

## Original Research Article

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## Detection and Molecular Characterization of Black Spot Disease of Papaya (*Carica papaya* L.) Incited by *Asperisporium caricae* (Speg.) Maubl

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### ABSTRACT

#### Keywords

Papaya, black spot, Molecular detection, *Asperisporium caricae*

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Black spot of papaya caused by *Asperisporium caricae* incidence was recorded in five different localities viz., Pasur, Telungupalayam, Thondamuthur, Vedapatti and TNAU orchard of Coimbatore district. Per cent disease severity (PDS) ranged from 1 to 29 per cent on leaves. The fungal pathogen associated with black spot of papaya was isolated and identified as *Asperisporium caricae* based on morphological characters. Severe disease incidence was observed from November to January and no disease incidence was recorded during summer. Pathogenicity test was proved on young papaya plants under glasshouse conditions twenty days after inoculation. Molecular confirmation of *Asperisporium caricae* through 18S rRNA gene sequencing gave an amplicon size of 560bp size. The isolate *Asperisporium caricae* was sequenced and submitted to NCBI database with the accession number MK879405. The present study was aimed to characterize the pathogen responsible for black spot of papaya and confirmation by molecular methods.

### Introduction

Papaya (*Caricae papaya* L.) is one of the most important fruit crop cultivated in many parts of world, rich in vitamins, minerals and digestive fibres required for human health. It belongs to the family Caricaceae which is cultivated mostly in tropical and subtropical regions. India stands first in papaya production which is cultivated over an area of 1.39 million hectares with production of 5831.00 Metric Tonnes (MT). Among the states, Andhra Pradesh occupied highest

production of 1288.58 Metric Tonnes followed by Gujarat (1241.25 MT) and Karnataka (527.86 MT) (Hort stat.2017). In Tamil Nadu the crop is cultivated in an area of 1.76 thousand hectares with the production of 403.19 MT in 2018. Popular varieties widely cultivated are Red lady, Sinta and Co 8.

Papaya crop is being affected by many fungal diseases (Anthracnose, powdery mildew, black spot, and brown spot), Bacterial diseases (Papaya dieback, Bacterial leaf

spot) and viral diseases (Leaf curl, Papaya ring spot, Mosaic). Among the diseases black spot of papaya was the recent emerging disease which was first observed in the variety “Honey dew” at Chettali, Karnataka and later it was observed in Co 1 variety in palani hills during cooler months (Ullasa *et al.*, 1978). Symptomatology of black spot of papaya were initially appeared on the upper surface of leaves as small water soaked spots, later these spots become greyish white in color and the corresponding lower side the leaves develop black velvety conidial mass around the lesions. Severely affected leaves shrivel, develop larger necrotic lesions, become brittle and finally die.

This leads to severe defoliation of the older leaves. In the fruits the symptoms were small dot like spots appear initially, later they enlarge up to 2-6 mm in diameter. The spots are confined to the outer rind of the fruit which reduce the market value of exported fruit (Thiribhuvanamala *et al.*, 2016). With this background information, the present study was carried out to isolate and characterize the papaya black spot pathogen *Asperisporium caricae*.

## **Materials and Methods**

### **Assessment of disease incidence**

Black leaf spot of papaya were recorded in five different localities of Coimbatore district during 2018-2019. Disease incidence was recorded in Thondamuthur (10.5923°N and 76.50274°E), Pasur (11.2732°N and 77.1204°E), Telungupalayam (10.5935°N and 76.5556°E), Vedaipatti (11.0849°N and 76.5332°E) and TNAU orchard (11.01038°N and 76.9333°E) in the cultivars Co 8 and Red lady. The per cent disease severity was determined using standard scoring system developed by Inglis *et al.*, 1988 using 0-5 scale.

The proportion of leaf area showing symptoms were recorded using a scale of 0 to 5 as follows, 0= No disease, 1= 1-10% leaflet area with lesions, 2 =11-25% leaflet area with lesions, 3= 26-50% leaflet area with lesions and limited chlorosis, 4= Over 50% or more of the leaflet area with lesions and extensive necrosis, 5= Defoliation.

### **Isolation and purification of pathogen**

The fungal pathogen was isolated from the black spot infected leaves of papaya var “Red lady” showing typical symptoms.

The infected leaves were cut into small pieces of 1.0-1.5 cm, surface sterilized with 0.1 % sodium hypochloride for one minute and washed thrice in the double distilled water.

The sterilized leaf bits were dried on the sterilized filter paper. The dried infected leaf bits were dried in a Petriplate containing potato dextrose agar amended with antibiotic. The culture plates were incubated at 24°C with a 12 h light/dark light conditions.

### **Morphological characterization of *A.caricae***

The pure culture of *A. caricae* was sub cultured on PDA medium on Petri dishes (90 mm in diameter) for phenotypic and growth characters. Colony colour and texture observations were recorded at twenty days after incubation. The colony character and conidia morphology of the isolated fungus were studied (Minnis *et al.*, 2011).

The conidia were scraped from infected leaves and mounted in lactophenol solution on a microscopic slide. Measurements of the conidia were made using a light microscope (Labomed-IVU 5100) and photographed using a Labomed camera model LX400 with an image analyser – pixelpro programme.

## Pathogenicity

Red lady papaya seedlings were raised under glasshouse condition in Tamil Nadu Agricultural University. For inoculation, 30 days old mycelium was carefully scrapped from culture plate and suspended in a sterile distilled water. The final concentration of conidia was adjusted to  $5 \times 10^5 \text{ ml}^{-1}$ .

The conidial suspension of 10 ml was taken and diluted in sterile water and it was applied on three month old papaya seedlings using hand sprayer during evening hours and the control plants were sprayed with sterile distilled water.

After inoculation, the plants were covered with pin pricked polyethene bags for 48 hours to maintain 80 per cent relative humidity. The symptoms were visible after 20-24 days of inoculation (Cumagun and Padilla, 2007).

## Molecular characterization

The fungus was subjected for molecular characterization by sequencing the 18S-28S rRNA genes. The genomic DNA was extracted by Cetyl Trimethyl Ammonium Bromide (CTAB) method. (Minnis *et al.*, 2011)

## Amplification by ITS universal primers

The genomic DNA was used as a template and subjected for PCR amplification of 18S-28S rRNA gene. The primers used were ITS1 (5'-AGAGTTTGATCCTGGCTCAG-3') and ITS4 (5'GGTACCTTGTTACGACTT-3') with an initial denaturation of 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 60 sec, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 3 min in nexus gradient Mastercycler showed the amplicon size of approximately 560 bp. (Minnis *et al.*, 2011)

## Results and Discussion

### Survey for the assessment of black spot of papaya

Field visits were conducted at the five different locations of Coimbatore district. Highest per cent disease severity of black spot was observed in Pasur *var* "Red lady" with a maximum disease severity of 31.67 PDI on leaves followed by 30.62 PDI in TNAU orchard. Lowest incidence was recorded in Thondamuthur with a PDI of 16.67 at "CO 8" papaya. Older leaves were found to be more susceptible than younger leaves. The pathogen responsible for this disease was isolated from the infected samples and it was confirmed as *A.caricae* which causes black spot. On the upper surface of the leaves a white spot with yellow margin was observed corresponding to the lower surface black colour spot was observed (Fig. 1a; 1b). Infection on the matured fruit showed black spots confined to the outer rind of fruit which reduces the market value (Fig. 1c).

### Cultural characters

The colonies on potato dextrose agar were near dark green to black in colour. Mycelium formed a raised mound covered with whitish, short erect hyphae or whitish aerial hyphae. Surface slightly velutinous with scattered black spherical structures. Hyphae are branched with walls smooth, hyaline to brownish, septate, 3–6.5  $\mu\text{m}$  diameter. The conidial production on potato dextrose agar were sparsely distributed. Sporulation usually occurred after a month at 24 °C with a 12 h light/ dark regimen.

### Morphological characters

Black spots are fruti colous scattered of 3-4 mm diam. Sporodochia were immersed on stromata. Sporodochia were erumpent,

punctiform, blackish in colour. Brownish septated hyphae of 3-6.5 µm diameter. On the darkly pigmental stromata, the sporodochia were arranged densely to loosely. Conidiophores were simply straight branched, brown coloured with smooth septum. Conidiogenous cell occurs at terminal portion

of conidiophores which were dark pigmented, cylindrical to clavate, polyblastic in nature. Conidia were initially hyaline later turn brown, 0-2 septate with verrucose margin. Hilum were thickened with dark in nature, usually present as base of conidia.

**Table.1** List of surveyed areas of severity of black spot of papaya

| S.no | Place of survey | Latitude               | Longitude              | Variety  | Age (months) | Per cent severity on leaves |
|------|-----------------|------------------------|------------------------|----------|--------------|-----------------------------|
| 1    | TNAU, orchard   | 11.0103 <sup>0</sup> N | 76.9333 <sup>0</sup> E | Co 7     | 4            | 23.33                       |
| 2    | TNAU, orchard   | 11.0103 <sup>0</sup> N | 76.9333 <sup>0</sup> E | Red lady | 6            | 30.62                       |
| 3    | TNAU, orchard   | 11.0103 <sup>0</sup> N | 76.9333 <sup>0</sup> E | Co 8     | 5            | 18.33                       |
| 3    | Pasur           | 11.2732 <sup>0</sup> N | 77.1204 <sup>0</sup> E | Red lady | 4            | 31.67                       |
| 4    | Telungupalayam  | 10.5935 <sup>0</sup> N | 76.5556 <sup>0</sup> E | Red lady | 5            | 25.00                       |
| 5    | Thondamuthur    | 10.5923 <sup>0</sup> N | 76.5027 <sup>0</sup> E | Co 8     | 5            | 16.67                       |
| 6    | Vedaipatti      | 11.0849 <sup>0</sup> N | 76.5336 <sup>0</sup> E | Red lady | 3            | 21.67                       |

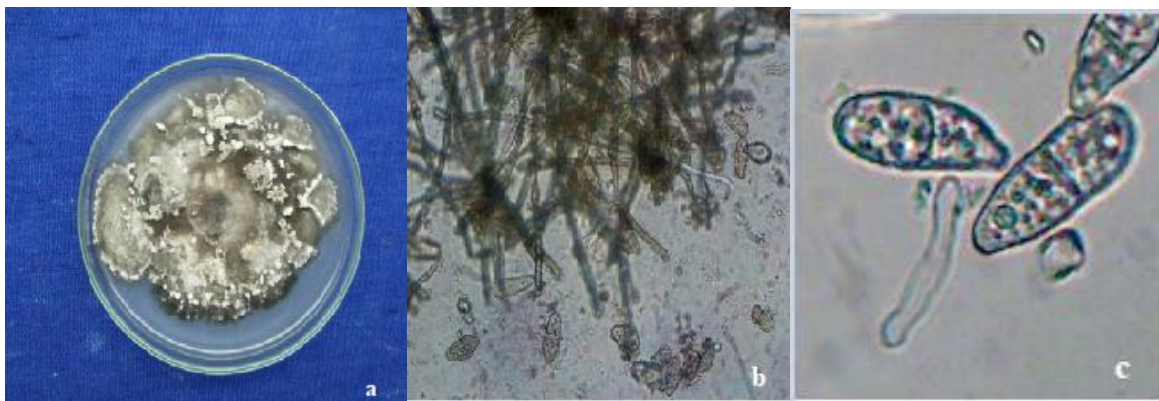
**Table.2** Pathogenicity test of *Asperisporium caricae* and symptom development on leaves up to 42 days

| Sl.no | Variety         | Disease severity %       |                            |                             |                             |                              |                           |
|-------|-----------------|--------------------------|----------------------------|-----------------------------|-----------------------------|------------------------------|---------------------------|
|       |                 | 7 DAI                    | 14 DAI                     | 21 DAI                      | 28 DAI                      | 35 DAI                       | 42 DAI                    |
| 1     | Co 7            | 0<br>(0.71) <sup>c</sup> | 0<br>(0.71) <sup>c</sup>   | 3.33<br>(1.96) <sup>c</sup> | 6.67<br>(2.68) <sup>c</sup> | 8.33<br>(2.97) <sup>c</sup>  | 18<br>(4.30) <sup>c</sup> |
| 2     | Co 8            | 0<br>(0.71) <sup>d</sup> | 0<br>(0.71) <sup>d</sup>   | 1.67<br>(1.47) <sup>d</sup> | 3.33<br>(1.95) <sup>d</sup> | 6.67<br>(2.68) <sup>d</sup>  | 15<br>(3.93) <sup>d</sup> |
| 3     | Red lady        | 0<br>(0.71) <sup>a</sup> | 2.8<br>(1.82) <sup>a</sup> | 6.67<br>(2.68) <sup>a</sup> | 10<br>(3.24) <sup>a</sup>   | 11.66<br>(3.49) <sup>a</sup> | 24<br>(4.95) <sup>a</sup> |
| 4     | Coorg honey dew | 0<br>(0.71) <sup>b</sup> | 2.2<br>(1.64) <sup>b</sup> | 5<br>(2.34) <sup>b</sup>    | 8.57<br>(3.01) <sup>b</sup> | 11.42<br>(3.45) <sup>b</sup> | 20<br>(4.52) <sup>b</sup> |
|       | SD              | -                        | 0.006                      | 0.009                       | 0.010                       | 0.011                        | 0.014                     |
|       | CD              | -                        | 0.32                       | 0.40                        | 0.131                       | 0.15                         | 0.16                      |

**Fig.1** Black spots on the lower surface of the leaf, (b) White spots with yellow margin on the upper surface of leaf (c) Black spots on the fruits



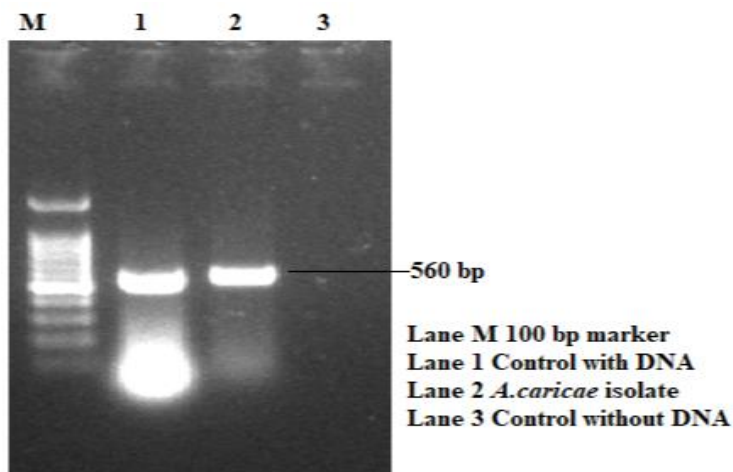
**Fig.2** Morphological and cultural characteristics of *Asperisporium caricae*.(a) Colony of *Asperisporium caricae* on PDA (b) Mycelium of *A.caricae* (c) Conidia and Spermata



**Fig.3** Pathogenicity test (b) symptom expression on the upper surface of leaf 20 DAI (c) symptoms on the lower surface the leaf at 20 DAI



Fig.4 PCR amplification of 18S-28S rRNA gene of *A.caricae*



### Pathogenicity

In order to prove the Koch's postulates, *A.caricae* isolate "Asp 1" was used to prove pathogenicity on two months old red lady papaya variety. Spore suspension was sprayed on the leaves and the inoculated plants were incubated in moist conditions by covering the plants with polythene covers (Fig. 3a). Inoculated plants showed typical yellow chlorotic lesions on the upper surface of the leaves twenty days after inoculation (Fig. 3b). Later the chlorotic lesions turned to white spots with yellow margin. On thirty days after inoculation numerous black spores were found on the lower surface which appeared as black spots (Fig. 3c).

### Amplification by universal primers

The *Asperisporium caricae* were subjected to polymerase chain reaction (PCR) for confirmation of pathogen at genus level using universal primers such as ITS 1 and ITS 4 with reaction mixture of 40 µl. The isolate showed the amplicon size of approximately 560 bp (Fig. 4). The isolate *Asperisporium caricae* was further sequenced and the same was submitted to NCBI Genbank. The accession number assigned was MK879405

In conclusion, the results of current study would be useful to fix parameters for exact identification of symptoms of black spot of papaya. Molecular confirmation could be made with universal primers ITS 1 and ITS4

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