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Phytopathogenicity of fungi associated with crown rot of Guava (*Psidium guajava*)

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Abstract

The present study determined the phytopathogenicity of eight species of fungi associated with the crown rot disease of guava fruits. These include *Aspergillus flavus*, *A. fumigatus*, *A. japonicus* var *japonicus*, *A. niger*, *A. tamarii*, *Fusarium sambucinum*, *F. verticillioides* and *Lasiodiplodia theobromae*. The study followed Koch's postulates for the *in vivo* infection of guava fruits and re-isolation of taxa for confirmation. Phytopathogenicity testing revealed that *Aspergillus flavus*, *A. fumigatus*, *A. japonicus*, *A. niger* and *A. tamarii*, were phytopathogenic causing crown rot of guava. Re-isolation of the phytopathogenic species on the infected plant tissue confirmed the identity of the fungal isolates.

Keywords – crown rot – fungi – guava – Koch's postulate – pathogen

Introduction

Guava (*Psidium guajava*) belonging to family Myrtaceae are well-known tropical fruits and are included among the "super fruits" due to its nutritive value. The medicinal properties of guava fruit, leaves, and other parts of the plant are also well-known in the traditional medicine (Joseph & Mini 2014). Its fruit comprises high amounts of vitamins A, B1, B2, dietary minerals, lutein, zeaxanthine, lycopene, folic acid, potassium, copper, and manganese (Rahman et al. 2003). As cited by Hassimotto & Genovese (2005), compared to other fruits, guava has a higher amount of minerals and pectin and about four times the amount of vitamin C as an orange. However, guava fruit ripens rapidly and are perishable due to their climacteric nature. Fruit ripening in guava is characterized by loss of green colour, softening, shrinkage, loss of brightness and rot development (Bassetto et al. 2005, Krishna & Rao 2014).

Fungi and plants have a long history of opportunities for co-evolution and exert reciprocal evolutionary effects on one another. Fungal traits involved in pathogenesis have variance and respond to selection by plants, as is regularly demonstrated when pathogens overcome the disease resistance bred into crop plants. In addition, plant populations vary in their resistance to fungi (Berbee 2001, Geoffroy et al. 1999). As a group, fungi are the most important plant pathogens, both from the great financial losses and the number of different diseases they cause. They exist in a wide range of habitats as in living plants and are often present as symptomless endophytes, as biotrophic or necrotic parasites. Fungal distribution and structure of communities are greatly affected by the

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specific and non-specific biologically active substrates that have the potential to inhibit fungi, which are common constituents of plant tissues (Cooke & Rayner 1984).

In guava fruits, fungi are the causal agents of anthracnose, light blight, stem rot, and crown rot. According to Misra (2006), guava is infected by around 177 pathogens and 167 of which infect various parts of the crop and about 17 are isolated with surface wash of fruits. Infection results in the appearance of discolored, malformed, or necrotic areas on the host plants. During infection, the pathogens grow, multiply, establish contact within the plant tissues, and procure nutrients from them (Streets 1969). As reported by Amusa et al. (2005), guava fruits infected with fungal disease had a significantly lower amount of carbohydrates, crude fibre, ash, fat, protein, calcium, iron, and phosphorus. More importantly, many fungal species produced mycotoxins which include deoxynivalenol, zearalenone, ochratoxin, aflatoxins, and fumonisins which can directly affect human health (Pittet 1998; Pitt 2000).

The present study was therefore initiated to determine which among the previously isolated molds are the primary post harvest phytopathogens causing crown rot in guava fruits. Results of the study would lead further to the utilization of the non-pathogenic fungi as biological control agents to numerous phytopathogens infecting various crops worldwide.

Materials and Methods

Phytopathogenicity Test

Phytopathogenicity test was guided by the Koch's postulates (Agrios 1997) which states that the pure culture of the organisms must produce the symptoms and signs of the disease when inoculated into the healthy plant and the suspected causal organisms must be re-isolated in pure culture from the inoculated plant and must be identical to the original organism.

Preparation of Spore Suspension

Spore suspensions of fungal isolates were prepared individually using a semi-solid Corn Meal Agar (0.2%) and standardized to 5×10^7 spores/ml using haemocytometer.

Sterilization

Guava fruits were subjected to surface disinfection prior to inoculation. Guava fruits were soaked in 10% sodium hypochlorite for 15 minutes and rinsed with sterilized distilled water. The fruits were soaked again in 10% sodium hypochlorite for another 10 minutes and rinsed thrice with sterile distilled water. Finally, to remove excess water, the fruits were blot dried with sterilized tissue paper.

Inoculation of Fungal Isolates

Drop inoculation technique (Singh 2000) was used for infecting the guava fruit. Artificial wounds were made using sterile blood lancets. Each guava fruit was pricked 30 times near the crown and 1 ml of fungal inoculum was inoculated on the artificial wounds. For the control, guava fruits were inoculated with sterile distilled water.

Incubation

Guava fruits were placed in clean culture bottles lined with moistened tissue paper to maintain the humidity inside the bottle. Then, culture bottles were covered with cheese cloth and were incubated at 28–30 °C for 7 days.

Observation

Close inspection on the fruits was made for the occurrence of crown rot infection. Symptoms diagnoses were based from Streets RT (1969), Agrios (1997), and Singh (2000). Symptoms are as follows: 1. The infection will occur through the cut ends and spreads into the crown of guava; 2. At first, it will appear soft, slightly discolored spots of varying size; 3. Molds will begin to grow at the center of the spots, mycelium of the fungus will be observed; 4. The diseased portion will turn black and dries; 4. Black spots are seen near the crown portion.

Re-isolation of the Fungi

Fungi were re-isolated from the advancing margin of the infected tissue of the guava fruit and were inoculated in PDA plates. Cultures were incubated at 28–30°C for 5 to 7 days.

Identification of the Isolated Fungi

This was done to prove that the isolated organism is the same organism that has been inoculated causing the specific disease. Identification was based on cultural and morphological characteristics of the fungal isolates. For the cultural characterization, fungal isolates were grown in PDA plates using the triple point inoculation technique. Cultures were incubated at 28–30°C for 5 to 7 days. The number of days before growth, the rapidity and luxuriance of growth, and color of the colony were noted. While for the morphological characterization, agar block technique and direct microscopic observation were employed. Taxonomic criteria and key by Frazier & Westhoff (1978) were used as basis for the confirmation of the identity of the fungal isolates.

Results and Discussions

There is a great diversity of fungal phytopathogens causing diseases to a wide range of agricultural crops which include species of genus *Alternaria, Aspergillus, Botrytis, Fusarium, Lasiodiplodia, Monilinia, Penicillium,* and *Rhizopus* (Ogawa et al. 1995). Li-Cohen & Bruhn (2002) and Singh & Sharma (2007) affirmed that the susceptibility of crops to fungal diseases is due to the high levels of sugars, carbohydrate, protein, fat and low pH values of fruits. Samson (1986) reported that the crude protein, carbohydrates, cruder fat content of the guava fruits were 7, 11, and 17.1%, respectively. Strobel & Mathre (1970) stated that fungal spores penetrate the surface of the fruits through artificial wounds; however, it does not always lead to infection and they cannot proceed beyond the stage of penetration and die without producing disease.

As presented in Table 1, among eight species of fungi associated with crown rot of guava, only five were found to be phytopathogenic causing crown rot on the wounded surface of guava fruits. These include all species *Aspergillus fumigatus, A. niger, A. tamarii, A. japonicus,* and *A. flavus.* The lesions caused by the phytopathogenic fungi consisted of dark brown to black discoloration on the infected area of the guava fruits after 7 days of incubation (Figs 1B-F).

Infections caused by the fungi vary in the appearance of symptoms and colonization of fungal mycelia. Crown rot disease in guava fruits was detected after 2 to 3 days of incubation. Brown discoloration on the crown of the fruits was observed on the second day of incubation, followed by the growth of mycelia on the 3rd day. Brown discoloration turned black on the 4th and 5th day of incubation. Depression on the surface of fruits was also noticeable in fruits inoculated with *Aspergillus niger* and *A. fumigatus* on the 4th to 5th day of incubation (Figs 1B & F). In addition, profuse sporulation of *A. niger* and *A. japonicus* on the surface of the infected guava fruits on the 5th day of incubation were also observed (Figs 1B & C). While the lesions produced by *A. flavus, A. fumigatus* and *A. tamarii* mummified and blackened on the 7th day of incubation (Figs 1D- F).

Accordingly, phytopathogenicity of genus *Aspergillus* in the recent study coincides with previous studies on Aspergilli as causal agents of fungal diseases. Kotan et al. (2009) characterized *Aspergillus* as wound-invading pathogen that causes decay on stored citrus fruits damaged by insects, animals, early splits, and mechanical harvesting. This also confirmed the reports of Chuku et al. (2008), Mathew et al. (2010), Akinmusire (2011) and Amadi et al. (2014), that *A. flavus, A. flumigatus* and *A. niger* are causal agents of postharvest spoilage in fruits including guava and tomatoes. Furthermore, members of genus *Aspergillus* are one of the major fungi species producing aflatoxin which were classified as toxin and carcinogenic compounds causing serious health implications, thus making fruits unfit for human and animal (Shenasi et al. 2002).

No signs of crown rot in guava were observed in fruits treated with *Fusarium sambucinum*, *F. verticilloides* and *Lasiodiplodia theobromae* (Figs 1G - I) indicating their non- pathogenicity. These fungi produce the same results with the control group wherein slight change in colour was observed, which can be attributed to the ripening of the fruits (Fig 1A). Although mycelia of these fungi were also observed, no sign of crown rot disease was observed.

According to Pitt & Hocking (1999), *Fusarium verticillioides* is an endophyte of maize. In addition, endophytic fungi are capable of living in host plants without causing any symptoms (Petrini et al. 1992). As stated by Munkvold & Desjardins (1997), this symptomless infection can exist throughout the plant in leaves, stems, roots, grains, and the presence of the fungus is in many cases ignored because it does not cause visible damage to the plant. In a previous study by Rubini (2005), species of *Fusarium* and *Lasiodiplodia* along with other species of fungi were isolated as endophytes of cacao and were identified as potential antagonist of *Crinipellis perniciosa*, a causal agent of Witches' Broom Disease.

Contrary to present results of phytopathogenicity test, Cardoso (2002) and Punithalingam (1980) cited *Lasiodiplodia* as one of the causal agent of stem end rot, dieback, root rot, fruit rot, blights, gummosis, stem necrosis, leaf spot, and witches' broom disease of tropical crops. Misra & Pandey (1999) also reported the phytopathogenicity of several *Fusarium* species causing wilt to guava plants. In addition, *Fusarium* are fumonisin producers, which are phytotoxic, damaging a wide variety of crops and have emerged as a highly visible animal and human health safety concern since they have been associated with many animal diseases such as leukoencephalomalacia and has been evaluated as possibly carcinogenic to humans (Fandohan et al. 2003). The results thus proved that the phytopathogenicity of fungi can be host and disease specific.

Table 1 Phytopathogenicity of fungal isolates

Fungal isolates	Phytopathogenicity
Aspergillus flavus Link	Phytopathogenic
Aspergillus fumigatus Fresenius	Phytopathogenic
Aspergillus niger van Tiegh	Phytopathogenic
Aspergillus tamarii Kita	Phytopathogenic
Aspergillus japonicus Saito var *japonicas*	Phytopathogenic
Fusarium sambucinum Fuckel	Non-phytopathogenic
*Fusarium verticilloides (*Saccardo) Niremberge	Non-phytopathogenic
Lasiodiplodia theobromae (Patouillard) Griffon and Maublanc	Non-phytopathogenic

Re-isolation of pathogenic fungi

Five species of phytopathogenic fungi were re-isolated from the infected area and cultural and morphological characteristics were observed for further verification. Slight changes on the cultural characteristics of the isolated molds were observed which can be associated with the interaction of fungi with the chemical composition and availability of nutrients of the host plant. However, morphological and cultural characterization of the fungal isolates based on Frazier & Westhoff (1978) proved the similarity of the initial inocula and the re-isolated pathogenic fungi. Thus, the confirmation of the fungal identity isolated from the infected fruits indicates that the five species of *Aspergillus* are the causal agents of crown rot of guava fruits.

In the study, the phytopathogenicity of the five species of *Aspergillus* in crown rot of guava fruits and the non-phytopathogenicity of *Fusarium sambucinum*, *F. verticillioides* and *Lasiodiplodia theobromae* were established. Thus, this would lead to the determination of the interaction and isolation of the bioactive compounds present in the pathogenic and the non-pathogenic species of fungi in search for biological control against fungal diseases in plants.

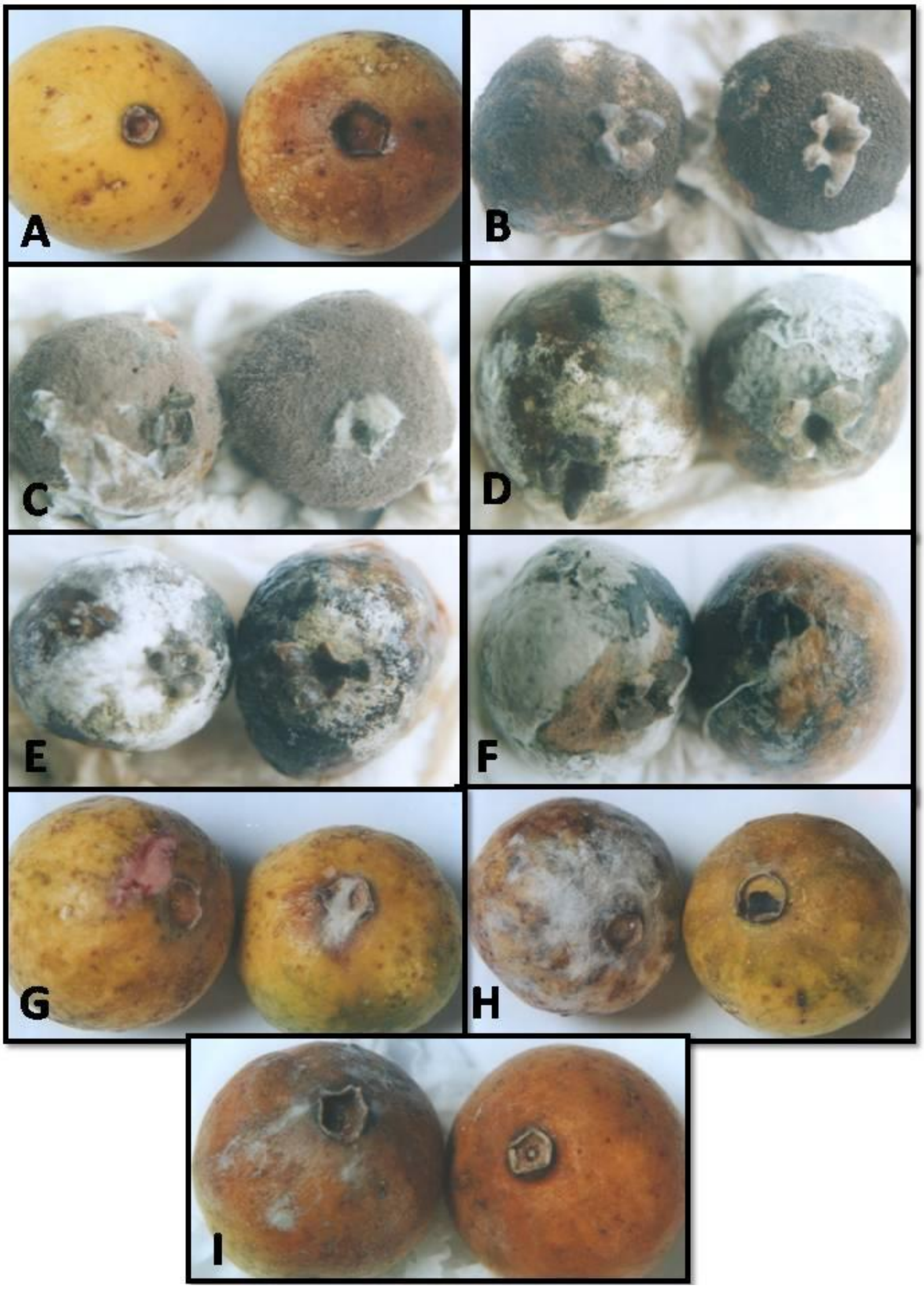

Figure 1 Guava fruits inoculated with (A) sterile distilled water, (B) *A. niger* (C) *A. japonicus* (D) *A. flavus* (E) *A. tamarii* (F) *A. fumigatus* (G) *F. verticilloides* (H) *F. sambucinum* (I) *L. theobromae* after 7 days of incubation

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