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Identification of Causative agents associated with decay of Trees Twig and Orchards Die-back and their Impacts on Vessels of Citrus, Date Palm and Ficus

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ABSTRACT

The present work is concerned with the studies of the organism causing wood decay of twigs and branches of citrus orchards, date palm *Phoenix dactylifera* L, and ficus trees. A survey for the occurrence of this disease in the main growing areas was achieved by the researchers. The following species of citrus orchards [lime (*C. aurantifolia*), sweet orange (*C. sinensis*), and mandarin (*C. reticulate*)] were surveyed, and so date palm and ficus trees. However, the results showed that the incidence of this disease was about 100%. Laboratory examinations data revealed mainly two fungal species causing the disease: *Physalospora rhodina (P.rhodina*) and *Diaporthe citri* (*D. citri*). In addition that, both fungi, which are *P. rhodina* and *D. citri* affected the vessels of tree tissues. According to the pathogenicity test, the fungus *P. rhodina* caused a breakdown of parenchyma cells, and the fungus *D. citri* caused the darkening of the xylem.

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Introduction

Citrus production occurs in tropical and sub-tropical countries (1, 2). Citrus products play an important role in human diets in the form of citrus fruits, which are highly rich in Vitamin C content and other important nutrients, such as folate and potassium. In fresh form, they are good sources of dietary fiber (3, 4). It is estimated that the citrus-processing industry, which has mainly focused on juice and essential oil production for a long time, uses 33% of the citrus harvest for fruit juice production (5).

Over history date, the palm tree has played a major role in the life of human beings. It has been used as a food source for building houses and landscaping (6). It has been cultivated alongside rivers, springs, and wherever water is available. The date palm tree has played and is still playing a significant role in Muslims' life in general. The role of the tree increased when growers realized that the tree is salt and drought-tolerant, in addition to its impact on combating desertification. The tree can decrease the atmospheric temperature and the level of pollutants resulting from industrial activities. The symmetric shape of the date palm tree has added another dimension to its impact on the future improvement of the environment (7).

Ficus trees, which are considered critically important components of tropical ecosystems, may be particularly attractive to seed dispersers in that they produce large and nutritionally rewarding fruit crops. Cottee-Jones (8) reported in his studies that large trees, and specifically large ficus trees, may be more effective forest restoration agents than other remnant trees in disturbed landscapes, and therefore the conservation of these trees should be prioritized.

The problem of these diseases in all parts of the world where commercial citrus cultivation is practiced is of sufficient importance to deserve careful attention (9). Many of these diseases are caused by pathogenic fungi. Some attack the fruit and either destroy it or make it useless much of its grade. Others damage different parts of the trees and interfere with their function, eventually bringing about the death of these parts and, in some cases, the whole tree succumbs. Among the major problems of the citrus tree, one of the highlighted problems is the formation of abundant gum accompanying or preceding large areas of dead bark, which constitute an important external feature that calls serious attention. The exudation of gum by citrus trees indicates an abnormal condition affecting the tree. Work reported by previous investigators had concluded that gum diseases of citrus trees originated independently of microorganisms (10). To date, the most serious problems of trees in commercial trees and shade trees cultivated are twig dieback and wood decay caused by two types of fungi: *D. citri* and *D. natalensis.*

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The presence of *Diplodia spp.* on *Citrus spp.* at Kadugli on dead twigs with several fungi, all probably and at Tong associated with dieback of citrus twigs (11, 12). Minessy et al. (1972) observed Declining in citrus orchards in the Northern States due to a decline in cultural practices (13, 14). So many factors may contribute to the citrus dieback infection, with the fungi regarded as the most important causal factor (15). Lime trees infected by these pathogens generally show leaf dryness, shedding, twigs, and, in turn, declines in tree growth, reducing their yield (16, 17). Furthermore, the two fungi, which are *P. rhodina* and *D. citri* affected the vessels of citrus tissues. There are no cases of plants bred or engineered specifically for resistance to diseases caused by *D. citri.*

So in this article all the reported work, field, and laboratory studies were conducted with main objectives such as surveying the incidence of dieback disease on citrus grown in trees, identifying the causal organisms of the disease, and proving that the two fungi can cause dieback of citrus twigs and branches, including histopathology investigations.

Materials and Methods

A survey was conducted randomly on the severe form of dieback on citrus trees of different species; sweet orange, mandarin, lime, Date palm and Ficus tree. The survey was carried out at different areas of twigs and branches containing both sound and disease tissues of recently killed regions located at the advancing margin of the darker area on the diseased part. Naturally, rotted fruits were also picked up for disease identification. The survey was conducted to collect specimens from different sites of severe dieback and wood decay. The collection included excised pieces of dying trees. The specimens of each tree and individual plant organs were separately maintained together with healthy citrus green leaves in plastic bags to avoid drying. The samples were carefully handled and brought to the laboratory for further examination and isolation.

Isolation

From these samples, fungi were isolated as follows: discs of approximately 0.5 mm in width were cut from the infected areas, sterilized with 0.01% mercuric chloride for the period of 1 minute, then were washed in sterilized distilled water and, after drying, plated in PDA+Rose Bengal. Slides were prepared from these sections and examined microscopically. The plates were then further incubated at 25±20°C. After 24 hours, fungal growth was observed. The fungi was identified based on growth characteristics, mycelia type, and the structure of acervuli, setae, and conidia following the procedure reported by Beales (2012) (18).

Physiological studies of isolates

1-Type of Culture Media: several sterilized Petri-dishes were poured with PDA, CMA, Czapek·s, Nutrient Agar, Salt Agar, Tap Water Agar, and Yeast extract glucose-chloramphenicol-Agar (see details in Appendix). Two diameters were drawn on the back of each petri-dish for centering the inoculums. Then a 7 mm disc was cut from the edge of the 7-day-old culture of *D. citri* and *P. rohodina*. The inoculum was placed with agar in a petri-dish. The Petri dishes were then incubated at 28 °C in four replicates. The fungal growth rate was estimated routinely by measuring its colony size along the two diameters drawn previously, and the mean colony diameter was calculated for each medium.

Carbon Sources

Effect of carbon source on linear growth of the pathogens under study

Four different carbon sources (dextrose, glucose, maltose, and sucrose) were used to study their effects on the linear growth of *P. rhodina* and *D. citri*. The amount of 20 g dextrose present in the PDA basal medium was substituted by an equal amount of glucose, maltose, and sucrose for the test. Four media have different carbon sources *viz*. PDA, PGA, PMA, and PSA were prepared in four 1000 ml flasks. The flasks were then plugged and sterilized. Each medium was poured into several sterilized Petri dishes. The Petri-dishes were inoculated with a 7 mm disc cut from the edge of a 7-day-old culture of the two fungi. The Petri-dishes were then incubated at 28 ± 2 °C for *P. rhodina* and at 20 ± 2 °C for *D. citri*. The fungal growth rate was estimated daily by measuring colony size along the two diameters on the back of each petri-dishes.

Histopathology studies: Sixteen earthen pots of 12 inches each in diameter were filled with a 7 kg sterilized mixture of clay and sand in a ratio of 2:1. Sterilized lime seedlings, eighteen months old, were transplanted into each pot. The plant seedlings were left to grow well under shade. Water was supplied regularly with foliar nutrients. The test pots were arranged in a completely randomized design with three replicates. Twenty months old seedlings were inoculated with the inoculums injected through a scratch made in the seedling stem. The experiment terminated three months after inoculation.

Histo-pathology studies were carried out on lime seedling's artificially infected and fungus-free stems [*C. aurantifolia* (Christm.) Swingle] Baladi variety. Thereafter, healthy lime trees were prepared and inoculated as previously mentioned. Different stages of disease development were investigated. Samples from treated stems were collected after three months of inoculation.

The collected materials were killed and fixed in a formalin-acetic acid-alcohol solution, followed by softening at 38 °C for at least 1 week in a 1:1 glycerin and 50% ethanol solution. The specimens were carefully washed with three changes of 50% ethanol and dehydrated in 100% ethanol. Stained sections were cleared in xylol and mounted in Canada balsam. Samples were embedded in paraffin wax, the melting point of which was 56-58°C, cut at 15 µm thickness and stained according to the Chamberlains (1932) safranin light green combination method was found more satisfactory than the other test stains.

Results

The examined trees were identified to have a high incidence of dieback of twigs and branches caused by *D. citri* and *P. rohodina*. The disease is widely spread among the citrus orchard, Date palm, and Ficus trees growing in various areas amounting to 100% infection. Within the tree species surveyed, the disease was predominant in Citrus sp., especially Lime, Sweet orange, and Mandarin (Table1).

The fungi, which have been encountered on the infec-

Table 1. The survey of dieback of citrus trees.

Citrus sp.	Age of examined trees	Total No. of examined trees	Infection%	Irrigation system	Kind of soil	Temperature (°C)	R.H.% annual mean
Lime	≥3	966	100	Basin	clay		
Sweet orange	≥6	466	100	Basin	clay	33	21
Mandarin	≥10	42	100	Basin	clay		

ted plants, using the blotter test method, were placed on Potato Dextrose Agar. Many successful isolates of the causal organism were made from diseased tissues: *D. citri* Wolf, conidial state *P. citri* Fawcett, and *P. rohodina* (Berk. & Curt.) were found. These fungi were identified depending on their cultural characteristics, such as the shape and size of conidia, false or prominent perithecial stages, and the presence or absence of chlamydospores. Accordingly, identification of the fungi from a series of isolates resulted as follows: *rhodina*, the spores of this fungus were about the same size as those of *D. phoenicum*. The hyaline spores may later become dark and septate. The pycnidia were commonly found on dead leaf parts and have not been found on the bases while they are still alive and attached to the mother tree (Figure 1).

These results indicated that *P. rhodina* thrives on PDA medium at 300 C, producing thick cottony mycelium at first, then forming many hyphal branches from different hyphae that grow toward a common point interweave to form pycnidial initials. From this point, the pycnidial wall develops and a cavity is formed in the center. The conidia are produced at the tip of the conidiophores Chlamydospores of the fungus were found (Figure 2).

Results also showed that the growth of *D. citri* was moderate on PDA medium at 20 ^{O}C, producing chlamydospores. Perithecia which are formed in stroma Ascospores are in ellipsoid, fusoid, two-celled and the imperfect state is Phomopsis, class Coelomycetes.

Influence of carbon sources on growth of *D. citri* and *P. rhodina* the growth of the two fungi was observed in four carbon sources in Potato Dextrose Broth. The four carbon sources were dextrose, maltose, glucose, and sucrose. Of the four-carbon sources tested, glucose in a liquid medium promoted better growth of the fungus *P. rhodina*, followed by maltose, sucrose, and dextrose. The best mycelial growth of *D. citri* was in glucose. The mycelial dry weight of both fungi was not significantly different between the four carbons sources, as shown in Table (2).

The physiological studies on the effects of different carbon sources on the growth of the two isolates of *P. rhodina* and *D. citri* showed that the best growth was obtained in the dextrose in Figures 3 and 4. In this connection, Qadri et al (2020) (19) reported that dextrose can easily be utilized by most fungi.

The Pathogenicity of *P. rhodina* in the seedling was inspected three months after the artificial infection. Sec-

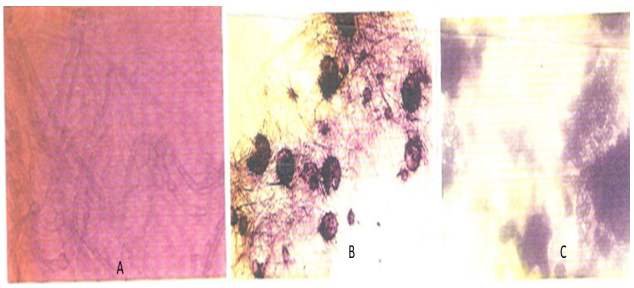

Figure 1. The fungus *P. rohodina*.(a) Separate mycelium with chlamydospores (b) Initial pycnidia (c) A mature pycnidium showing young ascospores.

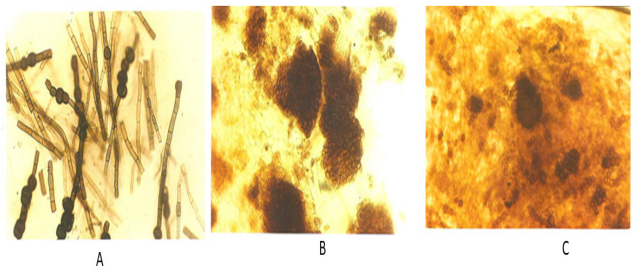

Figure 2. The fungus *D. citri*. (a) Septate mycelium with chlamydospores, (b) Cell-wall of pycnidia on the dead branch, (c) A pycnidia with flask-like structure.

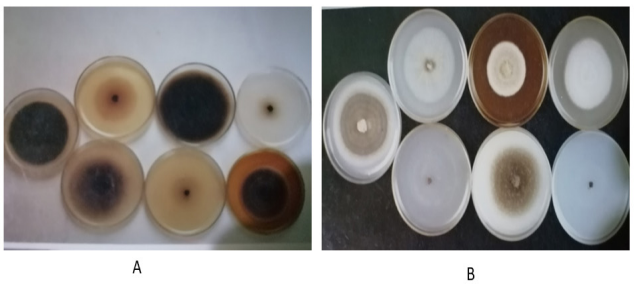

Figure 3. [A]: Growth of *D. citri* on media [B]: Growth of *P. rhodina* on media.

Table 2. Effect of carbon source on mycelia weight of *P. rhodina* and *D. citri* (cm).

Isolate	Carbon sources				Means
	PSB	PMB	PGB	PDB	
P. rhodina	4.896	5.750	6.113	4.85	5.402
D. citri	1.336	3.156	0.720	1.28	1.623
	3.116	4.453	3.416	3.065	Mean

Carbon source: (C.V. = 25.3%, L.S.D$_{0.05}$= 1.581, L.S.D$_{0.01}$= 2.217), Isolate: (C.V. = 1.27, L.S.D$_{0.05}$= 0.157, L.S.D$_{0.01}$= 0.362), Interaction between carbon sources and isolates S.E± =0.81

tions around the wound were inspected microscopically, and it was clear that the disease had progressed in the wood and the infection had proceeded toward the cortex. In the xylem parenchyma and xylem ray cells, there was an increase in cells, lacunae with gum secretion, as well as more cells showing the breakdown. The discoloration of the cell walls and the plugging of the intercellular spaces extended well in advance of the hyphae (Figure 5).

Pathogenicity of *Diaporthe citri* in the seedling as in the previous infection peg was usually formed and the fungus entered the host directly through the epidermis and invaded almost all tissues. It rarely entered open stomata. The fungus grew intracellular, rarely becoming intercellular. The epidermis, cortex, phloem, and cambium tissues disintegrated. The fungus grew through the xylem vessels to the pith cells. It interred the xylem vessels and a brown to dark material was produced, resulting in the plugging of the xylem vessels. Sometimes, in an advanced stage of the disease, the phloem and cambium tissues appeared to be with thick-walled cells loosely arranged perpendicular to the surface of the vascular cylinder.

The Histological study shows that the two isolates are *P. rhodina* and *D. citri*. In addition, the two fungi, *P. rhodina* and *D. citri* affected the vessels of tree tissues. According to the pathogenicity test, the fungus *P. rhodina* caused a breakdown of parenchyma cells, and the fungus *D. citri* caused a darkening xylem darkening plate (figure 5 B).

Discussion

The survey carried out indicated the progress of the disease infection and it is widespread during latent years.

The isolation was preferred. Those *Diaporthe citri pycnidia* were found on leaves, green and dead branches bark, and decaying fruits. They were scattered or clustered, immersed, erumpent, black conical to lenticular in shape. The pycnidial wall is usually many cells thick, composed of outer sclerotized cells with thin inner pseudoparenchyma lining the cavity.

Figure 4. [A] Pathogenicity test of *P. rhodina* on Lime seedlings [B] Pathogenicity test of *D. citri* on Lime seedlings.

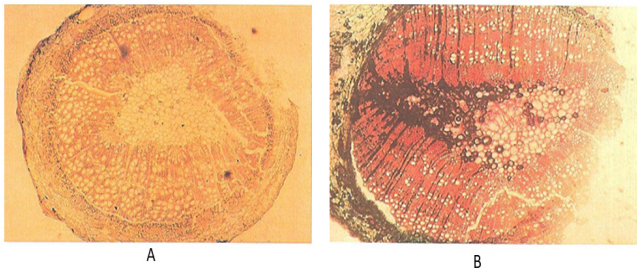

Figure 5. [A]: Histopathological effect of *Physalosphora rhodina* (parenchyma cells showing break-down [B]: Histopathological effect of *Diporthe citri* (darkening xylem vessels).

The growth of the fungi in PDA was compared with (20). This class of fungi imperfect comprises a form in which conidia develop within a pseudoparenchymatous cavity formed by hyphae and which may form a more or less open structure called acervulus, or a frequent flask–like closed structure.

Gottwald et al (2002) (21) stated that no visible symptoms were observed before three months when citrus was infected with any pathogen because seedlings of lime grew very slowly. Thus results of the pathogenicity test on lime seedlings showed visible symptoms after three months. To ensure the infection by the fungi *P.rhodina* and *D.citri*, the histopathological studies showed that the fungus *P. rhodina* had progressed in the wood and the infection had proceeded towards the cortex. These results are similar to those described by Madar et al. (1990). Therefore, the fungus *D. citri* grew through the xylem vessels to the pith cells, the results that are similar to those described by Hur et al (1999). Nevertheless, after three months, the visible symptoms of dieback were manifested on the lime seedling.

The general symptoms of infection by these minor forms of fungi are similar to those of brown rot gummosis. Still, the gumming is usually less profuse, and the extent and severity of the damage are usually less.

The cottony rot fungus, *Sclerotinia sclerotiorum*, may attack and kill all parts of the tree that have been injured. As in Botrytis gummosis, the affected bark is soft at first but turns gray to almost white and breaks up into shreds. Among the shreds of bark on the wood surface are black, irregular bodies of the resting stage of the fungus. Gumming may be rather profuse as in brown rot gummosis.

Both Botrytis and Sclerotinia fungi cause similar twig blights. starting in the ends of twigs or the blossoms, they kill the twigs back for a few inches. Their progress is arrested, however, by warm, dry weather.

D. citri and *Diplodia spp.* Attack bark weak end by shellbark disease and by other factors mentioned above, causing a gummosis. They and Botrytis will also invade growth cracks, pressure ridges, and wounds. These fungi are particularly severe when working together in shellbark areas, for they may kill the bark through to the cambium, and the Diplodia may invade the wood. Diplodia to advances in the wood of twig limbs injured by frost. Either *D. citri* or *Diplodia spp.* Alone can invade the inner bark, and the former (Phomopsis stage), although usually confined to bark, can invade the wood of weak trees. On the affected parts, the black spore-containing structures (ting black sphere embedded in the bark) of the two fungi may be found and they are characterized by the formation of elongated gum pockets in the inner bark, next to the wood surface of the affected inner bark is brown to black.

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