Field Survey and Fungicide Screening of Fungal Pathogens of Rambutan (*Nephelium lappaceum*) Fruit Rot in Hawaii

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Abstract. Rambutan (*Nephelium lappaceum* Linn.) is a tropical fruit in Hawaii that has increased in value in the niche market of exotic fruits. The primary limitation to preharvest and postharvest quality is the occurrence of fungal diseases of the fruit. A survey of rambutan disease was conducted at orchards 9.7 km south and 29.0 km north of Hilo, HI, to determine the main fungal pathogens affecting preharvest fruit quality. Pericarp of rambutan fruit revealed brown to black lesions that progressed to blackening and drying of the fruit with some fruits becoming totally mummified. Four fungi consistently isolated from symptomatic fruit included *Lasmenia*, *Pestalotiopsis*, *Phomopsis*, and *Colletotrichum* spp. Over the 2-year sampling period, disease incidence from more than 300 fruits sampled was 84.6%. Nine rambutan cultivars were evaluated for disease incidence under field conditions. Disease incidence was greater than 90% for ‘Sitangku’ and ‘R167’ but less than 60% for ‘Chompoo’. Twelve fungal isolates from infected fruit were selected for further characterization (six *Lasmenia* isolates and two isolates each of *Pestalotiopsis*, *Phomopsis*, and *Colletotrichum*). Morphology, colony characteristics, and pathogenicity of the isolates were examined. The optimum growth temperature for all fungal isolates ranged between 22 and 28 °C. Molecular methods were used to confirm the identity of the fungi. The fungal isolates were evaluated for in vitro baseline sensitivities for mycelial growth for fungicides registered for use in Hawaii (Abound® and Trilogy®). Abound® was more effective at inhibiting fungal growth than was Trilogy®; however, efficacy appeared to be influenced by fungal genera. Inhibition of fungal growth by Abound® ranged from a 76% reduction for *Lasmenia* to a 23% reduction for *Phomopsis* isolates.

Rambutan is a tropical tree in the Sapindaceae that produces delicious edible fruits, is native to Malaysia and Indonesia, and is commonly cultivated throughout Southeast Asia (Morton, 1987). Rambutan flourishes from sea level to 600 m in tropical, humid regions having well-distributed rainfall (Morton, 1987). Depending on location, rambutan can produce up to two crops a year

(Laksmi et al., 1987). Although these plants originated in Asia, they are now being grown for commercial production in other tropical regions. In Hawaii, ≈1 million kg of tropical specialty fruits (including longan and lychee) were grown in 2008 [National Agricultural Statistics Service (NASS)-USDA, Hawaii Field Office, 2009]. Valued at \$4.0 million, this group of tropical fruits garnered higher farm prices than other fruit crops in this niche exotic fruit market (NASS-USDA, Hawaii Field Office, 2009).

A number of fungal pathogens that attack rambutan affect both quality and quantity of the produce. Long-distance transportation of rambutan has been impeded by its very short shelf life resulting from fruit diseases, which may begin in the field or postharvest on fruit damaged during harvesting, packaging, or transportation (Farungsang et al., 1994a). Fruit rot can be caused by various fungal pathogens (Chayasombat and Sangchote,

1983; Visarathanonth and Ilag, 1987). *Lasmenia* and *Gliocephalotrichum* have been previously reported as fungal pathogens on rambutan in Hawaii (Nishijima et al., 2002). *Dolabra nepheliae* causes a stem canker disease on rambutan; although it is classified as a minor disease by the Food and Agriculture Organization, it may have the potential to cause damage to fruit production (Rossman et al., 2007). Information on the identity and biology of the main fungal species that cause disease and understanding the disease epidemiology and potential for cross-infection are important so that effective control measures can be developed and implemented (Hopkins and McQuilken, 2000).

Superior fruit quality is necessary for Hawaii’s specialty fruit industry to have a competitive advantage. To develop an integrated system of preharvest and postharvest practices that enable specialty tropical fruit growers to manage diseases, improve fruit quality, and extend shelf life, the economically important fungal pathogens must be identified, etiology information must be understood, and control measures must be developed. The objectives of this study were to: 1) identify the causal agent(s) of commonly observed symptoms of fruit rot of rambutan in Hawaii and establish pathogenicity by fulfilling Koch’s postulates; 2) compare fungal isolates by studying pathogenicity, morphological, cultural, and molecular characteristics; 3) examine the effect of temperature on growth of the pathogens; and 4) determine the in vitro efficacy of fungicides currently registered for use on rambutan in Hawaii.

Materials and Methods

Field observations and symptoms. During 2006 to 2007, rambutan trees with fruit were examined at the Tropical Plant Genetic Resource and Disease Research Unit orchard located at the University of Hawaii, Waikeae Agricultural Experiment Station, Hilo, HI, and at a nearby commercial farm in Paukaa, HI. The sites are 9.7 km south and 29.0 km north of Hilo, respectively, with an elevation ranging from 74 to 227 m. Maximum and minimum mean temperatures are 28 and 16 °C, respectively. Annual rainfall averages from 3632 to 4445 mm and is most abundant during October to February. The soil consists of an extremely stony Papai muck with organic soils formed over mostly fragmental a’u lava. Digital photographs of leaf and fruit symptoms were recorded using a Nikon Coolpix 995 model digital camera (Melville, NY).

Collection of isolates. Naturally infected, symptomatic fruit of nine rambutan cultivars were collected from fields in Hilo and the surrounding area. All fungi were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) at 24 °C under continuous fluorescent illumination, and single spore cultures were derived from each isolate and stored at room temperature (20 °C) in sterile distilled water (SDW).

Initial identification and cultural characteristics. The fungal isolates derived from

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single spores were maintained on PDA. Cultures were incubated at 24 °C in continuous light and cultural morphology was examined after 7 to 30 d. Colony color was defined according to Raynor (1970). For *Pestalotiopsis*, isolates were identified by comparing morphological and cultural characteristics (i.e., size of the conidia, color and length of median cells, length and number of apical appendages, and length of basal appendage) to those described in Guba's (1961) monograph of *Monochaetia* and *Pestalotia*. *Lasmenia*, *Phomopsis*, and *Colletotrichum* spp. were identified based on morphological and physiological characteristics and on literature reports (Farungsang et al., 1994b; Nishijima et al., 2002).

Temperature effects on mycelial growth. Five replicate, 10-cm diameter petri dishes containing PDA (25 mL) were inoculated centrally with an agar disc (5 mm diameter) of a subset of fungal isolates cut from the edge of an actively growing culture on PDA. The effect of temperature on colony diameter was determined after 4 and 7 d at 10, 15, 20, 26, 28, 32, and 37 °C under continuous fluorescent illumination. All temperature experiments were conducted twice.

Pathogenicity tests. Pathogenicity tests consisted of inoculations of harvested rambutan fruit (cultivar R134). Before inoculation, fruit were surface-disinfested by immersion in 10% bleach solution (0.5% sodium hypochlorite) for 2 min, rinsed in SDW, air-dried in a laminar flow hood, and placed in plastic chambers containing moistened paper towels. Fruit were wounded with a sterile cork borer and inoculated with mycelial discs (3 mm diameter) of fungal isolates grown for 5 d at 28 °C. Controls were inoculated with PDA discs only. Fruit were observed for symptoms over a period of 7 d. To fulfill Koch's postulates, diseased tissue was placed on water agar and PDA and observed for culture characteristics similar to that of the inoculum strains used for inoculations. Experiments were repeated at least twice with similar results.

Molecular identification. Total genomic DNA was extracted from fungal isolates following the protocol described in Keith et al. (2006). Polymerase chain reactions (PCRs) to amplify ITS Regions 1 and 2 were largely based on Caetano-Anolles et al. (2001) and White et al. (1990). PCR products were cloned (TA cloning kit; Invitrogen Co., San Diego, CA) and plasmid DNA for sequencing was prepared (Qiagen plasmid miniprep kit, Chatsworth, CA). DNA sequencing was performed at Eurofins MWG Operon (Huntsville, AL). Similarity searches of the GenBank database were performed with BLAST (Altschul et al., 1990).

Determination of sensitivity of fungal isolates to fungicides. Efficacy of two fungicides registered for use on rambutan in Hawaii to inhibit fungal growth was determined using amended media. For all studies, commercial formulations were used: azoxystrobin, Abound® 2.08 EC (Syngenta Crop Protection, Greensboro, NC) and clarified

hydrophobic extract of Neem Oil, Trilogy® (Certis USA, Columbia, MO). These fungicides were diluted in sterile water and added to molten PDA to obtain final concentrations as recommended by the manufacturer. Sensitivities of fungal isolates to Abound® and

Trilogy® were tested using radial growth (RG) assays. Each isolate (Table 1) was cultured on PDA at 24 °C for 7 d. Five-millimeter-diameter mycelial plugs were cut from the margins of colonies and transferred onto three PDA dishes amended with each

Table 1. Fungal isolates used in this study, their cultivar hosts, and sample origin.

Isolate	Name	Cultivar	Plant part
20-3B	<i>Lasmenia</i> sp.	Daun Hijau	Mature rambutan fruit, base of spintern
9-5A	<i>Lasmenia</i> sp.	Sileng Keng	Mature rambutan fruit, spintern
10-5	<i>Lasmenia</i> sp.	Sileng Keng	Mature rambutan fruit, spintern
25-2	<i>Lasmenia</i> sp.	Sitangku	Mature rambutan fruit, rind
9-2	<i>Lasmenia</i> sp.	Sileng Keng	Mature rambutan fruit, spintern
9-5B	<i>Lasmenia</i> sp.	Sileng Keng	Mature rambutan fruit, spintern
15-5 R12T12	<i>Pestalotiopsis</i> sp.	R167	Mature rambutan fruit, rind
15-2	<i>Pestalotiopsis</i> sp.	R167	Mature rambutan fruit, rind
21-4B	<i>Colletotrichum</i> sp.	Gula Batu	Mature rambutan fruit, base of spintern
25-4	<i>Colletotrichum</i> sp.	Sitangku	Mature rambutan fruit, spintern
7-2B	<i>Phomopsis</i> sp.	Chompoo	Mature rambutan fruit, rind
15-4	<i>Phomopsis</i> sp.	R167	Mature rambutan fruit, spintern

Fig. 1. Symptoms of fruit disease of rambutan seen in the field. (A) Discrete dark brown lesions on the fruit surface and dark brown to black, coalescing lesions giving rise to dried and cracked appearance (cultivar Jitlee). (B) Mature cultivar Binjai fruit with dark brown lesions on rind and spinterns.

Fig. 2. Disease incidence of the four main fungal pathogens (*Lasmenia*, *Pestalotiopsis*, *Phomopsis*, and *Colletotrichum* spp.) found on several rambutan fruit cultivars grown at orchards in Waiakea and Paukaa.

concentration of fungicide (Trilogy® = 1/3× or 3,380 ppm, 1× or 10,140 ppm, and 3× or 30,420 ppm; Abound® = 118.3 mL/378.5 L or 75 ppm, 183.4 mL/378.5 L or 116 ppm, and 455.4 mL/378.5 L or 289 ppm). Plates were maintained at 24 °C with continuous lighting. Colony diameter (RG) was measured across two axes, averaged, and the diameter of the mycelial plug subtracted from the average after 4 and 7 d. Growth inhibition in response to each fungicide at varying concentrations was compared with the control plates (PDA only). Each experiment was conducted twice.

Results

Field observations and symptoms. A pre-harvest fungal disease survey on rambutan was conducted at the Waiakea Agricultural Experiment Station and at a local farm. Disease symptoms were visible on leaves during non-fruiting seasons and on the exocarp of mature and immature fruits (pinhead size), which progressed as fruits got larger. Typical symptoms included tiny brown to black spots on the spinterns or on the surface of the fruit (Fig. 1A–B). The spots darkened in color and became crusty in appearance. The tiny spots progressively expanded to discrete, circular, dark brown to black spots, which dried out and developed into cracks in the pericarp.

Isolation and identification. Isolates were obtained over a 2-year period from more than 300 immature and mature fruit samples exhibiting disease symptoms. The single-spore fungal cultures were maintained on PDA and stored in SDW for additional studies. The study identified *Lasmenia*, *Colletotrichum*, *Pestalotiopsis*, and *Phomopsis* as the predominant pathogens in the Waiakea fields. The disease incidence varied among the nine cultivars of rambutan ('Sileng Keng', 'R167', 'R134', 'Daun Hijau', 'Gula Batu', 'Chompoo', 'Sitangku', 'Binjai', and 'Jitlee') (Fig. 2). *Lasmenia* was the most frequently isolated pathogen from lesions. Disease incidence for *Lasmenia* was greater than 88% for 'Sileng Keng' but less than 48% for 'R167'. Disease incidence for *Pestalotiopsis* was greater than 41% for 'R167' but less than 7% on 'Daun Hijau'. Disease incidence for *Phomopsis* was relatively low for 'R167', 'R134', and 'Chompoo' (less than 7%). Results were similar for *Colletotrichum* on 'Sitangku'.

'Jitlee', 'Binjai', and 'R167' rambutan trees were surveyed for incidence and etiology of fruit rot infection at a commercial grower's farm in Paukaa, and results were similar to those found at the Waiakea fields. Fruit rot incidence was greatest for *Lasmenia* at 64.3% of all fungi identified. 'Binjai' had less incidence to fungal infection compared with the other two cultivars. *Phomopsis* and *Pestalotiopsis* were also isolated along with *Gliocephalotrichum* and *Lasiodiplodia*, although incidence was less than 7%.

A second etiological survey of cultivars Binjai, Jitlee, and R167 was conducted in Paukaa, HI, and 72 isolations were obtained

from 24 fruits. Several fungal strains were isolated and included *Lasmenia* (48%), *Pestalotiopsis* (8%), *Gliocephalotrichum* (14.2%), and *Phomopsis* (1.4%). A cultivar effect was seen with certain pathogens. 'Binjai', 'Jitlee', and 'R167' were equally infected with *Lasmenia* (55% incidence). However, 'Binjai' and 'Jitlee' had lower incidences of *Pestalotiopsis* than R167 (9% compared with 41%, respectively).

Temperature effects. Temperature had an effect on the colony diameter of the 12 isolates examined (Fig. 3). When grown on PDA, most isolates grew at a temperature range of 10 to 32 °C with optimum growth between 20 and 28 °C. Within 7 d, the majority of the diameter of mycelial growth of the isolates measured ≈80 mm, the diameter of the PDA plate. The temperature range for growth of five of the six *Lasmenia* analyzed

was 10 to 32 °C with the optimum being 20 to 28 °C. The temperature range for growth of the majority of the *Pestalotiopsis*, *Phomopsis*, and *Colletotrichum* isolates was 10 to 32 °C. One isolate each of *Pestalotiopsis* (15-2) and *Colletotrichum* (25-4) was able to grow at 37 °C.

Pathogenicity tests. Mature rambutan fruit of cultivars Binjai, Jitlee, and R167 were inoculated in the laboratory with the six *Lasmenia* isolates, two *Pestalotiopsis* isolates, two *Phomopsis* isolates, and two *Colletotrichum* isolates to confirm pathogenicity and to determine if wounding was necessary for infection. Inoculated fruit were maintained in a moist chamber at room temperature for 7 to 10 d. Brown lesions resembling symptoms that occurred in the field were observed surrounding the inoculation sites beginning at Day 5 (data not shown). Symptoms

Fig. 3. Influence of temperature (10, 15, 20, 26, 28, 32, and 37 °C) on mycelial growth of (A) *Lasmenia* isolates and (B) *Pestalotiopsis* (15-5 R12T12 and 15-2), *Phomopsis* (7-2B and 15-4), and *Colletotrichum* (21-4B and 25-4) isolates from diseased rambutan fruit. Growth after 7 d on potato dextrose agar. Each bar represents mean ± SEM (n = 5).

were not observed on control fruit inoculated with agar media. Wounding was necessary for symptom development for *Pestalotiopsis*. Wounding was not necessary for the three other genera; however, it decreased the time necessary for onset of infection. The fungi were reisolated from the lesions of the diseased fruit and were identical to the original isolates, thus confirming Koch's postulates.

Determination of sensitivity of fungal isolates to fungicides. In vitro fungicide resistance screening tests were conducted with the 12 *Lasmenia*, *Phomopsis*, *Pestalotiopsis*, and *Colletotrichum* isolates. Trilogy® was used at concentrations equal to 1/3×, 1×, and 3× the recommended field concentration for tropical fruits (where 1× = 1% or 10,140 ppm) (Fig. 4). Fungi exhibited mild susceptibility to Trilogy® with only a 57% reduction in growth rate observed for *Lasmenia* and a 17% reduction for *Phomopsis*, *Pestalotiopsis*, and *Colletotrichum* depending on concentration, indicating that Trilogy® may be ineffective for control in the field. In vitro fungicide screening results for Abound® showed more promise (Fig. 5). *Lasmenia* growth rates were reduced by more than 76% using recommended rates for tropical fruits (6.2 to 15.4 fl. oz. product/A). The growth of the other strains (*Pestalotiopsis* and *Colletotrichum*) was reduced 46%. However, Abound® only resulted in a 23% reduction in growth for *Phomopsis*, indicating that not all fungi are inhibited to the same degree.

Discussion

Four pathogens, *Lasmenia*, *Pestalotiopsis*, *Phomopsis*, and *Colletotrichum* spp., were identified as the main fungal pathogens causing fruit rot on Hawaii-grown rambutan. Field symptoms were similar for all four genera (tiny brown to black spots on the spinterns or on the rind of the fruit, which expanded over time resulting in a dried out or crusty-looking appearance), which makes fungal identification extremely important for disease control. The fungi were isolated from lesions on fruit at all levels of maturity. In the Philippines, postharvest losses in rambutan resulting from disease have been reported to be ≈30% to 40% (Visarathanonth and Ilag, 1987). In a study by O'Hare et al. (1994), all organisms isolated from diseased rambutan were fungal; *Pestalotiopsis* sp., *Colletotrichum* sp., and *Phomopsis* sp. were detected on all cultivars analyzed, including 'R162', 'Jit Lee', and 'R156'. They have been previously identified as rambutan pathogens (Farungsang et al., 1991; Visarathanonth and Ilag, 1987) and are similar to the results of our study. In Thailand, *Pestalotiopsis* fruit rot is one of the most serious postharvest diseases of rambutan (Sangchote et al., 1998). *Pestalotiopsis* also causes gray spot disease on rambutan in China (He et al., 2010). For most rambutan fruit rots, fungicide applications before and after harvest help control the pathogens (Farungsang et al., 1994b; Saenyoung

Fig. 4. Influence of Trilogy® treatment on in vitro mycelial growth of (A) *Lasmenia* isolates and (B) *Pestalotiopsis* (15-5 R12T12 and 15-2), *Phomopsis* (7-2B and 15-4), and *Colletotrichum* (21-4B and 25-4) isolates from diseased rambutan fruit. Growth after 7 d at 24 °C on potato dextrose agar (PDA) only or PDA amended with different concentrations of Trilogy® (1/3× = 3,380 ppm, 1× = 10,140 ppm, and 3× = 30,420 ppm). Each bar represents mean ± SEM (n = 3).

and Visarathanonth, 1985). Abeyesekere et al. (1997) also found that fungicide applications could control the three major postharvest diseases of rambutan in Sri Lanka.

All the pathogens grew well at temperatures commonly found in Hawaii orchards. Sangchote et al. (1992) found that storage temperatures influenced the spectrum of fungi associated with postharvest rambutan decay. Koch's postulates were confirmed, and we found that wounding before inoculation was necessary for disease development for *Pestalotiopsis* sp. According to Visarathanonth and Ilag (1987), wounding was necessary for rapid penetration and severe infection of rambutan and explained why mechanically injured fruit were prone to fruit rot. In our study, wounding was not necessary to establish infection by *Lasmenia*, *Phomopsis*, and

Colletotrichum spp. suggesting that virulence is greater in these fungi and proper field management practices are crucial for disease control. Although the in vitro studies may not directly simulate the conditions of the natural environment, the results provide insight to the likely behavior and growth of the pathogens in nature. The in vitro growth studies may also indicate that disease development could be delayed during postharvest storage by the inhibition or slowing of fungal growth by low temperature (Johnson and Cooke, 1990).

Anthraxnose, caused by *Colletotrichum gloeosporioides*, is a serious disease of rambutan that affects leaves, flowers, and harvested fruit in high rainfall growing regions including the Philippines, Sri Lanka, and Thailand; fruit infection occurs in the field

Fig. 5. Influence of Abound® treatment on in vitro mycelial growth of (A) *Lasmenia* isolates and (B) *Pestalotiopsis* (15-5 R12T12 and 15-2), *Phomopsis* (7-2B and 15-4), and *Colletotrichum* (21-4B and 25-4) isolates from diseased Hawaiian rambutan fruit. Growth after 7 d 24 °C on potato dextrose agar (PDA) only or PDA amended with different concentrations of Abound® (4 oz/100 gal = 75 ppm, 6.2 oz/100 gal = 116 ppm, and 15.4 oz/100 gal = 289 ppm). Each bar represents mean $\pm$ SEM (n = 3).

but usually remains quiescent until fruit ripens or is harvested (Farungsang et al., 1991; Sivakumar, 1996; Visarathanonth and Ilag, 1987). Anthracnose is best controlled by a combination of sanitation; field applications of fungicides, including mancozeb, zineb, and/or benomyl from flowering to harvest; and optimum postharvest storage (Tindall, 1994; Visarathanonth and Ilag, 1987). In the study by Sivakumar et al. (1999), accumulation of leaf litter led to significantly higher levels of brown spot disease, indicating that the presence of leaf litter was a constant source of fungal inoculum, especially during wet weather when rain splash can carry the inoculum onto the leaf and fruit surface. Factors that favor fungal infection of rambutan include: inadequate field spraying to reduce latent infection and surface contamination, injuries that occur during harvesting, and high humidity and temperature during packaging,

storage, and transportation (Visarathanonth and Ilag, 1987). Most postharvest problems of rambutan are caused by fungi, which infect the fruit in the field. Proper management practices such as a program of field spraying, postharvest fungicide applications, and careful handling practices can help to minimize the development of fruit rots in rambutan, but spraying with the appropriate fungicides must begin at flower set and continue until harvest (Visarathanonth and Ilag, 1987). Farungsang et al. (1994b) found that preharvest chemical application could slightly reduce postharvest disease incidence with Carbendazim being the most effective fungicide. However, continuous application of fungicides can enhance fungal pathogen development of resistance against chemical fungicides (Griffie, 1973; Ogawa et al., 1983). The results of this study indicate that Trilogy® may not be effective as a preharvest

fungicide. Field studies are necessary to determine if additional fungicides currently registered for use in Hawaii can help control pre- and/or postharvest fruit rot caused by the four fungal pathogens identified in this study.

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