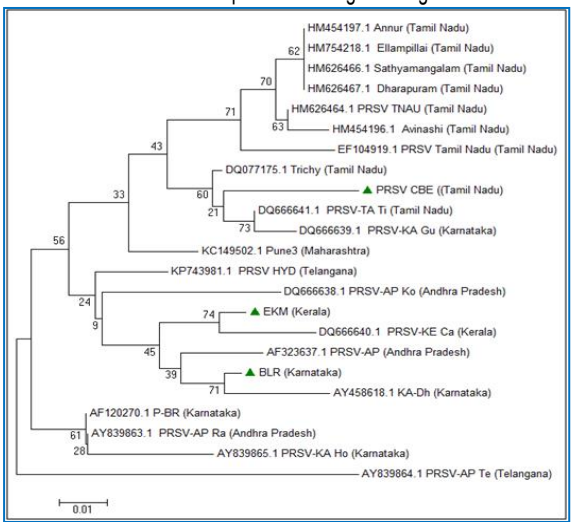

Fig-1 A Phylogenetic tree created based on the nucleotide sequences of the Coat protein gene of various south Indian PRSV isolates: Bootstrapped neighbour joining method using the software MEGA 6.06. was used to create the phylogenetic tree.

B

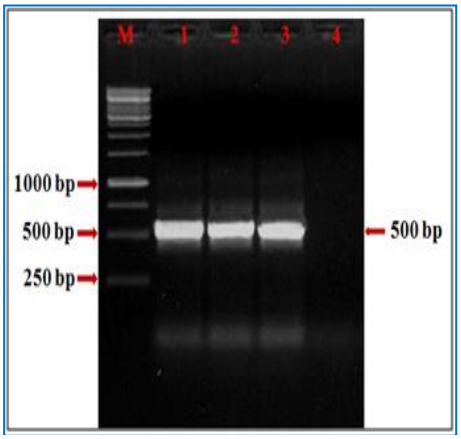

Fig-1 B Amplified PCR products of partial coat protein genes of PRSV isolates. The amplified products of PRSV-BLR isolate (1), PRSV-CBE isolate product (2), PRSV-EKM isolate (3) and healthy papaya sample. 1 Kb DNA ladder (NEB) was used as a reference (M).

b. Molecular variability of PRSV isolates based on CP gene sequence

The CP nucleotide sequences of three south Indian PRSV isolates *i.e.*, PRSV-BLR, PRSV-CBE and PRSV-EKM determined in the current study were compared to sequences of other PRSV isolates from different geographical locations of the southern Indian states [Table-1]. The nucleotide sequence identity of the three isolates used in the present study ranged from 97 per cent (PRSV-BLR) to 94 per cent (PRSV-CBE) with other Indian isolates deposited in the NCBI GenBank. PRSV-BLR isolate showed highest nucleotide identity of 94 per cent with CP gene sequences of accession AF323637 (AP isolate) followed by 91 per cent with PRSV-TA Ti (DQ666641) and isolate PRSV-KE (DQ666639). PRSV-CBE isolate showed highest identity (95 %) with PRSV-TA Ti (DQ666641) PRSV-EKM isolate showed highest identity (97 %) with isolate PRSV-KE (DQ666640) followed by 94 percent identity with PRSV-AP isolate (AF323637) and 91 per cent with HYD isolate (KP743981).

Phylogenetic tree [Fig-1] was constructed based on the CP nucleotides sequences of isolates from present investigation and sequences of twenty other PRSV isolates available in the NCBI GenBank [Table-1]. The close relationship of isolates from respective states was evident from the phylogenetic tree and clustering pattern of isolates correlated reasonably well with their geographical origins. Partial characterization of isolate BUH-1 by CP gene showed maximum homology of 98 per cent with south Indian and 87-92 per cent with Asian isolates [32]. PRSV-DEL (from New Delhi) has been reported to show a sequence identity of 83-89 per cent at the nucleotide level with other PRSV isolates [33]. The present study agrees with earlier reports that PRSV isolates from the Indian subcontinent are diverse [7, 14, 34-36]. The higher sequence divergence within the PRSV population of the Indian subcontinent has been related to wide range of cropping systems and cultivation practices followed in different geographical regions [7]. This diversity might have resulted in different levels of selection pressure on PRSV.

Genetic engineering is a viable option for managing viral diseases such as PRSV [37-39]. Knowledge of the nucleotide sequence and genetic diversity is necessary to select a virus gene for the development of pathogen derived resistance. Sequence variability has important implications for the use of genes to develop transgenic plants by pathogen derived resistance because such resistance could be highly sequence specific [12]. It is appropriate to note here that, due to variability in the coat protein genes, disease management using cross-protection and transgenic plants requires the selection of region specific virus isolates in each country [40]. In this context the coat protein gene sequences of three south Indian PRSV isolates discussed here would be valuable in designing transgenic papaya specifically resistant to PRSV across south Indian region

II. Vector diversity and epidemiology of PRSV incidence

a. Species composition of aphids trapped in papaya orchard

Monitoring of transitory aphids using yellow sticky traps in papaya orchard during August-2013 to July-2014, revealed trapping of eight major aphid species [Fig-2] & [Table-2]. *A. gossypii* was regularly trapped in large number (66.04%) followed by *A. craccivora* (26.80%) and *M. persicae* (2.12%) as predominant aphid vectors which contributed in large numbers (Table-2). while, Bamboo aphid, *Astegopteryx bambusae* (Buckton), Eupatorium aphid, *Hyperomyzus carduellinus* (Borner), cabbage aphid, *Brevicoryne brassicae* (Linnaeus) that have not been previously reported as PRSV vectors were also observed in small numbers at 1.02%, 1.15% and 0.55% respectively. Interestingly, *A. gossypii* (Glover) (64.22%), *M. persicae* (Sulzer) (9.88%) and *A. craccivora* (Koch) (9.66%) have been reported previously as major vectors of PRSV in India [20, 41]. And, *A. nerii* and *P. nigronervosa* that were also known previously as vectors of PRSV [42,43] were observed at small populations of 1.52 % and 0.82 % respectively. These observations are consistent with previous study by Cortez-Madrigal and Mora-Aguilera [44], who reported nearly 20 aphid species, of which *A. spiraecola* (80.51%), *Pentalonia nigronervosa* (1.67%), *A. gossypii* (1.52%), *A. craccivora* (1.36%), *A. fabae* (1.36%) and *Uroleucon* sp. (0.91%) as the major vectors of PRSV in a papaya orchard.

Table-1 *CP gene details of south Indian PRSV isolates obtained from NCBI GenBank*

Sl. No.	Isolate	Pathotype	Location	Accession no.	Host	Reference
1	PRSV-BLR	P	Bangalore (Karnataka)	-	Papaya	Present study
2	PRSV-CBE	P	Coimbatore (Tamil Nadu)	-	Papaya	Present study
3	PRSV-EKM	P	Ernakulam (Kerala)	-	Papaya	Present study
4	PRSV-AP	P	Andhra Pradesh	AF323637	NS	Unpublished
5	PRSV-TA Ti	P	Tiruvallur (Tamil Nadu)	DQ666641	Papaya	[14]
6	PRSV-KA Gu	P	Gulbarga (Karnataka)	DQ666639	Papaya	[14]
7	PRSV-KE-Ca	P	Calicut (Kerala)	DQ666640	Papaya	[14]
8	PRSV-HYD	P	Hyderabad (Telangana)	KP743981	Papaya	Unpublished
9	PRSV-AP-Ko	P	Kovvur (Andhra Pradesh)	DQ666638	Papaya	[14]
10	P-BR	P	Bangalore (Karnataka)	AF120270	Papaya	Unpublished
11	PRSV-AP-Te	P	Hyderabad (Telangana)	AY839864	Papaya	[14]
12	PRSV-AP-Ra	P	Rly Kodur (Andhra Pradesh)	AY839863	Papaya	[14]
13	PRSV-KA-Ho	P	Hospet (Karnataka)	AY839865	Papaya	[14]
14	KA-Dh	NS	Dharwad (Karnataka)	AY458618	NS	[7]
15	Pune3	P	Pune (Maharashtra)	KC149502	Papaya	Unpublished
16	TN-Tr	NS	Trichy (Tamil Nadu)	DQ077175	NS	Unpublished
17	TNAU	P	Coimbatore (Tamil Nadu)	HM626464	Papaya	Unpublished
18	Avinashi	NS	Tamil Nadu	HM454196	NS	Unpublished
19	PRSV Tamil Nadu	NS	Coimbatore (Tamil Nadu)	EF104919	NS	Unpublished
20	Annur	NS	Tamil Nadu	HM454197	NS	Unpublished
21	Ellampillai	NS	Tamil Nadu	HM754218	NS	Unpublished
22	Dharapuram	P	Tirupur (Tamil Nadu)	HM626467	Papaya	Unpublished
23	Sathyamangalam	P	Erode (Tamil Nadu)	HM626466	Papaya	Unpublished

NS: Not specified

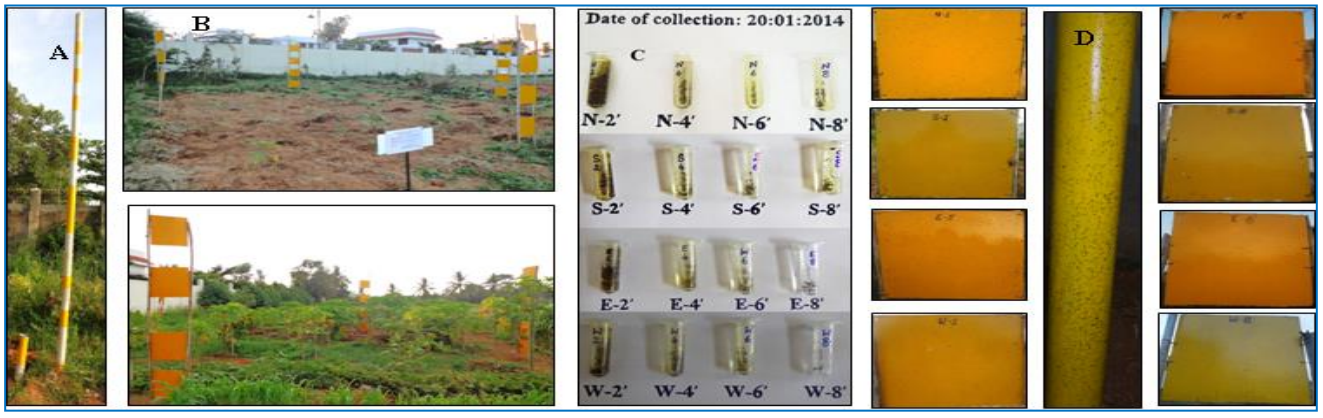

Fig-2 Monitoring of aphid vectors using yellow sticky traps for epidemiological studies

A. Yellow sticky trap installed at 15 feet height; B. Yellow sticky traps installed in N-S-E-W directions at 2, 4, 6, 8 feet in papaya orchard; C. Aphids collected from traps for identification; D. Aphids trapped on yellow sticky traps.

Table-2*Number and per cent contribution of different species of aphids trapped during August-2013 to July-2014*

Species	Aug-2013	Sep-2013	Oct-2013	Nov-2013	Dec-2013	Jan-2014	Feb-2014	Mar-2014	Apr-2014	May-2014	Jun-2014	Jul-2014	Total	Per cent contribution for PRSV infection
A. gossypii	1742	1202	1120	1447	1733	11418	4968	2767	1218	740	583	1248	**30186**	**66.04**
A. craccivora	800	575	635	572	998	3938	1499	1272	255	414	433	859	**12250**	**26.80**
A. nerii	46	41	55	44	60	135	90	125	11	17	35	35	**694**	**1.52**
A. bambusae	31	37	84	35	129	73	11	18	11	6	12	17	**464**	**1.02**
M. persicae	70	133	59	139	60	168	45	50	40	16	90	98	**968**	**2.12**
H. carduellinus	36	64	60	58	78	32	12	40	17	23	45	62	**527**	**1.15**
B. brassicae	10	24	21	24	25	18	19	29	44	2	8	26	**250**	**0.55**
P. nigronervosa	2	9	26	14	12	26	13	114	103	45	2	7	**373**	**0.82**
Total	**2737**	**2085**	**2060**	**2333**	**3095**	**15808**	**6657**	**4415**	**1699**	**1263**	**1208**	**2352**	**45712**	**100.00**

No. of traps installed = 32

Like many non-persistent virus transmission, PRSV transmission occurs by numerous species of aphids which do not colonize papaya [42]. Aphid species composition trapped in an area depends on the adjacent landscape including the extent of cropped area, diversity of crops, crop duration, cropping season etc. [20]. The crop diversity at MRS, Hebbal, Bangalore and surrounding area where this study was undertaken was characterized by the cultivation of oilseeds, cucurbits and legumes. *A. gossypii* is a common pest on these crops, while cowpea and beans have been regular hosts of *A. craccivora* and *M. persicae* is a polyphagous aphid species. On the other hand, the aphids such as *A. nerii*, *A. bambusae* and *Hyperomyzus* sp. habit non-host crops or weeds such as milkweed, bamboo and *Eupatorium* contributing to the diversity of vectors. Thus, weed composition potentially influences number and species composition of aphids in a papaya orchard.

b. PRSV incidence in relation to population dynamics of aphid vectors

After transplanting papaya seedlings to main field, the PRSV incidence was recorded at weekly intervals based on symptoms [Table-3]. It was noticed that disease incidence of PRSV (% DI) coincided with the major aphid vectors trapped after the first four weeks in yellow sticky traps suggesting a strong link between the aphid vector abundance and the PRSV incidence [Table-3]. The infection gradually increased from 13th week of transplanting and reached 100 per cent by 23rd week of transplanting. The number of major aphid vectors such as *A. gossypii*, *A. craccivora, M. persicae, A. nerii* and *P. nigronervosa* play a major role in increasing PRSV incidence. Highly significant positive correlation coefficient values of r=0.969**, 0.970**, 0.952**, 0.963** and 0.943**were obtained between per cent PRSV infection and cumulative number of *A. gossypii*, *A. craccivora, M. persicae*, *A. nerii*and *P. nigronervosa*, respectively [Table-4]. Although, none of the major aphid vectors colonized on papaya plants *A. gossypii*, *A. craccivora* and *M. persicae* that were regularly trapped in large numbers could be considered as potential vectors for large scale spread of PRSV. During the initial four weeks after transplanting no PRSV incidences were noticed. However, eventually the PRSV infection gradually increased as the number of trap catches increased. This could likely be due to three to four weeks of incubation period of PRSV in the fields [20, 42].

Non-persistent viruses are transmitted rapidly to number of plants in a manner related to the number of viruliferous vectors available [45]. The number of efficient vectors involved decides the epidemiology of PRSV incidence. Hence, a very high incidence of PRSV was observed between 14th week and 23rd week coincided with increased aphid population. These findings agree with previous studies by Cortez-Madrigal and Mora-Aguilera [44], who reported the occurrence of disease starting from 92 days after plantation with higher incidence after 260 days of plantation. The species composition of aphids involved in the field spread of PRSV under the South Indian field conditions discussed in this manuscript thus provide a systematic understanding of virus-vector and vector-crop relationship. Such epidemiological knowledge would be valuable in developing ecologically viable PRSV management strategies.

Table-3 *Weekly cumulative number of major vectors of Papaya ringspot virus (PRSV) contributing for increased per cent infection in papaya*

Month	Date of observation	Aphid species					No. of plants infected	Per cent infection
		A. gossypii	*A. craccivora*	*M.persicae*	*A. nerii*	*P. nigronervosa*		
Aug-13	1st week	408	212	23	11	0	0	0
	2nd week	830	421	42	27	0	0	0
	3rd week	1194	561	56	39	0	0	0
	4th week	1571	690	66	43	0	0	0
Sep-13	5th week	1860	859	90	51	4	2	4
	6th week	2146	1024	124	62	4	2	4
	7th week	2476	1202	150	74	4	5	10
	8th week	2712	1295	181	79	8	6	12
	9th week	2944	1375	203	87	11	8	16
Oct-13	10th week	3108	1485	208	94	18	9	18
	11th week	3337	1640	217	106	20	9	18
	12th week	3579	1774	226	123	24	11	22
	13th week	3918	1949	234	136	33	12	24
Nov-13	14th week	4225	2090	290	150	40	12	24
	15th week	4677	2295	329	166	44	18	36
	16th week	4985	2420	354	177	47	23	46
	17th week	5341	2523	388	181	50	26	52
Dec-13	18th week	5687	2712	405	194	54	30	60
	19th week	6092	2939	420	208	59	33	66
	20th week	6376	3123	430	219	59	36	72
	21st week	6618	3278	442	231	59	42	84
	22nd week	7244	3580	461	246	63	49	98
Jan-14	23rd week	8782	4129	500	256	64	50	100

No. of traps installed = 32

Table-4*Pearson's correlation coefficients and proportional contribution of major vector species of aphids on variation of per cent PRSV infection*

Pearson's Correlation		*A. gossypii*	*A.craccivora*	*M. persicae*	*A. nerii*	*P. nigronervosa*
PDI	Pearson's Correlation	0.969**	0.970**	0.952**	0.963**	0.943**
	N	23	23	23	23	23

** Correlation is significant at the 0.01 level (2-tailed)
N: Total number of weeks taken for 100% infection

III. Management of papaya ringspot virus disease

a. Integrated management approaches

The quick spread of the PRSV disease in the field necessitates the development of integrated management strategies. Hence, the present study was carried out to test different biological and physical methods on per cent disease control, per cent yield increase, growth and quality parameters of papaya. The consideration for choosing maize and banana as barrier crops is that these are not primary or secondary hosts of PRSV. Growing African Tall maize (1:1) [Fig-3]and Grand Naine banana (1:2) [Fig-4]as live barriers recorded 60-90 per cent disease control with maximum average fruits yield in Surya (15.78 kg and 14.34 kg/plant) and Red Lady (33.28 and 30.37 kg/plant) [Table-5& 6]. This is because, barrier crops have been shown to be effective in reducing virus transmission in crops by blocking aphids from reaching the target plant [21, 22]. Similarly, it was also concluded that intercropped maize barriers had a reducing effect on the incidence and final severity of PRSV in endemic areas [23, 24].

Since both the barrier crops can grow upto 10-11 feet, they can be suggested as intercrop with widely grown dwarf and semi dwarf varieties like, Red Lady (3-5 feet) and Arka Surya (5-7 feet), but cannot be recommended for tall variety like Sunrise Solo as it grows beyond 12 feet. During the peak aphid population (November to March) growing barrier crops across the wind direction (NE-E-SE) would be the best practice for avoiding viruliferous aphids into the main crop. These suggestions were made based on hypothesis provided byHooks and Fereres [46], who proposed that flora diversification can reduce the incidence of many non-persistent aphid-borne viruses. Because, non-host crops attenuate the spread of non-persistent viruses by avoiding influx of vector population on main crop [20]. Hence, barrier crops like Jowar/Maize should be sown densely along the perimeter one month before papaya transplanting. Previously, five rows of banana as border crop was shown to be effective in reducing aphid-vector population from 31 to 18 aphids/trap inside the papaya plantation when compared with outside the border crop [47].

Fig-3 Growing African Tall Maize with papaya as live barrier

Table-5*Effect of different treatments against Papaya ringspot virus (PRSV) and growth, yield, quality parameters of papaya var. Arka Surya*

Treatments	Av. plant height (feet)	Av. No. of fruits/plant	Av. yield/ plant	Yield increase (%)
T1: African Tall maize as live barrier (1:1)	7.4	29.9	15.78 (4.00)^{a}	91.90
T2: Grand Naine banana as live barrier (1:2)	7.6	25.1	14.33 (3.81)^{aa}	91.07
T3: Use of silver reflective mulch	7.1	20.8	12.17 (3.54)^{aa}	89.48
T4: Control (Untreated)	5.3	5.8	1.27 (1.29)^{e}	0.00
SEM ±	0.31	1.72	0.18	
CV (%)	14.02	28.79	18.77	
CD (5%)	0.88	4.83	0.52	

** Figures in parentheses are Sq.Rt. transformed values*

Fig-4 Growing Banana (Grand Naine) with papaya as live barrier

The physical approach of growing papaya with silver reflective row covers [Fig-5] recorded 90 and 100 per cent disease control in *var*. Arka Surya and Red Lady respectively [Table-7&8]. This may be because, reflective or floating row coverings delay the appearance of virus diseased plants by excluding or repelling the aphids by reflecting UV light [48, 49]. Compared to other colours of plastic mulches, silver reflective mulch has been observed superior in reducing aphid populations [50, 51]. Although, there are no reports of using reflective mulches in managing the PRSV in papaya, the plastic mulches have been used for control of *Papaya ring spot potyvirus* (PRSV-W) in zucchini (*Cucurbita pepo*) and it was found that the mulches with silver reflective surface reduced the hazard ratio from 1.0 in control to 0.32 by reducing aphid populations [52]. The 3–6 weeks of delayed onset symptoms of *cucumber mosaic cucumo virus*, *watermelon mosaic* and *zucchini yellow mosaic potyviruses* were also observed in plants growing over silver mulches [53].

Table-6 *Effect of different treatments against Papaya ringspot virus(PRSV) and growth, yield, quality parameters of papaya var. Red Lady*

Treatments	Av. plant height (feet)	Av. No. of fruits/plant	Av. yield/ plant	Yield increase (%)
T1: African Tall maize as live barrier (1:1)	4.5	10	33.27 (5.76)^{a}	92.27
T2: Grand Naine banana as live barrier (1:2)	3.9	9.6	30.37 (5.52)^{aa}	91.53
T3: Use of silver reflective mulch	4.4	7.1	24.64 (4.81)^{ab}	89.57
T4: Control (Untreated)	3.0	4.1	2.57 (1.66)^{e}	0.00
SEM ±	0.24	0.93	0.30	
CV (%)	19.11	46.00	23.29	
CD (5%)	0.68	2.63	0.85	

* Figures in parentheses are Sq.Rt. transformed values

Table-7*Effect of different treatments on PRSV incidence in papaya var. Arka Surya under field condition*

Treatments	Variety: Arka Surya*										No. of plants infected	Disease Control (%)
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10		
T1: African Tall maize as live barrier (1:1)	0.23	0.24	0.2	0.24	0.24	0.20	0.25	0.25	0.25	**0.38**	1	90.00
T2: Grand Naine banana as live barrier (1:2)	**0.31**	**1.39**	0.28	0.26	0.29	0.22	0.28	0.23	**0.41**	**0.49**	4	60.00
T3: Use of silver reflective mulch	**0.54**	0.2	0.23	0.17	0.15	0.17	0.16	0.19	0.16	0.15	1	90.00
T4: Control (Untreated)	**2.47**	**2.03**	**1.89**	**1.78**	**0.41**	**0.84**	**1.03**	**1.52**	**1.76**	**3.6**	10	0.00
Healthy	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.15	0.15	-----	-----
Buffer	0.10	0.12	0.14	0.13	0.10	0.12	0.10	0.10	0.12	0.10	-----	-----

Numbers in bold are plants infected with PRSV
* ELISA values

Table-8*Effect of different treatments on PRSV incidence in papaya var. Red Lady under field condition*

Treatments	Variety: Red Lady*										No. of plants infected	Disease Control (%)
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10		
T1: African Tall maize as live barrier (1:1)	0.23	0.19	0.18	**0.51**	0.16	0.17	**0.66**	0.15	0.2	0.17	2	80.00
T2: Grand Naine banana as live barrier (1:2)	**0.47**	0.21	0.2	0.2	0.22	0.22	0.22	0.24	**0.39**	**0.36**	3	70.00
T3: Use of silver reflective mulch	0.24	0.2	0.16	0.15	0.21	0.15	0.15	0.15	0.18	0.2	0	100.00
T4: Control (Untreated)	**1.09**	**1.15**	**0.77**	**1.45**	**1.89**	**0.44**	**3.23**	**3.01**	**0.39**	**0.63**	10	0.00
Healthy	0.15	0.16	0.14	0.15	0.15	0.15	0.16	0.15	0.15	0.16	-----	-----
Buffer	0.1	0.12	0.11	0.13	0.10	0.12	0.10	0.10	0.12	0.10	-----	-----

Numbers in bold are plants infected with PRSV
* ELISA values

Fig-5 T_3 -Use of silver reflective row mulch

b. Effect of foliar spray of seaweed extract (*Kappaphycus alvarezii)* in managing PRSV

The seaweed extracts have been previously shown to have beneficial effects on the overall growth and defense activity of plants. In this study the aqueous extract of a red seaweed, *Kappaphycus alvarezii*, in managing the PRSV disease of papaya was examined. The trial was conducted in a field at Kamalur village of Doddaballapura taluk in Bangalore Rural district. The trial was conducted in the 2016-2017 season. 60 days old seedlings of 'Red Lady' variety that were raised in an insect proof nylon mesh were used in this study. The seedlings were transplanted in the main field with a spacing of 6'X6'. The recommended package of practice was followed throughout the experiment. Five replicates of a set of 5 plants across different areas of the field were tagged as treated and untreated group for regular monitoring of growth parameters, disease symptoms and fruit yield. For every 15 days, all plants in the treated group received a foliar spray of aqueous extract of *K.alvarezii* at 4 mL/L dosage while the untreated control was sprayed with only water. The disease incidence was scored based on recording disease symptoms.

The plants that were treated with *K. alvarezii* extract were relatively taller in height with a dense foliar canopy compared to those in the untreated group. Moreover, the average number of fruits in case of the treated plants were higher than those in the untreated group. The average number of fruits per plant was 30 for treated group while the plants in the untreated group showed an average fruit setting of 15 [Fig-6]. Another striking difference was observed with respect to PRSV symptom expression. The PRSV incidence first appeared in the untreated plants and the percentage of infected plants slowly increased [Fig-6]. While in case of treated group disease onset was delayed and the percent incidence was relatively less than in the untreated group [Fig-6]. The most striking difference between the treated and the untreated group was with respect to the quality of fruits. Interestingly, despite showing symptoms of PRSV disease the fruits in the *K. alvarezii* treated group were less symptomatic and well formed.

The molecular details of the effect of *K. alvarezii* extract in delaying PRSV symptoms remain to be explored. However, it is likely that the sulphated oligosaccharides of *K. alvarezii* may serve a key role as defense elicitors. The oligosaccharides of *K. alvarezii* extract may activate plant's immune response like microbial elicitors such as bacterial peptidoglycans, flagellin, lipopolysaccharides and chitin of fungal cell wall that elicit MAMPs immune response [54,55]. Specific recognition of these elicitors and their subsequent transduction may trigger defense responses leading to downstream effects such as; thickening of plant cell walls, increased activity of defense enzymes and the production of phytoalexin like defense compounds etc. The brown algae derived laminarin was shown to stimulate phytoalexin accumulation in soybean seedlings [56-57], While the red algae derived sulphated carrageenans (kappa, iota, and lambda-carrageenans) have been reported to induce glucanase activity in *Rubus fruticosus* cell suspension cultures [58]. More recently, the red seaweed *Schyzimenia binderi* derived oligo-sulphated-galactan, poly-Ga, has been shown to induce long-term protection against TMV in tobacco plants [59].

During the process of cellular damage or infection induced necrosis, plant cells are known to produce few molecules that potentially activate plant's immune response termed as DAMPs (damage associated molecular patterns) immunity [60,61]. It is pertinent to note here that oligogalacturonides (OGs), the fragments of pectic polysaccharide, are well known DAMPs elicitors. The oligogalacturonide, a linear polymer of 1, 4-linked α-D galacturonic acid, was shown to bind to leucine rich repeat containing TLR (TLR2 and TLR4) receptors to induce immune

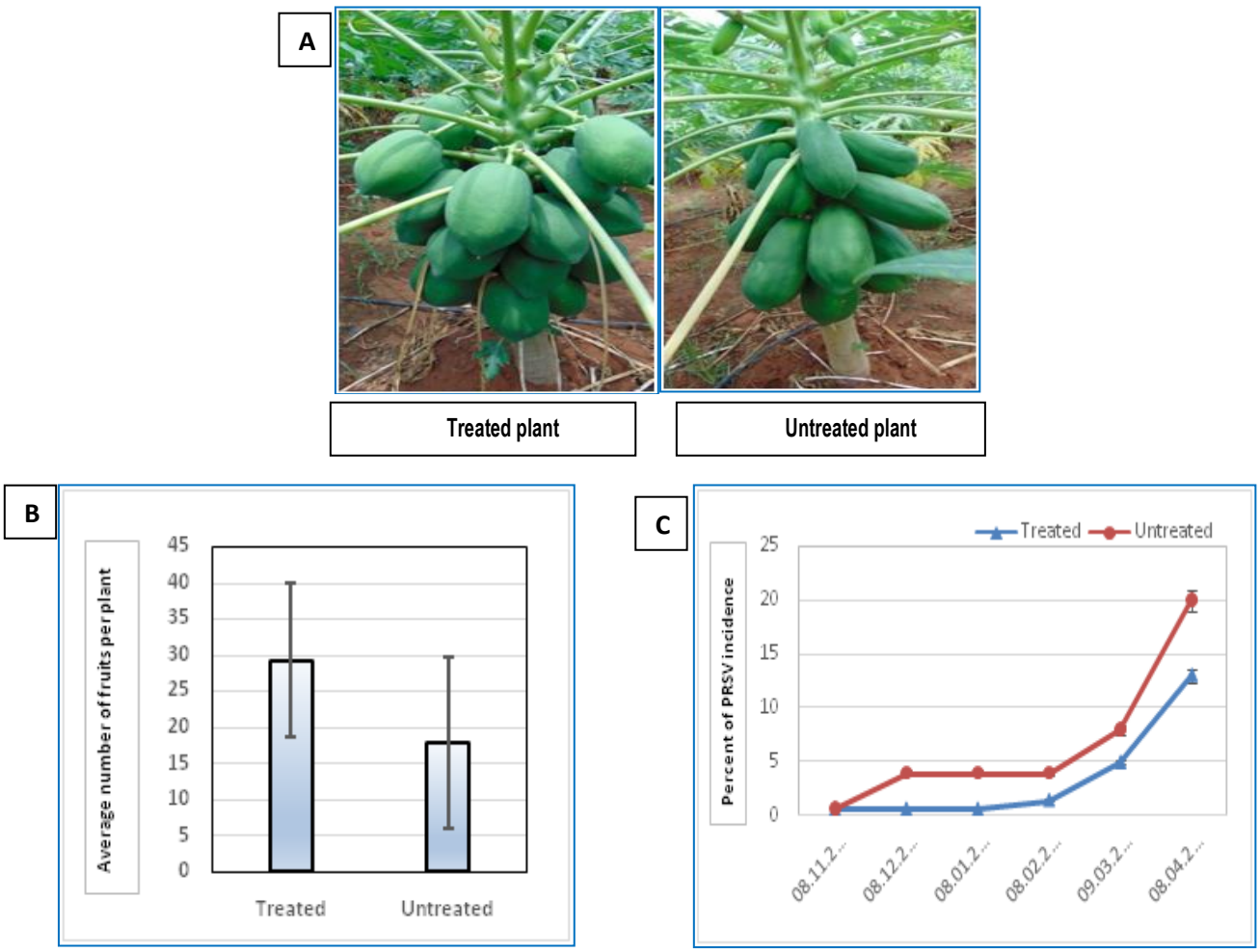

Fig-6 Effect of foliar spray of red seaweed extract *(Kappaphycus alvarezii)* in managing PRSV

A. Left panel shows picture of a plant in the treated group and on the right panel is the picture of a plant in the untreated group, notice the difference in the number of fruit setting.

B. Bar graph of average number of fruits in treated and untreated groups.

C. Percent of PRSV incidence plotted against different time points. The data recorded from 08-11-2016 to 08-04-2017.

responses such as MAPK activation, callose deposition, production of reactive oxygen species (ROS), elevated cytosolic Ca^{2+} and defense gene activation [62-64]. It is possible that the sulphated oligosugars or any unknown chemical compounds in *K. alvarezii* extract may mimic as DAMPs elicitors leading to activation of plant's immune response, however further studies in this direction are essential for detailed understanding of their molecular mechanisms.

The seaweeds typically grow in extreme conditions of high salinity, high temperatures and low light conditions. Because of such extreme growth conditions seaweeds have been reported to produce diverse secondary metabolites including antimicrobial, antifungal and antiviral compounds [27,28]. The polyphenol and flavonol metabolites in the seaweeds were reported to possess antioxidant activity. Thus, it is probable that the frequent foliar application of seaweed extract may either directly provide protection against the damages resulting from oxidative stress in PRSV infected cells or it may indirectly influence the activities of enzymes involved in maintaining cellular redox status. It is pertinent to note here that the brown seaweed extract was found to enhance antioxidant properties and tolerance to biotic and abiotic stresses [65]. The foliar spray of k-carrageenan on sweet basil parasitized with *Cuscuta campestris* was shown to improve the ROS (reactive oxygen species) scavenging enzyme activities and thereby reduce the ROS damaging effects [66].

Conclusion

Overall, the sequences of coat protein genes of three south Indian PRSV isolates discussed in this study provide insights towards understanding the PRSV genome variability in Indian conditions. The epidemiological studies suggest the dominant vector species and their impact on PSRV incidences. These insights would be valuable in designing strategies for aphid vector control in south Indian field conditions. The integrated practice described here emphasizes the beneficial effects of tall barrier crops in controlling PRSV spread. Further, the integrated management involving the growing of tall barrier crops combined with frequent foliar sprays of red seaweed extracts could serve as ideal strategy for effective management of PRSV in papaya orchards.

Application of research

The insights from the Coat Protein gene characterization studies would contribute to the understanding of the PRSV genome variability across the Indian state which is valued information towards designing the region specific transgenic papaya lines. Aphid vector diversity and PRSV management strategies examined in the current study would be beneficial to farming community directly towards effective management of PRSV in papaya under South-Indian field conditions.

Research Category: Plant Pathology and PRSV management

Abbreviations

MAMPs; Microbial Associated Molecular Patterns, DAMPs; Damage Associated Molecular Patterns, PRSV; Papaya RingSpot Virus, OG; oligogalacturonides, MAPK; Mitogen Activated Protein Kinase, MRS; Main Research Station, UAS; University of Agricultural Sciences, DEPC; Diethyl pyrocarbonate, DAS-ELISA; Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay, CP; Coat Protein. ROS; reactive Oxygen Species, TLR; Toll-like receptors.

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NCBI Submission
The nucleotide sequences of coat protein genes of PRSV-BLR, PRSV-CBE and PRSV-EKM isolates determined in the current study have been submitted to NCBI with submission ID of BankIt2077031, BankIt2077037, BankIt2077042 respectively.

Author Contributions: P.R.N., carried out epidemiology and integrated management studies, A.P carried out Coat Protein gene studies. Shantamma completed CP gene sequence analysis M.B. supervised the PRSV management studies using *K. Alvarezii* extact. S.K. prepared the Seaweed extract. R.K.T. helped to design the experiments and provided departmental support. T.R.G., S.B., Shantamma and N.N., designed and carried out PRSV management studies using Seaweed extract. N.N conceptualized and designed the experiments related to coat protein gene characterization, epidemiology and management studies. P.R.N., Shantamma, A.P., S.B., T.R.G. and N.N. contributed to manuscript writing.

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