Screening of *Carica papaya* x hybrids for resistance to papaya ringspot virus (PRSV).

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Abstract

***Carica papaya* x and intergeneric F1 hybrids of these species were screened for resistance to severely infected papaya ringspot virus isolates of papaya ringspot virus. Artificial screening for papaya ringspot virus was carried out 27 days after sap inoculation. Out of twenty nine F1 hybrid plants of CO 7 x *Vasconcellea cauliflora*, only six plants were found free from PRSV symptoms. Similarly, out of fifty five F1 hybrid plants of Pusa Nanha X *Vasconcellea cauliflora* only twenty three were found free from the symptoms and seventy plants out of 335 plants of CP50 x *Vasconcellea cauliflora* were found free from PRSV symptoms. The resistance of the hybrids and parents and their hybrids viz., CO 7 x, Pusa Nanha x and CP50 x *Vasconcellea cauliflora* were subjected to DAS ELISA test. ELISA titre value varied from 0.216 to 0.927. among the parents, the resistant male parent *Vasconcellea cauliflora* had recorded the lowest titre value of 0.216. However, the susceptible female parent CO 7 recorded the highest titre value of 0.972 followed by Pusa Nanha 0.952 and CP50 0.942. Among the hybrids ELISA titre value ranged from 0.218 to 0.29. Thus, the intergeneric hybrids and *Vasconcellea cauliflora* plants were resistant to PRSV. Molecular marker viz., ISSR markers were used to check and verify the hybridity. ISSR markers showed confirmity on three hybrid progenies viz., CO7V3, CO7V5 and CO7V6 from CO 7 x *Vasconcellea cauliflora*. Five female hybrid progenies viz., PNV3, PNV8, PNV9, PNV11and PNV13 and 3 male hybrid progenies viz., PNV1, PNV6 and PNV21 from Pusa Nanha X *Vasconcellea cauliflora* were confirmed through the ISSR markers. Three female hybrid progenies viz., CPV1, CPV23 and CPV56 and four male hybrid progenies viz., CPV12, CPV26, CPV31, and CPV39 from CP 50 x *Vasconcellea cauliflora* were also confirmed through the ISSR markers. Hybridity confirmed F1 hybrids are tolerant to PRSV at field conditions.**

Keywords: *Carica papaya, Vasconcellea cauliflora,* Intergeneric hybrids, Papaya ringspot virus.

Introduction

Papaya, a delicious fruit tree, is affected by number of diseases caused by various pathogens and viruses. Virus diseases are widespread and are very serious among the diseases damaging papaya. Most destructive disease of *Carica papaya* worldwide is papaya ring spot caused by papaya ring spot virus. Almost all cultivated varieties are found to be highly susceptible.

At present, it is cultivated throughout the world. India is the largest producer of papaya in the world has an area of about 98, 000 ha with an annual production of about 3629 million tonnes. In India, it is commercially cultivated in Andhra Pradesh, Gujarat, Maharashtra, Karnataka, West Bengal, Assam, Orissa, Madhya Pradesh, Manipur, Tamil Nadu and Bihar and certain extent in Kerala.

Papaya is affected by number of diseases caused by various pathogens and viruses. Nowdays the most destructive disease of *C. papaya* worldwide is papaya ring spot caused by papaya ring spot virus-type P, a definitive potyvirus species in the Potyviridae. PRSV is grouped into two types, Type P (PRSV – P) infects cucurbits and papaya and type W (PRSV-W) infects cucurbits but not papaya. Almost all cultivated varieties are highly susceptible. *Carica cauliflora* J., a wild species having non-edible fruits is known to be resistant for this viral disease. Now the species *Cauliflora* has been grouped under the genera *Vasconcellea.*

Incidence of PRSV has been reported to be more than 90 percent in India and rendering papaya orchards economically unviable. The results of the roving survey for papaya ringspot incidence in Karnataka revealed the presence of the disease in all the districts ranging from 75 to 100 per cent except Udupi, Hassan and Kodaku. In Tamil Nadu, the disease was first noticed in Coimbatore during 2003.

Trees infected with PRSV develop a wide range of symptoms which include ring spot on fruit, mosaic and chlorosis of leaf lamina, water soaked oily streaks on the stem and petiole

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and mottling and distortion of young leaves. Production and productivity are affected due to decreasing photosynthetic capacity of plant, which subsequently display stunted growth, deformed and inedible fruit, and leading to mortality due to this virus. When plants are infected at seedling stage or within two months after planting, the trees will not produce mature fruits. If trees are infected at later stage, fruit production is reduced along with poor quality of fruits because of ringspot on fruit with low sugar concentration[1-4].

Control measures to check the viral incidence against PRSV-P include cultural practices, cross-protection and planting of tolerant cultivars. None of these has been very successful and the development of virus resistant cultivars through conventional breeding is the only reliable tool for long term control. None of the *Carica papaya* cultivars has natural-resistance to PRSV-P. Several related wild species of Carica have been reported as resistant to PRSV-P. Even though interspecific hybridization of *Carica papaya* with other species attempted, a very little work has been done using *Vasconcellea cauliflora* which has the desirable gene for PRSV resistance. Under these circumstances, Screening of *Carica papaya* x *Vasconcellea cauliflora* hybrids for resistance to papaya ringspot virus (PRSV) is attempted.

Materials and Methods

Carica papaya and *Vasconcellea cauliflora* were produced *via* intergeneric hybridization. Seedlings thus raised were used for screening. All the seedlings were artificially inoculated with papaya ring spot virus through artificial inoculation method. The seedlings showing initial resistance alone were taken to field for further evaluation.

Mechanical inoculation of PRSV to parents, F1 progenies

One gram of infected leaves was ground in a pre-chilled mortar and pestle using 1 ml of 0.1M chilled sodium phosphate buffer (pH 7.2) containing β-mercaptoethanol and 0.01 M EDTA. The sap was rub inoculated using the pestle or glass rod on the young leaves of seedlings at 3 leaves stage previously dusted with carborundum powder 600 meshes [5-8]. After 5 minutes, the excess sap was washed off by distilled water. The disease incidence and intensity score was given using the scale developed. Details of the disease incidence and intensity score scale are presented in (Table 1).

Source of antiserum and positive sample

Antibody for PRSV and their positive samples were provided from DSMZ, Braunschweing, Germany. DAS-ELISA was performed for the detection of PRSV by following the manufacturer's instructions. Purified IgG was diluted in coating buffer (1:1000) and 200 µl was added to each well of a micro titer plate (Grainer). The plates were then incubated at 37oC for 2 to 4 hours and thereafter plates were washed with PBS-T using wash bottle, soaked for a few minutes and repeat washing for twice. Plates were blotted by tapping upside down on tissue paper. 200 µl aliquots of the test sample (extracted in sample extraction buffer) were added to duplicate wells. The plates were incubated overnight at 4oC. The plates were washed as in earlier and added with 200 µl of the anti-virus conjugate (1:500) to each well and incubated at 37°C for 2 hours. Then the plates were washed three times as done earlier. Finally, 200 µl of freshly prepared substrate (10 mg ρ-nitro phenyl phosphate (Sigma 104-105) dissolved in 10 ml of freshly prepared substrate buffer) was added to each well and incubated in dark at room temperature for 20 to 45 minutes or as long as necessary to obtain clear reactions. Spectrometric measurement of absorbance was then read at 405 nm (EL 800, BIO-TEK Instrument Inc., and USA). The reaction was stopped by adding 50 µl of 3 M NaOH. Buffer served as negative control.

Seedlings of three cross combinations viz., CO 7, Pusa Nanha, CP 50 and *Vasconcellea cauliflora* and their F1 progenies viz., CO 7 x *Vasconcellea cauliflora* (29 seedlings), Pusa Nanha x *Vasconcellea cauliflora* (55 seedlings) and CP 50 x *Vasconcellea cauliflora* (335 seedlings) were artificially inoculated with PRSV under glass house conditions. Apparently healthy six F1 hybrid seedlings of CO 7 x *Vasconcellea cauliflora*, 23 F1 hybrid seedling of Pusa Nanha x *Vasconcellea cauliflora* and 70 F1 hybrid seedlings of CP50 x *Vasconcellea cauliflora* along with parents (6 seedlings each) were transplanted in the main field for evaluation.

Cycling profile

Touch down protocol was followed for all the primers. Electrophoresis was performed in 1.5 per cent agarose with 120V for 2 hours. PAGE electrophoresis was carried out for SSR's silver staining protocol as performed.

Results and Discussion

Screening of F1 progenies through artificial inoculation against PRSV under glass house conditions. Intergeneric hybrid seedlings along with parents were raised and artificially inoculated with PRSV under glass house conditions for screening. Observation for PRSV was done 27 days after inoculation. Out of 29 intergeneric hybrid seedlings involving CO 7 x *Vasconcellea cauliflora*, six were found to be apparently free from the disease. Similarly in the cross combination Pusa Nanha x *Vasconcellea cauliflora*, out of 55 seedlings, 23 seedlings were found to be apparently free from PRSV. In the cross combination CP 50 x *Vasconcellea cauliflora*,

Table 1. *Scale of disease incidence and intensity score.*

Reactions	Intensity scores	Symptoms
Apparently healthy (AH)	0-1	0=No disease symptoms
Moderately resistant (MR)	1-2	1=Slight mosaic on leaves 2=Mosaic patches and / or necrotic spots on leaves
Moderately susceptible (MS)	2-3	3=Leaves near apical meristem deformed slightly, yellow, and reduced in size
Susceptible (S)	3-4	4=Apical meristem with mosaic and deformation
Highly susceptible (HS)	4 and above	5=Extensive mosaic and serious deformation of leaves, or plant death).

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out of 335 seedlings, 70 seedlings were apparently free from PRSV disease. However, all the parents except *Vasconcellea cauliflora* showed typical PRSV symptoms after artificial inoculation. In a perennial crop like papaya, field screening for diseases is very difficult since, it requires a larger area for planting. Hence, screening in glass houses in the nursery stage proved quick and rapid method.

A total number of 29 seedlings in CO 7 x *Vasconcellea cauliflora*, 55 plants in Pusa Nanha x *Vasconcellea cauliflora* and 335 plants in CP50 x *Vasconcellea cauliflora* were artificially inoculated with papaya ringspot virus through sap inoculation method. Typical PRSV symptom of mottling of leaves and water soaked lesions on stems were observed in the susceptible parents and the hybrids. However, six out of 29 seedlings in CO 7 x *Vasconcellea cauliflora*, 23 out of 55 in Pusa Nanha x *Vasconcellea cauliflora* and 70 out of 335 in CP 50 x *Vasconcellea cauliflora* were found to be completely free from PRSV symptoms. Regarding the female parents, all were found to exhibit the virus symptoms uniformly after sap inoculation. Symptom free F1 hybrids were transplanted in the main field for further evaluation. The failures of PRSV symptoms to develop on the manually inoculated hybrid plants indicate the incorporation of genes resistant to PRSV. Further, the wild genus *V. cauliflora* was found to be completely resistant to the strain PRSV prevalent in Coimbatore area of Tamil Nadu, India.

ELISA titre value for parents and F1 hybrids

The Enzyme Linked Immunosorbent Assay (ELISA), a powerful immunological test, is extensively used for detecting, identifying and quantifying viruses in many plant species. This test could be a component of a reliable method for screening *C. papaya* x *C. cauliflora* hybrid plants for PRSV resistance. In this study, the resistance of the hybrids and parents was assessed by serological test. Parents and their hybrids viz., CO 7 x *Vasconcellea cauliflora*, Pusa Nanha x *Vasconcellea cauliflora* and CP50 x *Vasconcellea cauliflora* were subjected to DAS- ELISA test [9-11].

Parents and F1 progenies involving CO 7 and *Vasconcellea cauliflora* were subjected to DAS-ELISA test. ELISA titre value varied from 0.216 to 0.972. Among the parents, the resistant male parent *Vasconcellea cauliflora* had recorded the lowest titre value of 0.216. However, the susceptible female parent CO 7 recorded the highest titre value of 0.972, followed by Pusa Nanha (0.952) and CP 50 (0.942). Among the hybrids involving CO7 and *Vasconcellea cauliflora*, ELISA titre value varied from 0.243 to 0.266. Among them, the cross combination CO7V3, confirmed hybrid through molecular markers, was found to record the lowest titre value of 0.243 followed hybrid CO7V5 (0.245) and CO7V6 (0.247). Among the hybrids involving Pusa Nanha x *Vasconcellea cauliflora*, ELISA titre value varied from 0.218 to 0.286. Among the hybrid combinations, the combinations PNV3 and PNV9 recorded the lowest titre value of 0.218 followed by PNV1 (0.219), PNV6 (0.220), PNV11 (0.220), PNV8 (0.222) and PNV13 (0.223). All the above said hybrid combinations were confirmed as true hybrids through molecular marker studies (Table 2).

Among the hybrids involving CP50 x *Vasconcellea cauliflora*, ELISA titre value varied from 0.218 to 0.299. Among them, the cross combination CPV23 a confirmed hybrid through molecular markers, was found to record the lowest titre value of 0.218 followed by other confirmed hybrids viz., CPV56 (0.219), CPV39 (0.220), CPV31 (0.221), CPV1 (0.222), CPV26 (0.226) and CPV12 (0.232). The results revealed that the lowest value of 0.216 was recorded by the resistant male parent *Vasconcellea cauliflora,* however; all the female parents used for this study recorded very high titre values proving their susceptibility. *V. cauliflora* registered the lowest titre value which clearly indicated its natural resistance to PRSV. They also reported that *V. cauliflora* is resistant to all the strains of PRSV which are prevalent in Coimbatore conditions.

Hybridity confirmation using ISSR markers

Three intergeneric hybrids of CO 7 x *Vasconcellea cauliflora* crosses out of six, eight intergeneric hybrids of Pusa Nanha x *Vasconcellea cauliflora* crosses out of 23 and seven intergeneric hybrids of CP 50 x *Vasconcellea cauliflora* crosses out 70 were tested for hybridity. The primer UBC - 856 produced unique banding patterns in *Vasconcellea cauliflora* (male parent) in which five bands were prominent, out of which third and fifth were absent in female parent [Figure 1] but present in CO 7 x Vasconcellea cauliflora (CO7V3). The same primer produced distingusible band between Pusa Nanha x *Vasconcellea cauliflora* (PNV9) which was used for the identification of true hybrid (Figure 1).

Lane 1. CO 7-Female; Lane 2. CO7V3-Hybrid; Lane 3. *Vasconcellea cauliflora*-Male; Lane 4. Pusa Nanha- Female;

Table 2. *ELISA titre value for parents and F1 population involving CO7 (apparently free from PRSV after inoculation).*

	Parents and their	
Sl.No	**Hybrids**	**OD value at 405nm**
1	*Vasconcellea cauliflora*	0.216
2	CO 7	0.972
3	Buffer	0.102
4	CO7V1	0.266
5	CO7V2	0.259
6	CO7V3	0.243
7	CO7V4	0.261
8	CO7V5	0.245
9	CO7V6	0.247
CO 7V (CO 7 x *Vasconcellea cauliflora)*		

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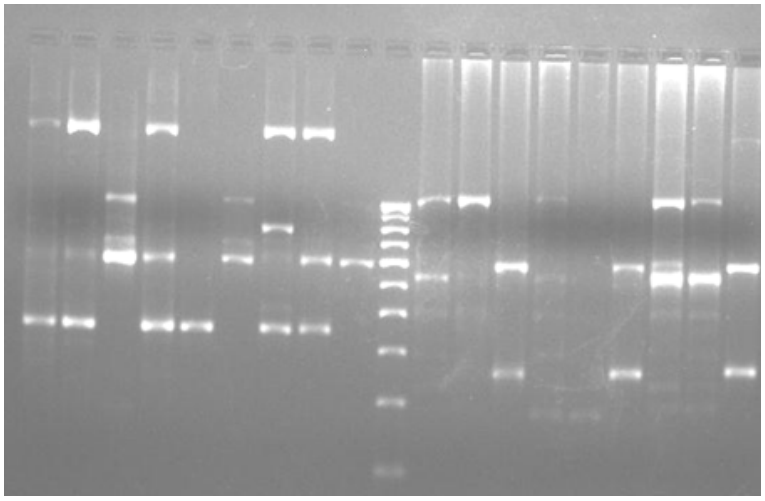

Figure 1. *ISSR marker profile for parents and F1s.*

Lane 5. PNV9-Hybrid; Lane 6. *Vasconcellea cauliflora*-Male; Lane 7. CP 50-Female; Lane 8. CPV23 – Hybrid; Lane 9. *Vasconcellea cauliflora*-Male; Lane 10. 100 bp ladder; Lane 11. CO 7-Female; Lane 12. CO7V3-Hybrid; Lane 13. *Vasconcellea cauliflora*-Male; Lane 14. Pusa Nanha-Female; Lane 15. PNV9 - Hybrid; Lane 16. *Vasconcellea cauliflora*-Male; Lane 17. CP 50-Female; Lane 18. CPV23-Hybrid; Lane 19. *Vasconcellea cauliflora*-Male.

Conclusion

These primers were helpful to identify F1's in cross CO 7 x *Vasconcellea cauliflora* (CO7V3, CO7V5 and CO7V6), Pusa Nanha x *Vasconcellea cauliflora* (PNV1, PNV3, PNV6, PNV8, PNV9, PNV11, PNV13 and PNV21) and CP 50 x *Vasconcellea cauliflora* (CPV1, CPV23, CPV12, CPV26, CPV31, CPV39 and CPV56). The hybridity confirmed F1 plants were forwarded to F2.

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