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Fungal quiescence in fruit: an attempt to avoid toxic substances?

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Fungal pathogens infecting fruits have to face challenges that the pathogens infecting vegetative plant organs do not generally encounter. Fungi such as *Colletotrichum* are known to infect fruit species at a very early stage of maturity in the orchard and remain quiescent. Such quiescent fungal infections have been observed in tropical and subtropical fruits. The transition of a quiescent fungal infection into a progressive one usually takes place during fruit ripening. Thus, in the context of postharvest diseases, a quiescent infection involves the inhibition of development of the pathogen through physiological conditions imposed by the host until some maturation has been accomplished. Quiescence of fungal infections may be resulted from inhibitory factors in the host. Unripe fruits are protected by chemical barriers in the superficial tissues, involving constitutive and inducible antifungal substances. These are believed to cause infections to remain localized and quiescent in unripe fruits. The onset of decay coincides with fruit ripening and concurrent decrease in the antifungal compounds to sub-toxic levels. Quiescence may therefore represent a mechanism for avoiding antifungal compounds present at toxic levels. In certain fruits in which the inducible defence system is weaker, the preformed antifungal compounds appear to perform a definitive protective role. In other fruits, the preformed antifungal substances appear to play a supportive role to their strong arsenal of inducible defences by excluding saprophytic and epiphytic microorganisms. Fungi causing quiescent infections are comparatively more difficult to control than wound invaders. Losses of fruits due to postharvest rotting could be reduced if fungal infections can be kept in their quiescent phase for extended periods during storage, transport and marketing. One of the possible ways to prolong quiescence is to maintain the natural antifungal barrier present in the unripe fruit at an inhibitory level into the post-climacteric phase. This paper will evaluate the antifungal systems in avocado (*Persea americana*) mango (*Mangifera indica* L.), papaya (*Carica papaya* L.) fruit in relation to quiescence of *Colletotrichum* species and subsequent rot development during fruit ripening.

Key words: Fungal quiescence, *Colletotrichum*, antifungal substances

Quiescent Colletotrichum infections

Ripe rots of tropical and subtropical fruit, during transit and storage, often originate from latent or quiescent infections by fungi long before harvest. The fungi most commonly associated with quiescent infections belong to the Genus *Colletotrichum*. The development of quiescent infections into progressive rotting generally takes place during fruit ripening. Shear and Wood (1913) were among the

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first to call attention to the occurrence of dormant infections of the type referred to here. They noted the development of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. on leaves and shoots of orange, pomelo, lemon and mandarin after penetrating the epidermis for only a short distance and remaining latent until the host became weakened. Since then many reports have been made of the occurrence of latent infections in various fruit crops and it is now a widely recognized phenomenon.

Appressoria as quiescent structures

The means by which quiescent infections are brought about by fungi have been studied and appressoria formation is believed to plays an important part in this process. Generally conidia germinate on the fruit surface by producing a short germ-tube followed by an appressorium. Species of *Colletrotrichum* which are most commonly forming quiescent infections produce particularly distinct appressoria (Emmett and Parberry, 1975). The typical mature appressorium as it occurs in this genus is a dark, melanized, irregularly-lobbed body with a thickened wall (Fig. 1) enclosing deeply staining protoplasmic contents. Younger appressoria appear hyaline (Muirhead and Deverall, 1981). The appressorium germinates to form an infection peg which pierces the cuticle and lodges between the cuticle and the epidermis and remains quiescent. The formation of appressoria while facilitating penetration of the host plays a role in survival under adverse conditions. Recent investigations have shown that appressoria either remain dormant on the surface of the host or the infection peg penetrates the cuticle but does not invade the epidermal cell.

Basis of quiescence

A common feature of quiescent infections is the inability of the pathogen to develop progressive lesions in immature fruit. This suggests that resistance mechanisms operate in the immature fruit in response to the presence of the pathogen. The transition of a quiescent relationship between a fungus and an immature fruit into a progressive one usually takes place during ripening of the fruit. The nature of resistance of immature fruit to progressive rotting has interested several workers, and was systematically studied by Simmonds (1963) with respect to banana anthracnose and reviewed by Verhoeff (1974), Swinburne (1983) and Prusky and Keen (1993).

Essentially four hypotheses have been explored to explain fungal quiescence, (i) Nutritional differences between immature and ripening fruit with respect to fungal development, (ii) Metabolic changes associated with ripening and alteration in respiratory pattern, (iii) The insufficient enzyme potential of the fungus to invade unripe fruit, and (iv) Presence of toxic compounds in unripe but not in ripening, or fully ripened fruit (Simmonds, 1963). Swinburne (1983), however, suggested postinfectional changes as a cause for temporary resistance expressed by some immature fruits forcing the infections to become quiescent. These postinfectional changes may be synthesis of lignin or more likely the synthesis of fungitoxic substances, phytoalexins.

Fungitoxic substances in the immature fruit

Quiescence of fungi in a number of fruit species has been implicated to the presence of preformed (Prusky and Keen, 1993; Adikaram *et al.*, 2010) or induced antifungal substances (Adikaram *et al.*, 1982). The onset of decay coincides with fruit ripening and concurrent decrease in the antifungal compounds to sub-toxic levels. Thus, quiescence may therefore represent a mechanism for avoiding toxic levels of antifungal plant compounds. There is considerable interest in determining mechanisms underlying the natural resistance of unripe fruits to fungal pathogens and extending fruit resistance to postharvest ripening phase. In fruits such as avocado, mango and wood apple (Adikaram *et al.*, 1989) where inducible defence system is weaker, preformed antifungal compounds appear to perform a protective role. In other fruits, in which inducible defences are prominent the preformed antifungal substances appear to play a supportive role to their arsenal of inducible defences by excluding saprophytic and epiphytic microorganisms.

Mango (Mangifera indica L.) anthracnose Causal agent/s

Anthracnose (Fig. 1) is the most common and predominant disease affecting the mango crop worldwide (Dodd *et al.*, 1991). The disease was long believed to be caused by *C. gloeosporioides* and more recently *C. acutatum* was also reported to be associated with the disease. However, in a recent study, DNA sequences of gene regions, glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) [308 bp] and â-tubulin 2 (TUB2)[716 bp], of *Colletotrichum* isolates obtained from four mango varieties in Sri Lanka were analysed. Aligning of gene sequences using NCBI BLAST>95% similarity revealed the isolates to be *C. asianum, C. fructicola* and *C. siamence*, indicating that these are the common causative agents of mango anthracnose in Sri Lanka. These species are grouped under Musae clade of *C. gloeosporioides* complex (Weir *et al.*, 2012). Most of the *Colletotrichum* isolates resembled, or were intermediate between, *C. gloeosporioides* and *C. acutatum*, in their conidial morphology. None of the sequences from 18 mango isolates matched with *C. acutatum* or with *C. gloeosporioides*. The study showed that the morphological features are not a reliable for identification of *Colletotrichum* species (Vithanage *et al.*, 2014 a,b).

resorcinols (Droby *et al.*, 1986), gallotannins (Fig. 2) and chitinases (Karunanayake *et al.*, 2011). Mango fruit peel contains a mixture of three closely related gallotannins with glycosidic linkages (Fig. 2). Gallotannins appear to be confined to the tissue of the fruit peel (Adikaram *et al.*, 2010). The gallotannins are directly inhibitory to the *Colletotrichum* isolates causing mango anthracnose. The methanol extract of fruit peel, when bioassayed with *Cladosporium cladosporioides* or *Colletotrichum* spp., produced a prominent inhibition zone at Rf. 0.00 (Fig. 2). Antifungal activity due to gallotannins was higher in the peel of unripe fruit at harvesting maturity and declined gradually during ripening. By colour break stage, the antifungal activity had declined by about 20% from what it was at harvest. At the ripe stage, when anthracnose development occurred in cultivar

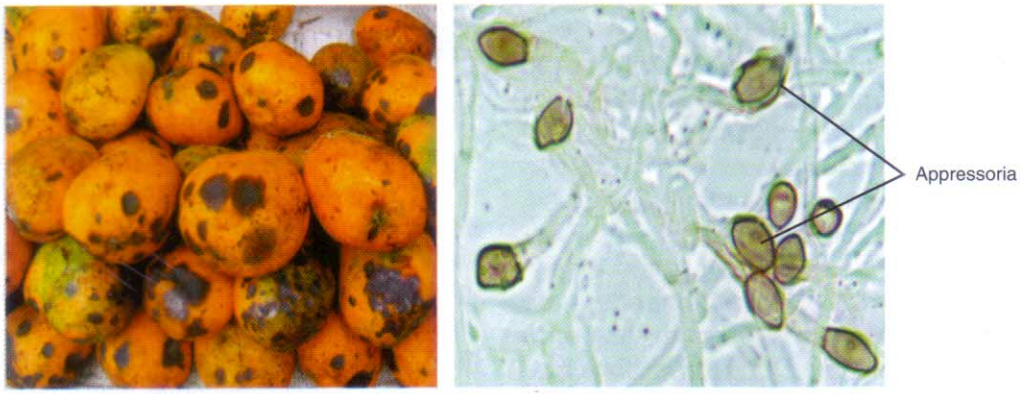

Fig. 1 : Ripe mango fruits affected by anthracnose disease (left); appressoria produced by *Colletotrichum* isolated from mango

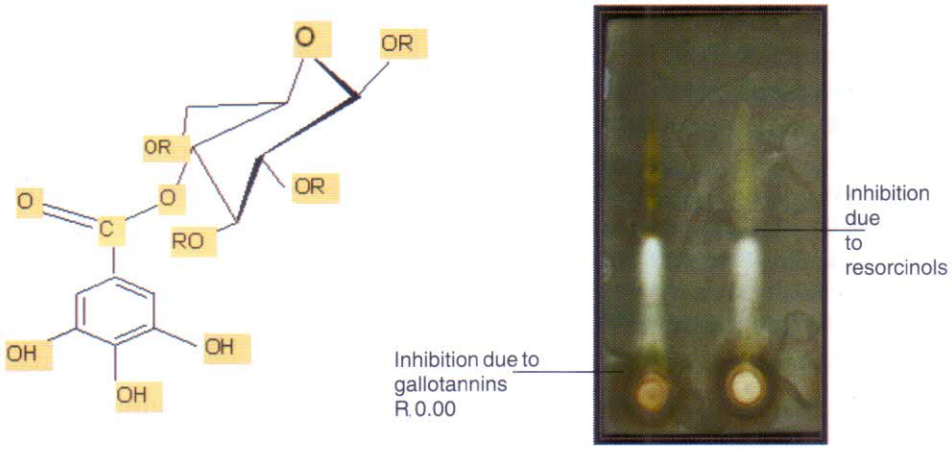

Fig. 2 : (a) Structure of gallotannins, (b) Thin layer chromatography bioassay of dichloromethane: methanol (1:1, v/v) extract of unripe mango fruit (Karunanayake *et al.*, 2011)

Preformed antifungal compounds in mango (Mangifera indica) fruit

Immature mango fruits have evolved a formidable defence system comprising constitutive antifungal

Karutha Colomban', the antifungal activity had declined to about 50% of the initial level (Karunanayake, 2008).

A significantly higher amount of antifungal resor-

cinols (Adikaram *et al.*, 2010) and chitinases are located in the latex which is distributed within a fine net work of minute canals. High Pressure Liquid Chromatography of the dichloromethane phase of peel extracts of two Sri Lankan cultivars, 'Karutha Colomban' and 'Willard', showed peeks corresponding to 5-(12-*cis*-heptadecenyl) resorcinol, 5-pentadecyl resorcinol and an additional peak due probably to a new resorcinol (Karunanayake, 2008). Two other resorcinols, 5(7, 12-heptadecadienyl) resorcinol from the fruit peel (Prusky *et al.,* 1996) and 5-(9, 12-heptadecadienyl) resorcinol from the latex (Oka *et al.*, 2004), have been reported. In the unripe fruit where the fungus forms quiescent infections, the resorcinols are present at higher concentrations and declined during fruit ripening to very low levels when anthracnose rot development commenced (Karunanayake *et al.*, 2011; Hassan *et al.*, 2007).

When the conidia of *Colletotrichum* species exposed to undiluted aqueous phase of mango latex, their walls were gradually digested. During early hours, a slight granulation was visible in the conidia and later the conidial wall was gradually dissolved. Three chitinases with molecular weights 47 KDa, 87 KDa and 97 KaD were present in the mango latex (Karunanayake, 2008). Chitinases were present in the aqueous phase of mango latex which hydrolyzes cell wall of *Colletotrichum* spp. (Karunanayake *et al.*, 2011). The level of chitinase activity in the aqueous phase varied with the mango cultivar. The chitinase activity was higher in the cultivar resistant to anthracnose, 'Rata' than in the more susceptible cultivar 'Willard'. The fact that in cultivars that are resistant to anthracnose disease, there was a higher concentration of 5-(12- *cis*-heptadecenyl), 5-pentadecyl and AR 21 resorcinol, than in susceptible ones (Karunanayake *et al.*, 2014) confirmed its involvement in fruit resistance. There was a strong positive correlation between the level of resorcinols and the degree of resistance to *Colletotrichum spp.* in different mango cultivars. A high correlation exists between the concentration of resorcinols in mango latex and the percentage (w/w) of the non-aqueous phase of mango latex (Hassan *et al.*, 2007; Hassan *et al.*, 2009). The concentration of the 5-substituted resorcinols decreased faster during ripening in cultivars like `*Willard*' susceptible to anthracnose than in somewhat resistant cultivars, `Karutha Colomban' (Karunanayake *et al.*, 2014). Mango cultivars more resistant to anthracnose such as `Gira' and `Rata' show greater gallotannin activity in their peel than more susceptible cultivars such as `Kohu' and `Willard'. The decline of gallotannin activity during ripening was greater in the more susceptible cultivars (Karunanayake *et al.*, 2014). Quiescence of *C. gloeosporioides* on mango was related to preformed antifungal substances, 5-substituted resorcinols' gallotannins and chitinases present in the unripe fruit peel and latex. Their decline during ripening leads to onset of progressive anthracnose development (Karunanayake, 2008).

Induced defences

Though constitutive antifungal substances have played a major role in the resistance of unripe mango (*Mangifera indica* L.) fruits to *Colletotrichum* infection, there is also evidence that unripe mango fruit responds to *C. gloeosporioides* challenge by inducing several defences (Sinniah *et al.*, 2013). Localized generation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) was observed within hours in the *Colletotrichum* challenged epidermal cells where O_2^- production was greater in the cultivar 'Karutha Colomban' more resistant to anthracnose than the susceptible 'Willard'. Superoxide (O_2^-) production was also greater in *C. gloeosporioides*-inoculated unripe fruits than in ripe fruits of both cultivars. Autofluorescence was first observed 12 h after inoculation indicating hypersensitive response (HR). Enhanced chitinase activity was detected in the inoculated peel due probably due induced chitinase (Sinniah *et al.*, 2013). mRNA differential display experiments revealed transcriptional activation of defence genes and differential gene expression in the fruits of two cultivars (Sinniah, 2010).

Inoculation of unripe fruits by *C. gloeosporioides* resulted in enhanced gallotannins (Sinnaih, Unpublished data) and the total soluble phenol content (Karunanayake, 2008). Concurrent histo-chemical tests carried out on inoculated fruit peel at different time intervals supported the findings of chemical tests that tissue phenolics increased following infection. Chitinase activity increased in the peel following inoculation with *C. gloeosporioides*, however, whether the increased activity is due the same chitinases found in the latex could not be ascertained (Karunanayake, 2008).

Avocado (*Persea americana* Miller) fruit

Anthracnose disease caused by *C. gloeosporioides* is recognized as a major disease in ripe avocado fruit. Young fruit are usually free from visible *symptoms* and characteristic decay lesions develop during fruit ripening. The anthracnose disease originates from quiescent infections in the immature fruit long before harvest (Binyamini and Schiffmann-Nadel, 1972). In unripe fruit the fungus produces an appressorium and an infection peg which ceases the growth in the cuticle (Coates *et al.*, 1993) before becoming quiescent.

The quiescence of *C. gloeosporioides* was attributed to the presence of substantial preformed antifungal activity in the immature fruit peel (Prusky *et al.*, 1982; Sivanathan and Adikaram, 1989a). Avocado peel contains antifungal monoene, 1-acetoxy-2,4-dihydroxy-*n*-heptadeca-16-ene and di-ene, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (Prusky *et al.*, 1982; Prusky *et al.*, 1991) and three other compounds 1,2,4-trihydroxyheptadec-16-yne,1,2,4-trihydroxyheptadex-16-ene and a-acetoxy-2,4-dihydroxyheptadec-16-yne (Adikaram *et al.*, 1992).

The levels of antifungal di-ene in peel of unripe avocados are subject to complex regulation and may be modulated by lipoxygenase, for which the di-ene is a substrate (Prusky *et al.*, 1983), and also by the flavan-3-ol epicatechin, an inhibitor of lipoxygenase (Prusky *et al.* 1982). Lipoxygenase activity increases during fruit ripening, while epicatechin levels decline, suggesting that these events are linked to the decrease in di-ene concentrations. In freshly harvested unripe avocado fruits, di-ene concentrations can be further enhanced by a variety of biotic and abiotic treatments. Latency of infection in unripe avocado by *C. gloeosporioides* was shown to be not due to the inadequate enzyme potential of the pathogen (Sivanathan and Adikaram, 1989b).

Papaya (*Carica papaya* L.)

Anthracnose in papaya fruit is caused by *C. gloeosporioides* and *C. capsici*. *C. gloeosporioides* forms quiescent infections in the immature fruit (Dharmasiri, 1988) and the progressive anthracnose lesion development takes place when the fruit attains fully ripe stage. Papaya fruit does not appear to contain preformed antifungal substances with sufficient toxicity or accumulate phytoalexins in response to fungal infection. Immature papaya fruit possess a milky latex, a complex mixture of enzymes, notably proteases, glycosidases, and lipases and simple sugars.

Papaya latex displays a high degree of fungitoxicity (Indrakeerthi and Adikaram, 2011; Adikaram *et al.*, 1998). Latex, when retrieved from the fruit, separates into a water soluble fraction (WSF) and insoluble semi-solid. The WSF of papaya latex can digest spores of several plant pathogenic fungi (e.g. *C. gloeosporioides*) upon brief exposure. The fungal species tested in this study responded to latex in different ways. The latex was highly destructive to certain species as their conidia become completely digested within a short period of time. *Colletotrichum gloeosporioides* is highly sensitive to papaya as when exposed the walls of conidia become digested gradually during the first 60 sec. Within about 10 min the walls of most conidia were completely disintegrated. The digestion of conidia of *C. gloeosporioides* took place even when papaya latex was diluted 100 times. The fungicidal effect of latex is due to the degradation of polysaccharide constituents in the fungal cell wall. Differential sensitivity of fungi to latex was related to their hyphal wall composition, particularly to the presence and amount of chitin. The conidia of *C. gloeosporioides*, which undergo complete digestion in the presence of latex, contain chitin and glucan in the wall and the chitin fraction is broken down by the enzyme N-acetyl-α-D-glucosaminidase in the papaya latex (Howard and Glazer, 1967). *Rhizopus* spp., which contain more chitosan, a substance which is not degraded by this enzyme, thrive well in latex. Immature papaya fruit contain latex in a minute network of canals (laticifers) and during ripening latex disappears. Those fungi that are sensitive to latex are unable to rot papaya fruit until ripening and *C. gloeosporioides* is an example of this, whereas fungi such as *Rhizopus arrhizus* which can grow profusely in the latex develop large rots in both unripe and ripe fruit.

In papaya latex N-acety1-α-D-glucosiminidase (chitinase) activity was found to be very high. Though chitinase activity has been found in a number of plants (Benhamau *et al.* 1994) the physiological role of the enzyme in general metabolism of plant cells has not been documented. It is difficult to envisage a general role for chitinase be-

cause its substrate, chitin, does not occur in higher plants. Chitinase in papaya latex may be important in the plant's defence against invasion of fungi, as a vast majority of fungal pathogens have a chitin-glucan cell wall, both in their mycelium and the spores (Bartnicki-Garcia, 1968).

Control of fruit rots that result from quiescent infections

Fungi causing latent or quiescent infections are more difficult to control than wound pathogens such as *Penicillium* which enter the fruit after harvest through wounds in the skin. Losses of fruits due to postharvest rotting could be reduced if fungal infections could be kept in their quiescent phase for extended periods after harvest. One possible way to prolong quiescence is to maintain the natural antifungal barrier present in the unripe fruit at an inhibitory level into the post-climacteric phase. Constitutive antifungal substances which provide basal resistance may be upregulated or new defences induced (Adikaram, 1990). On the other hand new defences can be induced by treatment with elicitors to enhance fruit defences and prolong the period of fungal quiescence consequently delaying progressive rot development. Certain sugars and oligosaccharides, produced during break down of host cuticle and cell wall components by pathogen-produced enzymes, function as elicitors of plant defence. Elicitors of mild or non-pathogen origin are believed to be more effective inducers of defence (Abayasekara *et al.*, 2013; Adikaram *et al.*, 1988).

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