RESEARCH ARTICLE

Differential defence responses expressed in mango (*Mangifera indica*) cultivars against *Colletotrichum gloeosporioides*

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**ABSTRACT:** This paper reports evidence that unripe mango fruit responds to *Colletotrichum gloeosporioides*, the anthracnose pathogen, by inducing several defence mechanisms. Localized generation of superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) was observed within hours in the pathogen-challenged epidermal cells, as early defence responses. O$_2^-$ production was greater in the cultivar 'Karutha Colomban' more resistant to anthracnose than the susceptible 'Willard'. O$_2^-$ production was also greater in *C. gloeosporioides* inoculated unripe fruits than in inoculated ripe fruits of both cultivars. Autofluorescence was first observed in challenged epidermal cells, 12 h after inoculation indicating hypersensitive response. Increased phenylalanine ammonia-lyase (PAL) activity and accumulation of lignin were observed in the infected peel. There was enhanced chitinase activity in the inoculated peel tissue probably resulting from induced chitinase isoforms with 59.4 kDa and 44.5 kDa molecular weight. In general, the early induced defence responses were more prominent in the resistant than in the susceptible cultivar. In both cultivars *C. gloeosporioides* becomes quiescent, due to the presence of constitutive antifungal substances in the fruit peel which decline during ripening. Greater resistance induced in the resistant cultivar, 'Karutha Colomban' may be restricting the spread (a susceptible plant). When constitutive defences are only slowly, and will be unsuccessful in preventing pathogen spreading throughout the plant (Morel and Dangle, 1997; Park, 2005). Accumulation of phytoalexins and synthesis of pathogenesis-related proteins (PR proteins) and cell wall strengthening proteins are also important induced defence mechanisms employed by plants against pathogens.

In mango, certain pre- and postharvest treatments have from time to time been reported to enhance defense responses. Bion®, salicylic acid, phosphonates (Zainuri et al., 2001) or BTH treatment (Zhu et al., 2007) increased alkylresorcinols, chitinase, β-1, 3-glucanase, polyphenoloxidase and PAL activity. Inoculation of the flesh of unripe mango fruit with *A. alternata* fruits of mango cultivars, resistant ('Karutha Colomban') and susceptible ('Willard') to anthracnose disease, at harvesting is characterized by rapid and transient generation of Reactive Oxygen Species (ROS), such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) or hydroxyl radicals (OH) (Wojtaszek, 1997). ROS are directly toxic to fungal pathogens and also activate other defenses such as HR (Lamb and Dixon, 1997).

**Key words:** Early defence responses, defence gene expression, mango, ROS

Mango anthracnose caused by *Colletotrichum gloeosporioides* Penz. originates from quiescent infections in the immature fruit. Resistance of unripe mango fruit to *C. gloeosporioides* has recently been shown to be due to a combined effect of preformed antifungal substances, 5-substituted resorcinols, gallotannins in the peel and chitinase in the latex (Karunanayake et al., 2011). Decline of resorcinols and gallotannins (Adikaram et al., 2010) during ripening makes the ripe fruits susceptible to anthracnose (Karunanayake et al., 2011). The variation in resorcinols (Hassan et al., 2007), gallotannin and chitinase (Adikaram et al., 2010) concentration explains the differential resistance of mango cultivars to *C. gloeosporioides* (Karunanayake, 2008). Quiescence of *A. alternata* in unripe mango was implicated to 5-substituted resorcinols in the fruit peel (Droby et al., 1986).

Although the preexisting barriers such as the cuticle, cell wall and preformed antifungal compounds are sufficient to block infection by most pathogens, the inhibition of pathogens that penetrate these barriers requires the timely recognition and activation of induced defenses. A plant that can recognize the pathogen and mount a rapid defense response will prevent pathogen proliferation (a resistant plant), whereas a plant that is incapable of recognizing the pathogen and does not induce a defense response, does so only slowly, and will be unsuccessful in preventing pathogen spread (a susceptible plant). When constitutive defences are breached, plants induce new defence responses at structural or biochemical level locally or systemically. Oxidative burst is such an early defence response which occurs just after pathogen recognition and signal transduction. Oxidative burst

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maturity were used for the experiments, unless otherwise stated. The fruits were brought to the laboratory within 24 h after harvest and blemish-free fruits of uniform size and maturity were selected and used. The fruits were washed under running tap water and air-dried at RT (26±2 °C). Fruits were then wiped with cotton wool soaked in 70% ethanol and allowed to air-dry before they were used for experiments.

**Pathogen**

*C. gloeosporioides* isolated from anthracnose lesions in fruits of cultivar, ‘Karutha Colomban’ and ‘Willard’, was used for inoculation of fruits of respective cultivars. For isolation, diseased tissue segments (5X5 mm²) were cut and surface sterilized in 1% NaOCl for 3 min and transferred aseptically to potato dextrose agar (PDA). The plates were incubated at RT and the pathogen was sub-cultured and maintained on PDA at RT.

**Fruit inoculation**

A suspension of conidia of *C. gloeosporioides* was prepared by suspending mycelium scraped from 10-14 d old cultures in sterile distilled water (SDW) and filtering through glass wool. The concentration in the filtrate was adjusted to 10⁸ conidia/ml. A set of 15 fruits from each cultivar was inoculated by applying 20-30 drops (20 µl) of the conidia suspension on the fruit surface and another set of fruits were treated with drops (20 µl) of SDW as controls. Inoculated and control fruits were maintained at 95-100% RH and RT.

Epidermal strips (30 µm thick) beneath the drop of inoculum were excised using a sharp sterile razor blade during a period of 3 - 24 h after inoculation at 3 h intervals, unless otherwise stated. A total of 20 epidermal strips cut from four randomly selected replicate fruits were used for each histochemical staining. In order to observe conidia germination and associated events, epidermal strips removed were cleared in boiling ethanol for 2-3 min and rinsed in distilled water. The fungal structures were stained in 10% blue ink (v/v) in 25% acetic acid for 1 min and the excess stain was removed by washing with distilled water (Huckelhoven and Kogel, 1998). The epidermal strips were mounted on glass slides and examined under a light microscope.

**Detection of H₂O₂ and O₂⁻**

To detect H₂O₂, the epidermal strips were immersed in 2 mg/ml 3, 3’-diaminobenzidine (DAB, Sigma) in 50 mM MES buffer, pH 3.8 for 2 h prior to sampling (Thordal-Christensen et al., 1997; Huckelhoven et al., 1999). For detection of O₂⁻, the epidermal strips were immersed in 0.1% nitroblue tetrazolium (NBT, Sigma) in 50 mM MES buffer pH 6.5 for 30 min (Heath, 1998). Ascorbic acid (10 mM) and 900U superoxide dismutase (SOD, Sigma) were used as the negative controls for H₂O₂ and O₂⁻, respectively. The epidermal strips were cleared in 95% boiling ethanol and observed under the light microscope (Olympus CX31 with Digital Camera DP20 and application software DP2-BSW build 2738, Olympus Corporation, 2006). The percentage epidermal cells stained with NBT were counted out of 100 appressoria-epidermal cell contact sites in 3 microscopic fields from an epidermal strip. Average value of 3 epidermal strips represented the frequency of interaction sites with NBT staining for each fruit. This experiment was carried out with unripe and ripe fruits of mango cultivars ‘Karutha Colomban’ and ‘Willard’.

**Detection of autofluorescence**

Epidermal strips were placed in a fixing solution (10% formaldehyde, 5% acetic acid, 45% ethanol, and H₂O) for 15 min and soaked in 50% ethanol for 20 min followed by 95% ethanol overnight (Bowling et al., 1997). The strips were mounted in 70% glycerol and observed under a fluorescence microscope [Nikon Ophtiphot, Japan, with EPI-FL filter block B-2A (BA 520, DM 510, Ex 450-490)] and photographed.

**Detection of lignin**

Epidermal strips and thin transverse sections cut through the fruit peel 12, 24, 36, 48 and 72 h after inoculation were observed under light microscope for red staining in the lignified cells (Siegel, 1953).

**Antifungal activity in the inoculated peel**

Fruits at harvesting maturity were inoculated with conidia of *C. gloeosporioides* and another set was treated with drops of SDW as a control. Peels (250 µm thick) beneath the inoculum/water drop were removed 48 h after inoculation or treatment with SDW. The peel tissues were placed in methanol: dichloromethane (50:50 v/v) in conical flasks (250 ml) with side arms connected to a vacuum pump and extracted by vacuum infiltration with continuous stirring (Karunanayake et al., 2011; Adikaram and Ratnayake Bandara, 1998). The extracts were concentrated and subjected to Thin Layer Chromatography bioassay using *Cladosporium cladosporioides* as the test fungus (Karunanayake et al., 2011). The area of inhibition zones was measured and their Rf values were recorded.

**Chitinase activity in the inoculated fruit peel**

Fruits at harvesting maturity were inoculated with conidia of *C. gloeosporioides* and another set was treated with drops of SDW as a control. Chitinase was extracted by homogenizing peel tissue (1.5 g, 250 µm thick) cut from the inoculated sites at 3 h intervals over a 24 h period after inoculation in 3 ml 60 mM phosphate buffer (pH 7) containing 0.1g polyvinylpolypyrrolidone (PVPP) and 30 µl phenylmethylsulfonyl fluoride (PMSF). Extracts were centrifuged at 9000 g (Sigma 3K30, DJB Labcare, Buckinghamshire, UK) for 5 min and the supernatant was used as the enzyme source. Chitinase activity was determined by gel diffusion assay using glycol chitin as a substrate (Zou et al., 2002). Aliquots (10 ml) of the extracts and the enzyme standards (Chitinase EC3.2.1.14, from *Serratia marcescens*, Sigma) were pipetted into individual wells cut out in the gel. The plates were incubated at RT for 48 h and the gel was stained with 10 ml of 0.1% (w/v)
calcofluor white (Fluorescent brightener 28, Sigma) in 500 mM Tris-HCl (pH 8.9) for 10 min, washed in SDW with orbital shaking for 2-3 h and viewed under UV light (365 nm). The diameter of the dark lytic zones, was recorded. The enzyme activity was calculated using a regression equation derived using the lytic zones of known enzyme standards.

**SDS-PAGE**: Extracts of peel (10 g) cut from inoculated or control fruits (cultivar 'Willard') 15 h after inoculation were subjected to SDS-PAGE in 12% acrylamide gels containing 0.1% (w/v) SDS and 0.01% glycol chitin at 50 V for 2-3 h. The gel was stained with calcofluor white in 500 mM Tris-HCl buffer (pH 8.9) for 10 min. The stain was removed and lytic zones were visualized by placing the gel on a transilluminator (SXT 20M UVitec, France) (Trudel and Asselin, 1989).

**Assay for PAL**

Fruits at harvesting maturity were inoculated with conidia of *C. gloeosporioides* and another set was treated with drops of SDW as a control. Peel tissues (1.0 g, 250 µm thick) were removed from fruits inoculated with *C. gloeosporioides* and controls, at every two-day intervals for 8 days and homogenized in 10 ml cold acetone (-20 °C) for 2 min at 11,000 rpm in a homogenizer (ULTRA TURAX® model T25 basic, IKA LABORTECNIK, Selangor, Malaysia). The homogenate was filtered through Whatman No 1 paper and the powder retained was homogenized twice more in cold acetone and air-dried. PAL was extracted from the powder (100 mg) by stirring for 2 h at 4 °C with 5 ml of 0.1 M sodium borate buffer (pH 8.8). The extract was centrifuged at 10,000 g (Sigma 3K30, DJB Labcare, Buckinghamshire, UK) for 20 min at 4 °C and the supernatant was used as the enzyme source. The reaction mixture consisted of 10 mM L-phenylalanine (Sigma), 0.1 M borate buffer (pH 8.8) and 1.5 ml of enzyme extract in a final volume of 5 ml. The reaction was carried out at 37 °C for 60 min and terminated by adding 0.1 ml of 5 N HCl. Cinnamic acid produced was extracted into diethyl ether (7 ml) and quantified by measuring the trans-cinnamic acid formed at 269 nm (Cintra 10e-UV visible spectrophotometer; GBC Spectral, Victoria, Australia). trans-4-hydroxy-3-methoxycinnamic acid (99%, ALDRICH®) was used as the standard. The amount of cinnamic acid in the test samples was calculated from a standard curve prepared using absorbance values of a series of solutions (10-50 µg/ml) in diethyl ether and expressed as µg cinnamic acid in 1.0 g peel (Beno-Moualem and Prusky, 2000).

**Statistical analysis**

Each experiment was repeated at least twice. One-way ANOVA was performed to find out significant difference in O$_2^-$ production between the two mango cultivars and the two development stages at a particular experimental time point. Chitinase and PAL activity data obtained were analyzed by two-way ANOVA and the means were separated by Duncan’s Multiple Range test using SAS computer software at 95% significant level (SAS Corporation, Release 6.12).

**RESULTS**

**Germination and appressoria formation by *C. gloeosporioides* on unripe mango fruit**

The conidia germinated on the unripe fruit surface of both resistant and susceptible cultivars 3 h after inoculation. A septum was formed in the conidium prior to emergence of germ-tube. Within 6 h non-melanized appressoria were observed and all appressoria appeared melanized 12 h after inoculation and the lobes were clearly visible in them. Infection hyphae that emerged beneath the appressoria were clearly visible in the TS taken through the peel, 24 h after inoculation. A considerable number of epidermal cells in contact with infection hyphae had turned brown (Fig. 1a).

**Formation of H$_2$O$_2$ and O$_2^-$**

Production of H$_2$O$_2$ was observed as brownish and diffusible DAB polymers and O$_2^-$ as blue colour, granulated formazan (Figs. 1b & c). First evidence of H$_2$O$_2$ was observed 12 h after inoculation, however, at subsequent time points it was difficult to distinguish the DAB stain from induced cell browning.

Superoxide generation was evident in the infected cells, 9 h after inoculation, in both cultivars tested. The percentage of infected cells showing superoxides increased with time and reached a peak at 15-21 h after inoculation and decreased sharply. About 50% of the infected cells showed superoxide generation 15-18 h after inoculation in the unripe resistant cultivar ‘Karutha Colomban’. Only 29-35% of the infected cells showed superoxide generation 15-18 h after inoculation in the ripe fruits. In susceptible ‘Willard’, the percentage cells that generated superoxides was significantly lower than ‘Karutha Colomban’ and a maximum of 33% cells produced superoxides 21 h after inoculation. Superoxide generation by the ripe ‘Willard’ cells was very low and the
percentage of infected cells showing NBT staining was less than 10% at all the time points tested. The difference in percentage of interaction sites (epidermal cells in contact with appressoria) that produced \( \text{O}_2 \) between cultivars as well as between unripe and ripe stages was statistically significant (\( P>0.05 \)) (Fig. 2).

**Autofluorescence**

In both cultivars, the epidermal cells that were penetrated by infection hyphae and had turned brown after 24 h, showed autofluorescence as localized bright yellow-green fluorescence (Fig. 1d). The first evidence of autofluorescence was observed in the cell walls 12 h after inoculation. With time autofluorescence was observed in cells surrounding the pathogen invaded cell.

**Histochemical test for lignin**

In both cultivars, the infected brown cells and the cells immediately surrounding these gave positive results for lignin. Red staining was first observed 36-48 h after inoculation and continued at latter time points examined.

**Antifungal activity in the fruit peel inoculated with \( C. \) gloeosporioides**

Several inhibition zones were visible at \( Rf \) 0.16-0.18, 0.21-0.22, 0.26-0.28, 0.48-0.50 and 0.53-0.59 in the chromatograms (Fig. 3) with dichloromethane phase of peel extract from inoculated or healthy fruits of both cultivars. However, the area of inhibition produced by the extract of inoculated fruit tissue was slightly larger and stronger. The size of the inhibition zones produced by the compounds at \( Rf \) 0.48-0.59 were 157±32 mm² and 305±15 mm² in healthy and inoculated ‘Karutha Colomban’ fruit peel, respectively. In ‘Willard’ the area of inhibition was 175 ±20 mm² and 287±30 mm² respectively, in healthy and inoculated fruit peel. There were no additional inhibition areas in the chromatograms with inoculated fruit extract compared to the uninoculated controls (Fig. 3). The size of the inhibition area produced by the methanol fraction of both inoculated and control tissue extracts corresponding to gallotannins was similar in the cultivar ‘Karutha Colomban’. However, a slight increase in gallotannin activity was observed in the inoculated peel extract of the cultivar ‘Willard’ (data not shown).

**Chitinase activity**

Peel chitinase activity fluctuated with time after inoculation in unripe fruits of both cultivars. The peak activity was observed 12 h after inoculation in ‘Karutha Colomban’ (Fig. 4). However, the difference in chitinase activity observed at different time points was not significant (\( P>0.05 \)) in the peel of unripe fruit of cultivar ‘Willard’. Four chitinase bands corresponding to molecular weights 69.7, 65.4, 44.5 and 34.2 kDa were visible in peel extract of healthy fruit of cultivar ‘Willard’. The inoculated peel extract showed an additional band corresponding to 59.4 kDa. The band corresponding to 44.5 kDa was more prominent in the inoculated peel extract than that of the healthy peel extract.

**PAL activity**

The inoculated peels showed about two fold increase in PAL activity (\( P<0.05 \)) than the control peels in both ‘Karutha Colomban’ and ‘Willard’ following inoculation with \( C. \) gloeosporioides.
Coloban' and 'Willard' at all the time points tested (Fig.5). PAL activity was higher in the cultivar 'Karutha Colomban' than that of 'Willard' throughout the period. There was an increase in PAL activity with time from the initial level (Day 0) in the inoculated and control fruit peel of the both cultivars.

**DISCUSSION**

The resistance of unripe mango fruit to *C. gloeosporioides* has been implicated to the presence of constitutive resorcinols (Hassan et al., 2007) and gallotannins in the peel and chitinase in the latex (Karunanayake et al., 2011). The nature of immature mango fruits to respond to infection by inducing defence responses remained relatively unknown. There were occasional reports of PR-proteins in mango in inducing defence responses remained relatively unknown. There were occasional reports of PR-proteins in mango in response to defence elicitor treatment (Zainuri et al., 2001). The present study provides clear evidence for induced defence responses in mango during *C. gloeosporioides* infection.

Light microscopic observations showed no morphological and temporal difference in the pattern of germination and appressorium formation by conidia of *C. gloeosporioides* on the unripe fruit between 'Karutha Colomban' and 'Willard'. This suggests that the pre-penetration events are similar in both resistant and susceptible cultivars and that the resistance responses trigger following penetration. Similarly, there were no morphological differences in pre-penetration events of *C. acutatum* between the resistant and susceptible blueberry cultivars (Wharton and Schilder, 2008).

Accumulation of *H₂O₂* and *O₂⁻* was observed in the epidermal cells as an early defence response (6-9 h after inoculation) in mango fruit peel following infection by *C. gloeosporioides*. *O₂⁻* generation was clearly identified by the formation of blue colour formazan when stained with NBT. However, pathogen-induced cell browning interfered with yellow-brown DAB staining of *H₂O₂* and made the quantification difficult. The rapid and higher level of *O₂⁻* generation in the resistant 'Karutha Colomban' compared to the susceptible 'Willard' suggests an active role of *O₂⁻* in defence. The highly localized accumulation of ROS may have a direct antimicrobial effect (Thordal-Christensen et al., 1997) or trigger other defence responses such as papillae formation (Huckelhoven et al., 1999; Thordal-Christensen et al., 1997), protein cross-linking and accumulation of phenolic compounds associated with plant defence (Borden and Higgins, 2002). Further, ROS production in mango fruit peel seems to be dependent on the physiological stage of the fruit development. In both cultivars, the percentage of interaction sites that produce *O₂⁻* was higher in the unripe than ripe fruits. This result suggests that ROS production decreases with ripening which probably makes ripe fruits more susceptible to pathogen attack.

**H₂O₂** and **O₂⁻** accumulation 12 h after inoculation was restricted to the sites where host cell browning had occurred. This was indicated by intense autofluorescence in the infected brown cells and neighbouring host cells. The occurrence of autofluorescence was due to accumulation of polyphenols at the site of infection. Towards 36-48 h after infection, the accumulation of lignin was also evident in the brown cells. Accumulation of autofluorescent compounds is associated with hypersensitive response (HR) and browning and accumulation of phenols are thought to be responses that follow cell death (Park, 2005). It appears that the epidermal cells of mango fruit peel undergo HR like cell death in response to infection by *C. gloeosporioides* though visible necrotic symptoms appeared 3-4 days after inoculation in 'Willard' and 6-7 days in 'Karutha Colomban' (G.D. Sinniah, unpublished data). Oxidative burst is considered as one of the earliest events in the HR (Jabs et al., 1997; Able et al., 2003) and localization of *H₂O₂* and *O₂⁻* at the site of cell browning and autofluorescence suggests that ROS may have a role in the accumulation of polyphenols and associated cell death in mango-*C. gloeosporioides* interaction.

There was a significant increase in PAL activity, a key enzyme in phenolic biosynthesis, after inoculation in both cultivars. The increased PAL activity corresponds with the accumulation of phenolic compounds and lignification at the inoculated sites. These results provide further evidence for enhanced localized production of secondary metabolites that are needed to inhibit pathogen infection, directly or indirectly. Increased PAL activity and phenolic content have also been reported in freckle infected banana (Abayasekara et al., 1998), *Fusarium* infected banana (De Ascensao and Dubery, 2000) and *C. gloeosporioides* infected avocado pericarp (Beno-Moualem and Prusky, 2000).

Accumulation of PR-proteins is an important feature of plant defence responses upon infection by pathogens (Ferreira et al., 2007). The rapid enhancement of chitinase activity in mango peel was observed after infection by *C. gloeosporioides*. The induction of chitinase activity was common to both resistant 'Karutha Colomban' and susceptible 'Willard' cultivars. However, the increase was significant only in the resistant cultivar. Higher chitinase activity observed in the peel of 'Willard' is a cultivar effect. It was reported that chitinase activity per unit area of peel and in a given volume of latex was higher in 'Willard' than that of 'Karutha Colomban' (Karunanayake, 2008). Preformed chinatinas present in the mango latex have been shown to hydrolyze the walls of conidia of *C. gloeosporioides in vitro* within 2-3 h after exposure (Adikaram et al., 2010). The
induction of chitinase following infection may enhance hydrolytic degradation of mycelial walls of invading fungi. The molecular weights of induced chitinase isoforms were shown to be 59.4 kDa and 44.5 kDa. Induction of chitinase enzymes was also shown in Vicia faba during Botrytis fabae infection (Attia et al., 2007). Two chitinase isoforms with molecular weights, 18 kDa and 12 kDa, were induced in freckle infected banana fruits var. ‘Embul’ (Wanigasekara, 2009). In rice cells treated with an elicitor from Rhizoctonia solani induced a banana fruits var. ‘Embul’ (Wanigasekara, 2009). In rice cells earlier and in greater magnitude than susceptible cultivar anthracnose ‘Karutha Colomban’ showed defense responses generated more in unripe mango fruits than in ripe fruits in phenolics and lignification of cell walls. These responses were increased activity of chitinase, PAL and accumulation of several early defences. These responses are followed by biochemical defence mechanisms (Djamni-Tchatchou et al., 2012).

In conclusion, the unripe mango fruit responded to inoculation with conidia of C. gloeosporioides by inducing several early defences. These responses are followed by increased activity of chitinase, PAL and accumulation of phenolics and lignification of cell walls. These responses were generated more in unripe mango fruits than in ripe fruits in both mango cultivars tested and the resistant cultivar to anthracnose ‘Karutha Colomban’ showed defense responses earlier and in greater magnitude than susceptible cultivar ‘Willard’ The study revealed that inductive defences also play an important role in the resistance of unripe mango to C. gloeosporioides when the preformed defences are overcome by the pathogen.

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