International Journal of Plant & Soil Science

Volume 35, Issue 23, Page 97-105, 2023; Article no.IJPSS.110624
ISSN: 2320-7035

Distribution of *Papaya Ring Spot Virus* Infecting Papaya in Kerala, India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2023/v35i234221

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/110624

Original Research Article

Received: 09/10/2023
Accepted: 11/12/2023
Published: 16/12/2023

ABSTRACT

In the midst of the rising interest of papaya cultivation in Kerala, it is inevitable to study the current status of the Papaya ring spot disease in the state. Knowing the severity and distribution of PRSV isolates, causing ring spot disease, helps in the better formulation of effective management strategies against it. Purposive sampling surveys carried out in 2021-22 across five agro ecological units (AEUs) of Kerala, recorded papaya ring spot disease incidence (PRSD) ranging from 50 to 100 per cent in the cultivated areas with vulnerability index of 33.54 to 98.22. Highest disease incidence was recorded from AEU 8- Southern Laterites: Thiruvananthapuram (100.0%) and

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highest vulnerability index was recorded from Kalliyoor (98.22). Twenty symptomatic samples tested positive for PRSV in double antibody sandwich- ELISA (DAS-ELISA) using PRSV polyclonal antiserum. RT-PCR using coat protein gene specific primers RKJ 52 and 3 yielded amplicons of size approximately ~890bp in all the samples. Virulence of the Kerala isolates was evaluated based on the symptom expression, disease incidence and vulnerability index, upon mechanical transmission of PRSV on two months old papaya plants (variety- Red Lady). PRSV isolate from Kalliyoor (TVM1) inoculated on papaya plants expressed chlorosis, mottling, malformation of leaves and stunting with 96.80 vulnerability index. Maximum vulnerability index and severe symptoms including stunting in the inoculated plants were observed in isolates from southern and central Kerala, which include Thiruvananthapuram, Alappuzha and Thrissur had the most virulent PRSV isolates compared to north Kerala.

Keywords: Papaya ring spot virus; Agro Ecological Units (AEUs); DAS-ELISA; RT- PCR; Coat protein; vulnerability index.

1. INTRODUCTION

Papaya ringspot virus (PRSV) is a member of the family *'Potyviridae'* and the genus *'Potyvirus'*. The virus's propensity for mutation is likely a contributing factor to its widespread geographical distribution and thus it poses a significant challenge to papaya production worldwide, including the India [1]. PRSV isolates are primarily categorized into two serologically indistinct groups: PRSV-W, which infects plants in the *Chenopodiaceae* and *Cucurbitaceae* families, and PRSV-P, which additionally affects members of the *Caricaceae* family [2,3,4]. PRSV produces a range of symptoms such as leaf mosaic and chlorosis, water-soaked oily streaks on the petiole and upper parts of the trunk, distortion of young leaves leads to shoestring-like symptoms, stunting of infected plants and flower abortion. In addition to these symptoms, the infection is characterized by a typical ring spot symptom on fruits. When the plants are infected at nursery stage or early vegetative stage, infected trees do not produce fruits, whereas delayed infection leads to reduced yield with altered fruit quality causing losses ranging from 85.0 to 90.0 per cent [4]. Papaya ringspot disease (PRSD) was initially documented in the island of Ohau in the state of Hawaii by Parris [5]. Subsequently, Jensen [6] introduced the term *'Papaya ring spot virus*.' Since then, reports on the distribution and incidence of PRSD, caused by PRSV, have surfaced from nearly every country worldwide.

India is one of the largest producer and exporter of papaya in the world [7]. Incidence of PRSD in India was first reported by [8] and subsequently documented in various geographical regions across the country [9]. In Kerala, area of cultivation of papaya increased over the years due to the increased demand of papaya attributed to its high nutritional value, albeit the productivity decreased from 6.6 to 5.8 MT ha^{-1} during 2015-2018 [10]. In the midst of the rising interest of papaya cultivation in Kerala, it is inevitable to study the current status of the Papaya ring spot disease in the state. Knowing the severity, distribution of PRSV isolates helps in the better formulation of effective management strategies against it.

2. MATERIALS AND METHODS

2.1 Survey and Collection of Papaya Ring Spot Disease Affected Samples

Purposive Sampling surveys were carried out across five agro ecological units (AEUs) of Kerala, India and recorded the Per cent Disease incidence (PDI) and vulnerability index (VI) of papaya ring spot disease (PRSD).

$$\text{Per cent Disease incidence (PDI)} = \frac{\text{No. of plants infected}}{\text{Total No. of plants}}\ x\ 100$$

List 1. VI was calculated using the following scale [11]

Score	Description
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

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

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

Leaves from papaya plants showing symptoms such as vein clearing, mild mottle, leaf blistering and leaf distortion were collected from Kalliyoor, Venganoor, Balaramapuram, and Pallichal of Thiruvananthapuram district, Harippad and Kayamkulam of Alappuzha district, Muriyad of Thrissur district, Pattambi and Ottapalam of Palakkadu district as well as from Kinanoor-Karindalam and Kayyur- Cheemeni of Kasaragod district. The identity of the virus was confirmed by pathogenicity tests on papaya. The presence of PRSV in the different isolates was also verified by DAS-ELISA using polyclonal antiserum of PRSV-P. It was performed for detection of the virus isolates following the manufacturer's instruction (DSMZ Gmbh, Braunschweig, Germany).

2.2 RNA Isolation, RT-PCR and Sequencing

The total RNA from 100 mg of fresh healthy and PRSV-infected papaya leaf tissue was isolated using RNeasy plant mini kit according to the manufacturer's instructions (Qiagen). RT and PCR were performed in two tubes. First strand cDNA synthesis was performed using Verso cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Resulting cDNA was stored at -20°C. Later PCR was performed using TaKaRa PCR mastermix with a set of CP gene specific primers- HRP 52 and RKJ3 [1]. The mixture was subjected to one cycle of initial denaturation at 95°C for 2 minutes followed by 35 cycles of denaturation at 95 °C for 1minute, annealing at 52°C for 45 seconds, extension at 72°C for 2 minutes 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 and resulted in the expected PCR amplicon of ~890bp.

2.3 Screening for Virulence Amongst the PRSV Isolates

Two-month-old papaya seedlings of variety Red Lady were used for mechanical inoculations following standard protocols [12]. For inoculum preparation, 500 mg of fresh infected tissue were ground and homogenized in 5 ml phosphate buffer 0.05 M, pH 7.0 using a mortar and pestle. Two fully developed young leaves were dusted with silicon carbide (mesh 320) and rubbed with the homogenate using a cotton. For each PRSV isolate, five plants were inoculated. Inoculated plants were kept in an insect-proof greenhouse and monitored for symptom development. Vulnerability index and disease incidence were calculated as well as severity of symptoms were compared.

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3. RESULTS AND DISCUSSION

3.1 Distribution and Symptomatology of PRSD

Red Lady, the commercially popular variety known for its high yield and red flesh was found highly susceptible to PRSV in all the surveyed areas. The disease incidence ranged from 50 to 100 per cent; highest incidence of PRSD was recorded from AEU 8- Southern Laterites: Thiruvananthapuram (100.0%) followed by AEU 3- Onattukara Sandy Plain: Alappuzha, AEU 6- Kole Lands: Thrissur, AEU 10- North Central Laterites: Palakkkad and AEU11- Northern Laterites: Kasaragod respectively. The least sease incidence was recorded in the fields at Kayyur- Cheemeni (50.25%) followed by Badiyadkka (56.25%) and Chengala (60.33%). Kalliyoor (98.22) of Thiruvananthapuram district (AEU 8) recorded highest vulnerability index followed by Velukkara (95.69), Venganoor (93.25). Lowest VI was recorded from Badiyadkka (33.54) followed by Chengala (35.48). Studies conducted by Harish [13] has reported that papaya ring spot disease incidence in Thrissur district of Kerala ranged from 25 per cent (Muringoor) to 99.60 per cent (Vellikulangara) and the vulnerability index ranged from 39.33 (Muringoor) to 99.67 (Puthur). Our study is in concurrence with the above results wherein the continuous co-cultivation of papaya and cucurbits attributes to the high incidence and vulnerability. This may also lead to the persistence of PRSV inoculum in the fields.

There are previous reports from different parts of India on PRSV occurrence. Surekha et al. [14] recorded occurrence of PRSV in Udaipur of Rajasthan and Marathwada region of Maharashtra. Khurana and Bhargava [15] during their survey observed 75%–100% incidence of PRSV in and around Gorakhpur district of Uttar Pradesh. Occurrence of PRSV from other

regions of India, like Tamil Nadu [16], Andhra Pradesh, Himachal Pradesh, Jharkhand, and Karnataka Maharashtra [1], have also been reported previously. Disease incidence was noted from the southern part of West Bengal [1] and sub-Himalayan West Bengal [17]. Premchand et al. [18] observed to PRSV incidence ranged from 50.5 to 100.0 percent across different districts surveyed in Karnataka. But the reports on PRSV prevalence from Kerala is scanty.

Although PRSV occurs in different countries, higher levels of diversity were observed among Indian isolates compared to the rest of the world [19,20]. This might be due to a lack of resistant varieties, the fast evolution of the new strains of PRSV through recombination, and the occurrence of different aphid species [21].

Typical symptoms of PRSD recorded were chlorotic spots, mosaic appearance, wavy leaf margins, puckering of leaves, green islands, vein thickening, complete malformation of leaf, elongated oily streaks on stem, rosetting of leaves in canopy, stunting of infected plant, shoestring, circular ring spots on fruits, malformed fruits and uneven ripening of fruits *etc.* (Fig. 2). Plants which are PRSD affected in the seedling stage, exhibited severe symptoms like shoestring leaves, malformation of leaves, resetting of leaves, severe stunting and yield loss. Those plants got affected in the vegetative or reproductive stages showed mild symptoms like mild chlorosis and foliar symptoms. Prominent ring spots were seen on all the fruits produced on the infected papaya plants. In severe cases the fruits were malformed and caused uneven ripening and thus led to the reduced market value of papaya.

Similarly, Premchand et al. [18] observed that younger plants (below five months) at the pre-vegetative stage recorded only green mosaic symptoms. However, symptoms such as a yellow mosaic, leaf curling, stunted growth, puckering, mottling, blistering on leaves, and shoestring symptoms were common in plants in the pre-reproductive stage (above five months old plants). Singh and Shukla [22] reported that symptoms were typically ringspot and mild to severe distortion of leaf lamina. In some samples, foliage showed mild mottling visible as chlorosis. Deformed fruits with ring spots were found. Plants showed crowning of top leaves and denuded appearance due to defoliation, reduced fruit setting and also severe stunting in some areas. Srinivasulu and Saigopal [23] that all isolates collected from different parts of south India induced symptoms typical of PRSV infection on papaya. Isolates TA-Ti, KA-Gu, AP-Ra, AP-Te and KE-Ca induced mild mosaic symptoms. Whereas isolates AP-Ko and KA-Ho caused mild mosaic and slight leaf distortion symptoms. The observed symptoms were very much similar to that of previously reported PRSV isolates [17,24].

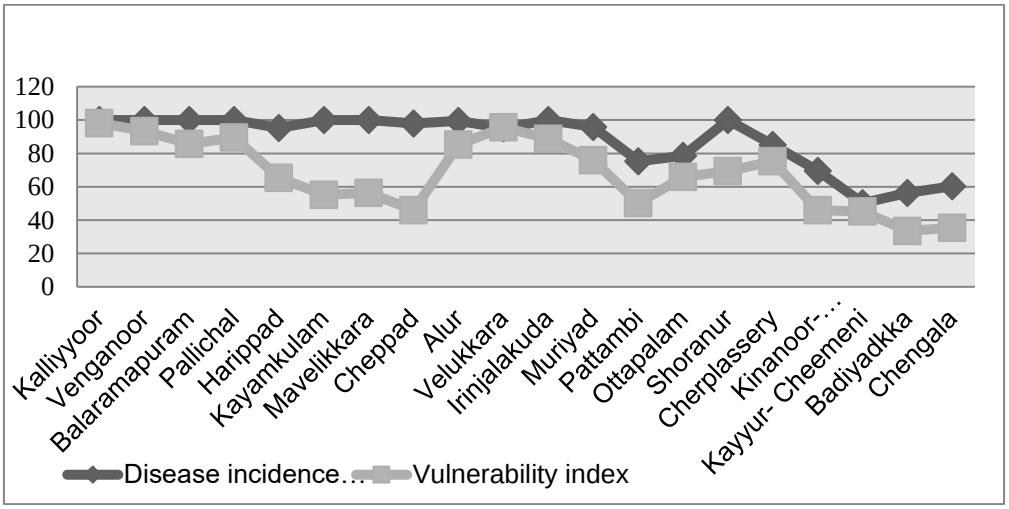

Fig. 1. Disease incidence and vulnerability index of PRSD recorded from surveyed areas

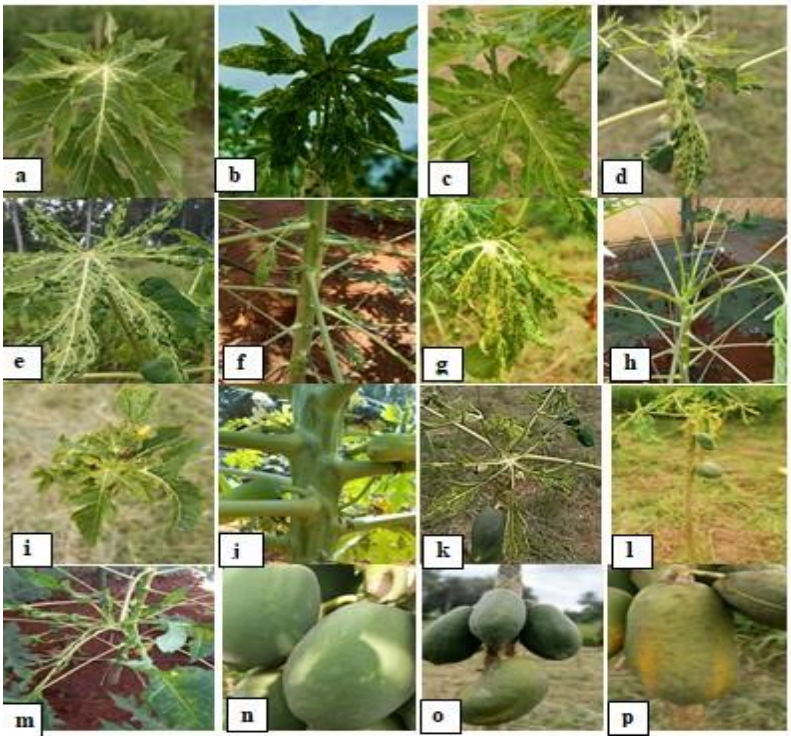

Fig. 2. Symptomatology; a. Chlorotic spots, b.Mosaic appearance, c. Wavy leaf margins, d. Puckering of leaves, e. Green islands, f. malformed young leaves, g. vein thickening, h. malformed apical leaves, i. complete malformation of leaf, j. Elongated oily streaks on stem, k. Rosetting of leaves in canopy, l. stunted plant, m. Shoestring, n. Circular ring spots on fruits, o. Malformed fruits, p. uneven ripening of fruits

3.2 Serological and Molecular Detection

All the samples tested positive in DAS-ELISA. Maximum absorbance value with highest titre of PRSV was observed in sample collected from Kalliyoor (1.181) followed by Venganoor (1.172) and Balaramapuram (1.121). All these three samples belonged to Thiruvananthapuram district of Kerala which also showed maximum disease incidence and VI. The lowest absorbance value was recorded in the sample collected from Kinanoor (0.436) followed by Badiyadkka (0.441) and Cherpulassery (0.452). Thus, isolates from north Kerala (Kasaragod (AEU11) and Palakkad (AEU 10) districts) recorded comparatively less absorbance values during ELISA which directs to the lower PRSV titer in the samples collected. The southern and central Kerala showed comparatively higher virus titer (AEU 3 (Thrissur), 6 (Allappuzha) and 8 (Thiruvananthapuram). This accounts for the higher disease incidence and vulnerability index in the southern Kerala.

All 20 symptomatic samples collected from different farmers' fields gave amplification in RT-PCR with CP gene specific primers (HRP 52, RKJ3) resulted in the expected PCR amplicon of ~890bp CP gene. Likewise in a study conducted by Singh and Shukla [22], all the collected samples from surveyed districts of eastern Uttar Pradesh (representing middle Gangetic plains of India) showed positive reaction for PRSV in DAC-ELISA. In a study carried out by Basawaraj et al. [25] in different climatic zone of India, amongst 41 papaya samples, 23 showed positive reaction with the PAbs to PRSV alone in DAC-ELISA. Srinivasulu and Saigopal [23] reported that all isolates from south India strongly reacted in DAC-ELISA with PRSV-W polyclonal antibodies. Pushpa et al. [26], reported that 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 and also the PCR reactions of Bangalore (PRSV-BLR), Coimbatore (PRSV-CBE) and Ernakulam (PRSV-EKM) isolates resulted a product of about 500 bp, a part of the coat protein gene while the Tirupati isolate (PRSV-TPT) did not result in a product.

Table 1. Absorbance values of collected Kerala PRSV isolates

Location	Absorbance at 405nm	Location	Absorbance at 405nm
Kalliyoor (TVM1)	1.181	Irinjalakuda (TCR4)	1.060
Venganoor (TVM2)	1.172	Pattambi (PKD1)	0.542
Balaramapuram (TVM3)	1.121	Ottapalam (PKD2)	0.453
Pallichal (TVM4)	0.981	Shoranur (PKD3)	0.494
Harippad (ALP1)	0.462	Cherpulassery (PKD4)	0.452
Kayamkulam (ALP2)	0.453	Kinanoor (KGD1)	0.436
Mavelikkara (ALP3)	0.458	Kayyur- Cheemeni (KGD2)	0.453
Cheppad (ALP4)	0.467	Badiyadkka (KGD3)	0.441
Muriyad (TCR1)	0.626	Chengala (KGD4)	0.461
Alur (TCR2)	0.527	NC	0.22
Velukkara (TCR3)	1.07	PC	1.114

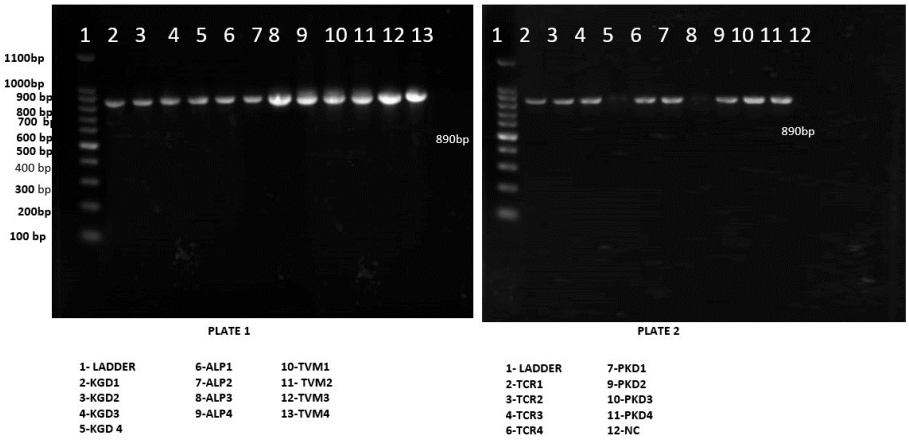

Fig. 3. Nucleotide detection using RT-PCR for CP gene specific amplification in the collected Kerala PRSV isolates

3.3 Screening for Virulence Amongst the PRSV Isolates

Virulence of the Kerala isolates was assessed based on the symptom production, disease incidence and vulnerability index upon mechanical transmission on 2 months old papaya plants. In the mechanically evaluated plants, highest vulnerability index was observed in the isolates from Thiruvananthapuram district of AEU 8. Maximum vulnerability index was recorded in isolates from Kalliyoor (TVM1) followed by Balaramapuram (TVM3), Pallichal (TVM4), Venganoor (TVM2) with VI values 96.80, 95.60, 95.60, and 93.45 respectively, which indicates that the isolates from Thiruvananthapuram district were the most virulent. Further Mavelikkara (ALP3) PRSV strain was more virulent with VI of 92.85 as well as showed chlorosis, mottling, malformation of leaves and stunting of the inoculated plants. PRSV isolates from Alur (TCR2) and Irinjalakuda (TCR4) of Thrissur district (AEU) also showed high virulence with VI of 91.50 and 92.54 respectively. These isolates also showed foliar symptoms and stunting in the inoculated plants.

The least virulence was shown by the Cherpulassery (PKD4) with milder foliar symptoms and lowest VI (56.34). This was followed by Badiyadkka (KGD3), Ottapalam (PKD2), Kayyur- Cheemeni (KGD2), Pattambi (PKD1), Chengala (KGD4) with VI values 64.75, 65.56, 65.89, 75.35 and 75.68 respectively. Severe symptoms including stunting was not observed in the inoculated plants for these isolates. All the isolates produced 100 per cent disease incidence except in Ottapalam (80%) and Badiyadkka (80%). Thus, these findings implied that the southern and Central Kerala, which includes AEU 3, 6, and 8 had the most virulent PRSV isolates compared to the central and northern Kerala with maximum VI and severe symptoms.

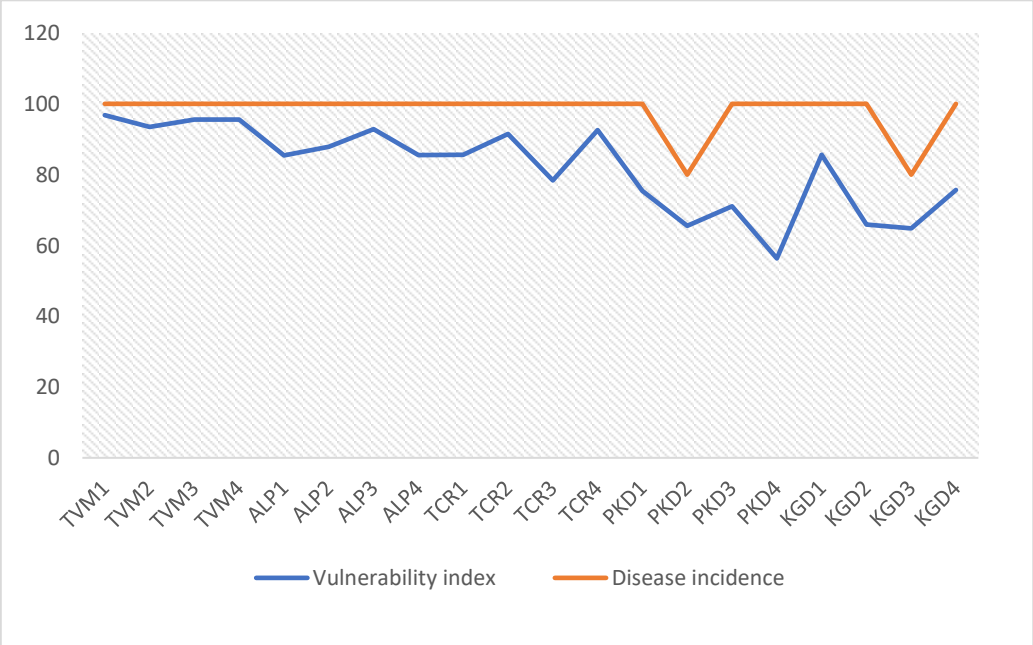

Fig. 4. Vulnerability index and disease incidence of the screened PRSV isolates for virulence evaluation

4. CONCLUSION

From the sampling surveys carried out in 2021-22 across five agro ecological units (AEUs) of Kerala, all the surveyed papaya fields had higher PRSV presence. The average disease incidence ranged from 50 to 100 per cent in the surveyed areas and the vulnerability index ranged from 33.54 to 98.22. All the 20 samples tested positive for PRSV in DAS-ELISA using PRSV specific antiserum and RT-PCR using CP gene specific primers RKJ 52 and 3. Northern Kerala with AEU 11 (Kasaragod) and AEU 10 (Palakkad) recorded comparatively less absorbance values during ELISA which directs to the lower PRSV titer in the samples collected. The southern and central Kerala showed comparatively higher virus titer (Thrissur, Alappuzha and Thiruvananthapuram). This accounts for the higher disease incidence and vulnerability index in the south and central Kerala. Also, south and central Kerala, which includes AEU 3, 6, and 8 had the most virulent PRSV isolates compared to the central and north Kerala with maximum vulnerability index and severe symptoms including stunting in the PRSV inoculated plants.

ACKNOWLEDGEMENTS

The current study is a part of the doctoral research program of the first author. The authors are thankful to Kerala Agricultural University for providing financial support and essential facilities for the timely completion of the research work.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/110624