Diplodia natalensis Pole Evans, the causal agent of citrus gummosis disease in Ghana

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SUMMARY

Isolations were made from the barks of gummosis-infected citrus trees from orchards of the University of Ghana Agricultural Research Station at Kade. The isolation media used were 1.5 per cent water agar, 1.5 per cent water agar + nystatin, and 1.5 per cent water agar + benomyl. Four isolates including *Diplodia natalensis* Pole Evans, *Fusarium solani* Appel + Wr., and two other identified fungi were obtained. The two fungi were unidentified on Potato Dextrose Agar (PDA). When the isolates were tested for pathogenicity, only *D. natalensis* induced the disease symptoms in the inoculated 18-month-old rough lemon seedlings which were incubated after inoculation in a screenhouse of 30-37 °C and 55-75 per cent relative humidity. This result strongly suggests that *D. natalensis* is one of the pathogens causing citrus gummosis disease in Ghana.

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RÉSUMÉ

ASSUAH, M. K., ODURO, K. A. & OFOSU-BUDU, K. G.: Diplodia natalensis *Pole Evans l' agent causal de citrus gommose au Ghana.* Isolement était fait à partir de l'écorce de l'arbre de citrus gummose-infectée tiré des vergers de la Station de Recherche Agricole d' Université du Ghana à Kade. Les milieux d' isolement employés étaient 1.5 pour cent agar d' eau, 1.5 pour cent agar d' eau + nystatin et 1.5 pour cent agar d'eau + benomyl. Quatre isolats y compris *Diplodia natalensis* Pole Evans, *Fusarium solani* Appel +Wr. et deux autres fongus non identifiés étaient obtenus. L' identification des deux fongus était possible sur Agar Dextrose de Patate (ADP). Lorsque les isolements étaient mis à l'essai pour la pathogénie, *D. natalensis* seulement déclenchait les symptômes de la maladie dans les semis de citron rugueux inoculé à l' âge de 18-mois, qui étaient incubés après inoculation dans la cage de Faraday de 30-37°C et 55-75 pour cent d'humidité relative. Ce résultat suggére fortement que *D. natalensis* est l'un des pathogènes causant la maladie de citrus gommose au Ghana.

Introduction

The gummosis disease of citrus had been known in Ghana for some time now (Eady, 1930; Auchinleck, 1932). The disease is characterized by the exudation of gum from an infected area on the tree, mainly the trunk. The oozed gum may be absorbed in the soil, if the infected area is close to the ground, or may harden on the surface of the bark (Fig.1). The affected bark becomes necrotic, firm and darker than the surrounding healthy tissue. If the infected bark is removed, the diseased wood surface is dark brown which reduces in intensity through brown to light brown toward the healthy, cream surface. The infected bark later peels or flakes off when the disease advances in age. Other symptoms include chlorosis and die-bark, with the trees producing an exceptionally heavy crop before eventually dying when the infection girdles greater part of the circumference of the tree trunk. Cultivars of lime (*Citrus aurantifolia* (Christm) Swingle), lemon (*C. limon* (L.) Burm, grapefruit (*C. paradisi* Blanco), sweet orange (*C. sinensis* (L.) Osbeck), and tangerine (*C. resticulata* Blanco) had been infected with the disease in Ghana (Eady, 1930).

The disease is still the major citrus disease of economic importance in Ghana (Auchinleck, 1932; Gyamera-Antwi, 1966; Ofosu-Busu & Oduro,

Fig. 1. Field symptoms of the gummosis disease showing hardened gum on a late valencia sweet orange budded on rough lemon rootstock.

1995). However, an outbreak of the more deadly virus disease, the quick decline or tristeza between 1938 and 1948 overshadowed gummosis in Ghana (Lister, 1947; 1948; Hughes & Lister, 1949).

These two diseases had influenced the choice of rootstocks in the citrus industry in Ghana. However, the current rootstocks in use, namely, rough lemon (*C. limon* (L.) Burm), cleopatra mandarin (*C. reticulata* Blanco), and rangpur lime were selected for their resistance to the deadly tristeza rather than to the gummosis disease (Hugnes & Lister, 1953; Sam-Aggrey, 1971). The rationale for this selection was that gummosis, being a fungal disease, could be easily controlled with appropriate fungicides than the viral disease whose incidence is normally more destructive and difficult to control. Thus, the common rootstocks in use in Ghana are tolerant to tristeza but susceptible to gummosis. The disease is thus prevalent and severe in all citrus-growing regions in Ghana (Leather, 1959; Ofosu-Budu & Oduro, 1995).

A less expensive control measure is to screen for rootstocks which will be tolerant to possibly both diseases. This requires the isolation of the pure culture of the pathogen. In Ghana, *Phytophthora parasitica* Dast has been reported as the pathogen of the disease (Leather, 1959; Clerk, 1974). Isolation of the pathogen can, therefore, be used in screening rootstocks for tolerance to the disease.

Several attempts, including the use of selective media, were made to isolate the reported causal organism but with no success. Instead, four other fungi isolates were obtained. Two were identified as *Diplodia natalensis* Pole Evans and *Fusarium solani* Appel + Wr; the remaining two did not sporulate and could not be identified. All the four isolates were tested for pathogenicity.

This study reports a new pathogen for the disease in Ghana.

Materials and methods

Isolation

Specimen of diseased materials were collected from diseased plants in the orchards of the University of Ghana Agricultural Research Station at Kade for the isolation process.

About 0.5-cm^2 pieces of bark and wood, excised from the leading margins of the disease on the isolation media, were plated. Before plating, the excised tissues were surface sterilized in different concentrations (1, 5, 10, and 20 per cent) of sodium hypochlorite solution for 1, 2, and 5 min, rinsed in sterile distilled water and blotted on sterile filter paper. The isolation media consisted of 1.5 per cent water agar (WA), 1.5 per cent WA + 100 ppm of nystatin (500 000 iu/5 ml; 20:1, v/v), and 1.5 per cent WA + 100 ppm of benomyl (WP, 50 per cent ai; 20:1, v/v). These are antimycotic, antibiotic and fungicide, respectively, with no inhibitory or fungicidal activity against pythiaceous fungi; and were used to enhance the growth of the reported

causal organism whilst suppressing possible distractants. The plates were incubated between 25 and 28 °C in the laboratory and examined for mycelial growth daily. Mycelia which originated from the tissues were transferred onto PDA plates for further growth and sporulation.

Slide preparations were observed under the microscope to identify the isolates, and the observed characteristics compared to those of other researchers.

Pathogenicity test

Eighteen-month-old potted rough lemon seedlings were used for the test. The inocula consisted of 5-day-old PDA-grown cultures of the four isolates and sterile PDA for control seedlings. Inoculation sites on test-citrus seedlings were disinfected with 10 per cent sodium hypochlorite solution. The inoculation involved inserting a small block of the inoculum of each of the isolates under the flap of bark made by a vertical slit and covering it with a moistened sterile cotton wool. The wounds were taped with a parafilm and the seedlings were arranged in a completely randomized design with two replicates (four seedlings in a replicate) and incubated in a screenhouse of 30-37 °C and 55-75 per cent relative humidity.

Results

Identification of isolates

Mycelia growth were observed from specimen in most of the plates except specimen on WA-benomyl medium.

Four fungi isolates were obtained. Two were persistent and were frequently observed in most of the plates. They also sporulated readily *in vitro* and were identified using growth rate, colour, and morphology of mycelia, conidia, and sporulating structures as seen under the microscope. The other two isolates did not sporulate and could not be identified.

One isolate was identified as *Diplodia natalensis* Pole Evans (*Botryodiplodia theobromae* Pat) because of its abundant fluffy aerial septate mycelium and grey colour which later turned black (Fig. 2 and 3). It filled 9-cm diameter Petri plates in 2 days when a 5-day-old inoculum of mycelium cut with a 2-mm cork borer was put at the centre of the plates. On the reverse, a greyish green colour preceded the final black colour, with the culturing medium remaining colourless. The isolate produced several conspicuous stromata (Fig. 4) on corn meal agar. Simple and separate

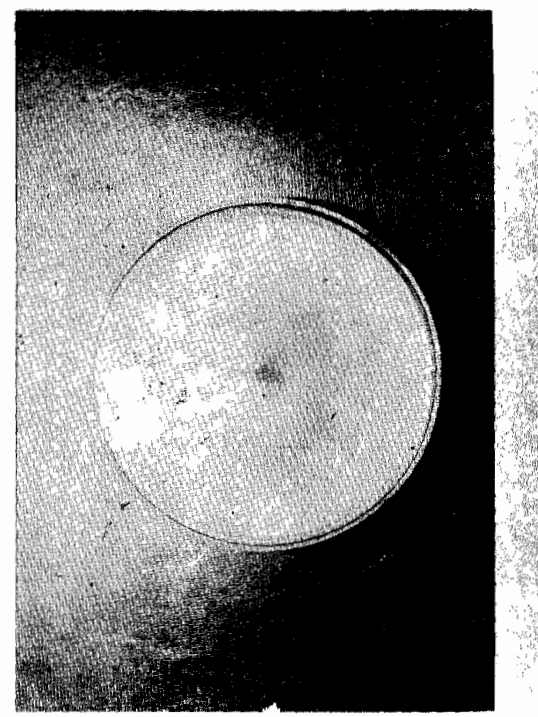

Fig. 2. 4-day-old grey and fluffy culture of *D. natalensis*.

pycnidia formed in the stromata (Fig. 5) produced numerous ellipsoidal conidia (pycnidiospores) of different ages. The immature conidia were hyaline and aseptate (Fig. 6) whilst the mature were thick-walled, dark-brown, 2-celled (septate), and longitudinally striated (Fig. 7). A PDA culture of the organism kept under a filament bulb (60 watts) in the laboratory turned red or pink in the medium. The observed cultural and morphological

Fig. 3. Ten-day-old darkened culture of *D. natalensis.*

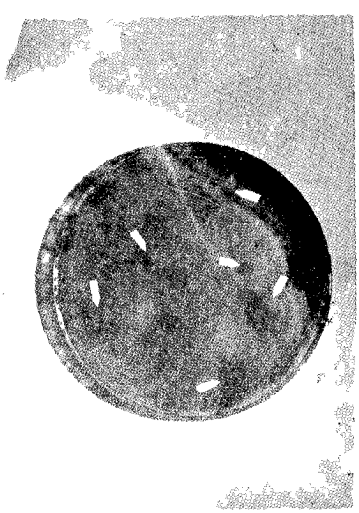

Fig. 4. Conspicuous stromata of *D. natalensis* (arrowed) developed on corn meal agar.

characteristics are similar to those observed for *Diplodia natalensis* by other researchers (Stevens & Wilcox, 1925; Alasoadura, 1970; Udeobo, 1974; Holiday, 1980).

The second isolate had a whitish or creamy culture and took over a week to fill the PDA plates. It formed chlamydospores and countless

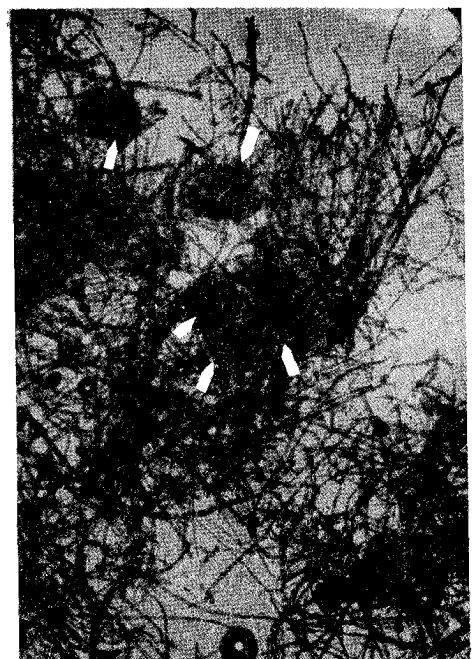

Fig. 5. Simple and separate pycnidia (arrowed) of *D. natalensis* (× 125).

Fig. 6. Immature aseptate pycnidiospores of *D. natalensis* (× 500).

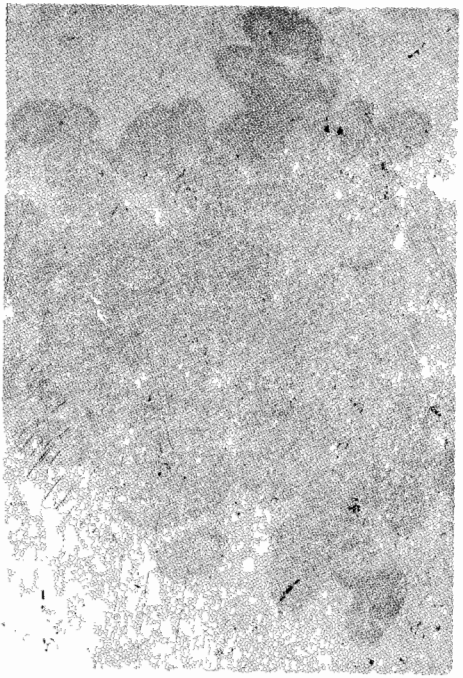

Fig. 7. Mature septate pycnidiospores of *D. natalensis* with longitudinal striations (× 500).

microconidia. Macroconidia were few, boat-shaped, and had one or two septations as well as blunt ends. The microconidia were not in chains and the culture turned brown on aging. These characteristics conform to those reported for *Fusarium solani* according to Snyder and Hansen classification of the genera (cited by Messiaen, 1959). The other two isolates had white colonies on PDA and their mycelia were septate.

Pathogenicity test

Rough lemon seedlings inoculated with *D. natalensis* were infected because by the 4th day, gum oozed out from the inoculated site. When the wound was opened 2 weeks after inoculation by removing the parafilm and the cotton wool, a necrotic canker was observed at the site. On removal of the bark below the inoculated site, the infection was seen to have developed further on the wood than on the bark. More gum oozed out and mycelium of the pathogen developed at the wounded site 3 days after exposure (Fig. 8). To satisfy Koch's postulates, *D. natalensis* was re-isolated from the artificially inoculated, diseased seedlings.

Seedlings inoculated with *Fusarium solani,* the two unidentified fungi, and blocks of PDA (control) did not show any symptom of the gummosis disease.

Discussion

The causal organism of the gummosis disease in Ghana has been attributed to *Phytophthora parasitica* (Leather, 1959; Clerk, 1974) and *P. citrophthora* (R.E. Sm. Y.E.H. Sm.) Leonian and / or *P. parasitica* in other citrus-growing countries such as the USA and South Africa (Fawcett, 1913; Klotz & Calavan, 1969). Leather (1959) and Clerk (1974) reported that *P. parasitica* was the causal organism of the citrus gummosis disease. Elsewhere, the disease has also been associated with other fungi species such as *Botrytis cinera,*

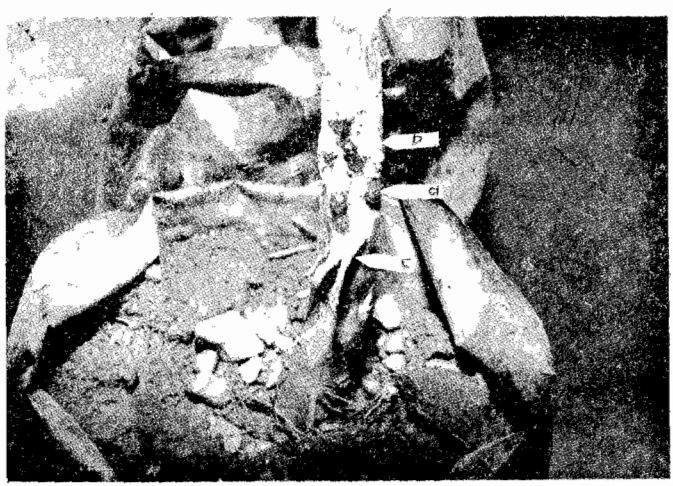

Fig. 8. Eighteen-month-old rough lemon seedling with gummosis disease symptoms 17 days after inoculation with *D. natalensis:* a - droplets of hardened gum, b - mycelium of *D. natalensis,* c - the flap of bark.

Sclerotinia sclerotiorum (Lib) d By., *Diaporthe citri* (Wolf.), *Diplodia natalensis* (Pole Evans.), and a *Dothiorella* species (Fawcett, 1913; Childs, 1953; Klotz & Calavan, 1969; Rodriguez, 1978; Mukhopadhyay, 1985; Padmanabhan & Chandhury, 1989).

The absence of *P. parasitica* on the selective media (WA + nystatin; WA + benomyl) used suggest that *P. parasitica* was not the causal organism of the gummosis diseased condition observed. The ability of *D. natalensis*, which was one of the four isolates, to induce similar disease symptoms in the inoculated seedlings, makes it more convincing that *D. natalensis* is involved in causing the disease in Ghana. Similar observations have been made in Cuba (Rodriquez, 1978). The implication of *D. natalensis* as the causal organism of the gummosis disease in Ghana in this study does not rule out the possibility of *P. parasitica* being involved in causing the disease. In fact, Rodriquez (1978) reported that both *Phytophthora* spp. and *D. natalensis* are involved in causing the gummosis disease of citrus in Cuba. It is, therefore, important to continue with more isolation studies on diseased materials from other citrus-growing areas in Ghana to check the involvement of *Phytophthora* spp. in causing the disease in Ghana.

Diplodia natalensis is a common tropical pathogen which has been identified as causing rot on several crops including yam, banana, and sweet oranges (Holiday, 1980). On sweet orange trees, *D. natalensis* also causes die-back (Bunting & Dade, 1925). In spite of the several studies done on this pathogen, this study is the first report indicating that *D. natalensis* is the cause of gummosis disease of citrus in Ghana.

The implication of *D. natalensis* as the causal organism of the disease suggests an additional strategy in the control of the disease. The benzimidazole group of fungicides such as benomyl, which could not control *Phytophthora* spp., but controls *Diplodia* spp., may be considered as a cheaper fungicide for the control of gummosis disease of citrus in Ghana.

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