Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(4): 3434-3437
Received: 19-05-2020
Accepted: 21-06-2020

Mahesh M Chaudhary
Ph.D. Scholar, Department of Plant Pathology, C. P. College of Agriculture, S. D. Agricultural University, S. K. Nagar, Uttar Pradesh, India

DS Patel
Department of Plant Pathology, C. P. College of Agriculture, S. D. Agricultural University, S. K. Nagar, Uttar Pradesh, India

Dinesh H Chaudhary
Polytechnic in Agriculture, Navsari Agricultural University, Vyara, Gujarat, India

Sandip B Dighule
Department of Plant Pathology, C. P. College of Agriculture, S. D. Agricultural University, S. K. Nagar, Uttar Pradesh, India

Corresponding Author:
Mahesh M Chaudhary
Ph.D. Scholar, Department of Plant Pathology, C. P. College of Agriculture, S. D. Agricultural University, S. K. Nagar, Uttar Pradesh, India

Isolation and characterization of fungi associated with deterioration of papaya fruits

Mahesh M Chaudhary, DS Patel, Dinesh H Chaudhary and Sandip B Dighule

Abstract
The isolated eight fungi were found associated with fruit rot of papaya and differentiated in cultural and morphological characteristics. The fungi were identified on the basis of cultural and morphological characters. The fungi were *Colletotrichum gloeosporiodes*, *Graphium* sp., *Fusarium pallidoroseum*, *Alternaria alternata*, *Fusarium solani*, *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus microspora*. The pathogenicity of isolated fungi was proved by knife injury, cork borer injury, and pin prick injury methods on papaya fruits.

Keywords: Fungi, morphological character, pathogenicity, symptomatology

Introduction
Papaya (*Carica papaya* L.) is an important fruit crop, which belongs to family *Caricaceae. Carica* is the largest group of the four genera with 48 species, among which *Carica papaya* L. is most important and cultivated all over the world (Badillo, 1971 and Waller, 1992) [1, 2]. The popularity of papaya fruit has made it ubiquitous in tropical and subtropical regions of the world. Papaya is the native of tropical America (Singh, 1990) [3]. It is now commercially grown in many parts of the world like Hawaii, Taiwan, Peru, Florida, Texas, California, various parts of Central and South Africa, India, Pakistan and Bangladesh and is also known as pawpaw, papam, papaya in local languages.

Cultivated papaya is prone to many diseases incited by fungi, bacteria, nematodes and viruses leading to heavy losses. Especially fungal pathogens gregariously attack various parts of plant from roots to fruits. Approximately, 171 different fungi attack papaya in the world. Papaya fruits are highly perishable commodity suffering from heavy post harvest losses, of which papaya anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. appears to be more severe causing heavy devastation of papaya fruits during transit and storage. Papaya anthracnose is the most important disease throughout the year in India and a major limiting factor in transit and storage. It is important in many other tropical regions where papaya is grown (Bolkan *et al.*, 1976) [4].

Fruits are living entities and are highly perishable commodities that are affected by number of factors leading to be postharvest spoilage and hence postharvest losses are major ones.

Alvarez and Nishijima (1987) [5] described three types of post-harvest diseases in papaya. They are, *viz.*,

1. Fruit surface rots occurred by fungi that infect intact, immature, green fruits still attached to the tree (anthracnose, chocolate spot, *Cercospora* black spot and *Phytophthora* fruit rot). The organisms involved typically weak pathogen (*Phomopsis*, *Mycosphaerella*, *Alternaria*, *Stemphylium* and *Fusarium*) caused diseases by fungi that infect fruits through wound occurring before and after harvest.
2. Stem-end rots occurs after harvest (*Ascochyta* sp., *Botryodiplodia* and *Fusarium*).
3. Internal fruit infection occurs sporadically when the blossom end of the fruit is not completely sealed (*Cladosporium* sp., *Penicillium* sp., and *Fusarium* sp., and bacteria such as *Erwinia herbicola* and *Enterobacter cloacae*).

Material and Methods
Collection of diseased samples
Papaya fruits with typical rotting symptoms were collected from fruit markets of Palanpur and Deesa. Such samples were brought to the laboratory for further examination. The samples were examined visually and recorded the growth and morphological characteristics of the pathogens causing rots.

The fresh samples of diseased fruits, showing typical rotting symptoms were gently washed under distilled sterilized water to remove exogenous material. Temporary mounts was prepared from diseased specimens to know the microorganisms associated with the rotted fruits. In case of small rotting lesions incubation of diseased sample was done by taking small pieces (approx. 1 sq.cm) of infected fruits which was placed on the surface of sterilized micro slide. Each micro slide was kept on a pair of glass rods in a sterilized rod in a sterilized Petri plate lined with layer of sterilized moist blotter paper. These plates were then incubated at room temperature (27 ± 2 ºC) for 48 hours and later on examined under microscope to identify the associated pathogens. The diseased fruits with typical symptoms were kept for rotting and to record the changes in the symptoms in the rotting and the growth of the pathogen.

Isolation of the pathogens

Direct isolation

The fresh infected papaya fruits showing typical rotting symptoms were used for isolation of the pathogen. Fungal growth on rotted fruit surfaces was directly transferred by sterilized wire loop in sterilized Petri plates containing 20 ml sterilized potato dextrose agar (PDA) medium under aseptic condition. The plates were incubated at 27 ± 2ºC temperature for 48 hours to obtain fungal growth. The individual fungal colonies were aseptically transferred to PDA slants. Fungi were used for further investigation. The pure culture was obtained by single spore method.

Tissue isolation

Papaya fruits showing typical symptoms were cut into small pieces along with some healthy un-rotten portion and surface sterilized with mercuric chloride 0.1 per cent solution for one minute followed by three subsequent washes with distilled sterilized water to remove all the traces of mercuric chloride. The pieces were aseptically transferred to sterilized Petri plates containing 20 ml sterilized potato dextrose agar (PDA) medium and were incubated at room temperature (27 ± 2ºC). The growth of the different fungi were observed periodically. The culture obtained was further purified by aerial mycelial tip technique. Pure colonies developed from the mycelial bit was transferred to PDA slant.

Identification of the pathogens

Identification of the pathogens associated with papaya fruit rot was carried out by studying the cultural characters *viz.*, colour, pattern of mycelial growth and morphological characters of spores *viz*., length and diameter. These were measured under low power magnification of the microscope from actively sporulating 10 days old cultures of different fruit rotting fungi using stage and occular micrometer and were compared with those given in the literature. The cultures were identified.

Pathogenicity and symptomatology of associated pathogens

To prove the pathogenicity of different fungi isolated from rotted papaya fruits, five healthy half matured papaya fruits were selected for each fungus isolated from infected papaya fruits. The fruits were disinfected by dipping in sodium hypo chlorite one per cent solution for one minute. The surface inoculation was done on three disinfected fruits by different following methods.

Knife injury

Sterilized sharp edged knife was used to make one cm diameter wound of about 0.5 cm depth on sterilized fruit surface under aseptic condition. Sterilized cotton pad of about 5.0 sq. cm was dipped in spore suspension of test fungi (1×10^8 cfu/ml) and swabbed over the wounded surface of the fruits. The inoculated fruits were kept along with cotton plug dipped in distilled sterilized water, to maintain the humidity and incubated at room temperature.

Cork borer injury

Wound of one cm depth was made on the healthy fruits surface with the help of sterilized cork borer under aseptic condition. The mycelial disc of half centimeter diameter of the test fungus (1×10^8 cfu/ml) was inoculated at the place of the wound. Sterilized cotton pad of about 3.0 sq. cm was dipped in distilled sterilized distilled water and swabbed over the inoculated surface of the fruits. The inoculated fruits were kept along with cotton plug dipped in water, to maintain the humidity and incubated at room temperature.

Pin prick injury:

The sterilized fruit surface was injured by pricking with the help of a sterilized needle. Sterilized cotton pad of about 5.0 sq. cm was dipped in spore suspension of test fungus and swabbed over the wounded surface of the fruits. The inoculated fruits were kept along with cotton plug dipped in water, to maintain the humidity and incubated at room temperature.

Suitable control with healthy fruits uninjured and un-inoculated were kept under the high moisture condition and incubated at room temperature in all the three artificial inoculation methods.

Symptoms produced were recorded and compared with the earlier literature. The fungi were re-isolated from the inoculated diseased portion of the fruits and the morphological and cultural characters were compared with original cultures to prove the Koch's postulate's. The fungus was sub-cultured on PDA slants and allowed to grow at 27 ± 1 ºC for one week. Such slants were preserved in refrigerator at 5 ºC and sub-cultured once in two months.

Results and Discussion

Collection of diseased samples

The infected papaya fruits samples of cv. Taiwan red lady were collected randomly on the basis of differentiated typical fruit rotting symptoms from fruit and vegetable markets of Palanpur and Deesa during survey and were brought to the laboratory of the Department of Plant Pathology, C. P. College of Agriculture, SDAU, Sardarkrushinagar. Symptoms and sign were observed visually and the presence of association of the pathogen was confirmed by microscopic observation.

Isolation of the pathogens

The fruits showing presence of any fungal attachment to the fruit rot was subjected to the isolation by both, direct isolation and tissue isolation. It was found that eight fungal pathogens associated with rotting and were differentiated on the basis of cultural characteristics. Out of eight, three fungi were isolated by direct fungal growth on fruits transferred to potato dextrose agar (PDA) medium and another five were isolated by the tissue isolation. These cultures were further purified by single spore isolation technique and were maintained on PDA slants for further studies. The periodical sub-culturing and

multiplication were done on the PDA to keep cultures fresh and were used throughout the investigations.

Identification of the pathogens

For identification of the pathogens, microscopic observations were carried out on the basis of cultural characters *viz.*, colour, pattern of mycelial growth and morphological characters of spores of all the eight pathogens and were primarily identified as *Colletotricum* sp., *Graphium* sp., *Fusarium* sp., *Alternaria* sp., *Fusarium* sp., *Aspergillus niger*, *Aspergillus flavus* and *Rhizopus* sp. The cultures were sent to Indian Type Culture Collection, New Delhi for confirmation and identified as *Colletotrichum gloeosporiodes*, *Graphium* sp., *Fusarium pallidoroseum*, *Alternaria alternata*, *Fusarium solani*, *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus microspora.*

Cultural and morphological characteristics of the isolated isolated fruit rot fungi

The fungi isolated from symptomatically differentiated fruit rots of papaya revealed variation in their cultural and morphological characteristics are depicted in Table 1.

Table 1: Cultural and morphological characteristics of isolated fruit rot fungi

Casual organism	Cultural characteristics	Morphological characteristics
Colletotrichum gloeosporioides	White to dull white mycelium with smooth margins, sporulation maximum with fruiting body at centre as compared to periphery.	Mycelium: Hyaline, septate and branched, superficial Acervulus: Black colour, circular measured 122 - 261.2µm Conodia: Single celled with smooth walled, hyaline, cylindrical or oblong measured 4 – 10.1 X 3 – 4 µm
Graphium sp.	Mycelium cottony and woolly, gray in colour.	Mycelium: septate Conidiophore: simple, long and dark in colour, Conidia: one celled, hyaline, oval shaped and form in cluster Synnemata: erect, black, bearing a single, terminal one celled hyaline conidia
Fusarium pallidoroseum	Flat colony with superficial mycelial growth and circular margin, red colour colonies	Mycelium: septate, red coloured and branched Macro conidia: conidia with 3-5 sapta and measured 27.45 X 2.15 µm Micro conidia: unicellular conidia measured 8.75 X 1.24 µm
Alternaria alternata	Fungus produced dull white profuse mycelial growth initially, but afterwards turned light brown to dark brown due to sporulation.	Mycelium: Profusely branched and septate, light to dark brown Conidia: Yellowish to dark brown, rounded at basal portions ovoid to ellipsoid, long beak at apical end in 2 – 6 transverse and 1 – 3 longitudinal septa and measuring 13.20 – 53.25 µm in length X 4.8 – 16.25 µm in diameter.
Fusarium solani	Initially fungus colony was flat with spares mycelial growth, which gradually showed profuse growth with fluffy, cottony to dull white mycelium.	Mycelium: Hyaline, septate, branched Micro conidia: Elliptical, hyaline, unicellular conidia without septation measured 10.2 – 14.35 X 2.30 – 3.15 µm. Macro conidia: Fusiform, hyaline, 1 – 3 septate and measured 20.16 – 33.22 X 2.98 – 4.72 µm.
Aspergillus niger	Black colonies with white edges	Conidia: Globose, large heads biseriate and phialides, dark brown. Conidiophore: Conidial heads were born on metula, smooth walled.
Aspergillus flavus	White spreaded flat growth of mycelium, later turn light yellow – green with balls appeared on upper surface of colony.	Radiate conidial head splitting into several loose columns. Conidiophores hyaline, vesicle become globose and flask shaped phialides that produce chains of conidia which borne directly on vesicles.
Rhizopus microspora	Rapid growing, initially white woolly and later turned gray mycelium with small dot shaped sporangia	Mycelium: hyaline, broad ribbon like irregular branched, aseptate to sparsely septate Sporangiophore: umbranched, yellowish brown to dark brown, produced singly or in groups and bear globose sporangia with slightly elongated to pear shaped columellae.

Pathogenicity and Symptomatology of associated pathogens

Pathogenicity test

The fruit rot fungi were inoculated on papaya fruits (cv. Taiwan Red Lady) by three methods *viz.*, knife injury, cork borer injury, and pin prick injury as mentioned in point 4. The results are presented in Table 2.

The cent per cent symptom was observed in knife injury in all the fungi. Cork borer injury was showed cent per cent symptom for *Colletotrichum gloeosporoides*, *Fusarium pallidoroseum*, *Alternaria alternata*, *Aspergillus niger* and 80 per cent infection in *Graphium* sp., *Fusarium solani*, *Aspergillus flavus* and *Rhizopus microspora*. In pin prick injury *C. gloeosporioides*, *F. solani*, *A. niger*, *A. flavus* and *R. microspora* were produced cent per cent symptom but *Graphium* sp., *F. pallidoroseum* and *A. alternaria* recorded 80 per cent symptom.

Table 2: Pathogenicity of different isolated fungi on papaya fruits inoculated by different methods

Sr. No.	Inoculation technique	Diseased fruits(%)							
		C. gloeosporioides	***Graphium* sp.**	***F. pallidoroseum***	***A. alternata***	***F. solani***	***A. niger***	***A. flavus***	***R. microspora***
1.	Knife injury	100	100	100	100	100	100	100	100
2.	Cork borer injury	100	80	100	100	80	100	80	80
3.	Pin prick injury	100	80	80	80	100	100	100	100
4.	Control (Uninoculated)	00	00	00	00	00	00	00	00

Symptomatology

The symptoms produced in pathogenicity test were compared with naturally occurring symptoms by the different fruit rot fungi and are presented in Table 3.

Table 3: Symptoms of different fruit rot fungi

Causal organism	Symptoms
Colletotrichum gloeosporioides	The symptoms on a fruits first appear as a brown circular spot superficial discoloration of the skin which developed into slightly sunken area of 1 to 3 cm in diameter. These lesions coalesced and sparse mycelial growth appear on the margins of the spots. Finally, the whole fruit turn dirty dark brown and rotted. In immature fruits resulted in mummification deformation.
Graphium sp.	At initial stage woolly to cottony whitish spots of mycelial growth on fruits epidermis. Gradually turned in to dark gray to blackish colour. Finally spots collapsed and resulted into whole fruit rot.
Fusarium pallidoroseum	At initial stage water soaked, slightly sunken lesions with whitish to pinkish mycelial growth on the fruit epidermis. Gradually lesions collapsed and finally resulted in fruit rot.
Alternaria alternata	At initial stage brown spot appeared on the fruit epidermis. Gradually change into circular to oval, and become gray brown lesions. The lesions covered with mycelium and conidiophores. Such lesions coalesced as the expanded and cover the entire fruit surface laterally.
Fusarium solani	At initial stage small circular to irregular, brownish water soaked lesions appeared on the fruit epidermis. These lesions gradually spread into large area which becomes necrotic. In heavy infection stage lesion got depressed, caused soft rot. At last, whole fruit got rotted with whitish lesions on fruits
Aspergillus niger	At early stage of infection black small spots appeared on the fruit epidermis. These spots laterally grow to the dry, blackish patch. Gradually all these patches collapsed and gave rise to brownish colour with fluffy growth of mycelium and the spores. At the last stage whole fruit got rotted and covered with blackish mycelial growth.
Aspergillus flavus	Initially small sunken spots with dark colour appeared on the fruit epidermis. The spots enlarged to depressed large patches. Gradually produce whitish fluffy growth of mycelium which turn into pale to greenish, producing rotting odour
Rhizopus microspora	Initially irregular water soaked lesions which enlarged and got cover by white and dark brown mycelial growth and sporangiophores. Laterally the lesions became water soaked and emitted foul odor. Finally whole fruit collapsed watery exudates come out.

Thus, The present results are in conformity with the finding of Bolkan *et al*. (1976) found that fully matured and immature papaya fruits inoculated with *Colletotrichum gloeosporioides*, *Alternaria* sp., *Fusarium solani*, *Penicillium* sp., *Cladosporium* sp. and *Rhizopus stolonifer* produced a water soaked condition at wound site three days after inoculation and typical lesions developed by the fifth day. Rahman *et al.* (2008) [6] proved the pathogenicity of *Colletotrichum gloeosporioides* isolated from the surface of fully matured papaya fruits *cv.* Sekaki. The test showed that both wounded and unwounded fruits inoculated with conidial suspension of *Colletotrichum gloeosporioides* developed distinct symptoms of anthracnose after three and five days of inoculation, respectively. Zakaria *et al*. (2012) [7] carried out pathogenicity test of *Fusarium semitectum*, *Fusarium solani*, *Fusarium verticillioides* and *Fusarium oxysporum* on banana (*Musa* spp.), papaya (*Carica papaya*). From pathogenicity tests, *F. solani* and *F. semitectum* were pathogenic to both banana and papaya and *F. verticillioides* to banana. Kelechi *et al.* (2019) [8] carried out the pathogenicity test of presumptively identified *Aspergillus niger* and *Alternaria* spp. responsible for the spoilage of papaya. *Aspergillus niger* and *Alterneria* spp. were capable of causing spoilage on healthy papaya fruit by the 5th day of inoculation. Signs of spoilage showed a white rot at the point of the inoculation and brownish deterioration.

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