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Research article

Expression of chitinase with antifungal activities in ripening Banana fruit

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Abstract: The banana (*Musa acuminata*) is a climacteric fruit of a great economic importance. In this present study, the expression of chitinases and their antifungal activities were investigated. The ripening stages of banana were defined according to the peel colour index (PCI). In the dye-labelled assay with carboxymethyl chitin remazol brilliant violet 5R (CM-chitin- RBV 5R), chitinase activity was detected in both pulp and peel extracts. The activities increased markedly at the onset of ripening and peaked at PCI 5 stage. On sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) activity gels, two chitinases of apparent molecular masses of 26 kDa and 31 kDa were detected in both pulp and peel extracts and they showed an accumulation during the subsequent ripening. This result was in consistent with the pattern of chitinase activity obtained in dye-labelled assay. Interestingly, both pulp and peel extracts inhibited the growth of the fungus *Botrytis cinerea* on plates and caused lysis of hyphal tips. In some instances, with bursting of the fungal tips, flowing out of the cell contents could be observed.

Keywords: Banana (*Musa acuminata* L. AAA) - Fruit ripening - Chitinase - Hyphal tip lysis.

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INTRODUCTION

Fruit ripening is a complex metabolic process. After a phase of active cell division followed by cell expansion, the growth and development of the fruit decline and the ripening begins. This is accompanied by some physiological changes including changes in aroma, texture, colour and flavour and some biochemical changes including the synthesis of phytoalexins (Hammerschmidt 1999, Fraser *et al*. 2007) and pathogenesis-related (PR) proteins (Van Loon 1997, Datta & Muthukrishnan 1999). Such changes make the fruits more attractive to animals and more likely to be attacked by pathogens (Derckel *et al*. 1998). Since fruit ripening is a tightly regulated process (Giovannoni 2001), all these changes are very likely brought about by activation or repression of specific genes.

Systemic acquired resistance (SAR) is a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens. SAR pathway is induced by most pathogens that cause necrosis of tissues (Anfoka & Buchenauer 1997). SAR provides protection in uninfected parts of the plant against pathogens and it is correlated with the expression of pathogenesis-related (PR) proteins (Mauch *et al*. 1984, Lawrence *et al*. 1996, Daulagala & Allan 2003). Among the PR proteins, the most intensively studied and well characterized in plant-pathogen interactions are chitinases and β-1, 3-glucanases. Although chitin, the substrate of the chitinase enzyme does not exist in plant cell walls, chitinases are widely distributed in plants (Neuhaus 1999, Kitajima *et al*. 2010). Further, β-1, 3-glucanses are another group of PR proteins that attack glucans cross-linked with chitin in fungal cell walls.

A defensive role of PR proteins in plant systems has been suggested based on the induction of their synthesis upon pathogen infection, and on their in vitro and in vivo antifungal activities. Expression of chitinase-encoding genes is thought to be an important biochemical mechanism of plant defense against fungal pathogens as chitinases can degrade chitin in fungal cell walls and inhibit fungal growth (Mauch *et al*. 1984, Lawrence *et al*. 1996). Some plant chitinases have direct antifungal activity, causing rapid lysis of fungal hyphal tips and germinating spores. This antifungal activity of chitinases is synergistically potentiated by β-1, 3-glucanses.

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Chitinases (EC 3.2.1.14) are hydrolytic enzymes. Basically there are three activity classes of chitinases; β-(1,4)-*N*-acetylglucosaminidases, exochitinases (chitobiosidases) and endochitinases. β-(1,4)-*N*-acetylglucosaminidases cleave *N*-acetylglucosamine (NAG) oligomers of the chitin chain and generate *N*-acetylglucosamine monomers. Chitobiosidases cleave diacetylchitobiose units from non-reducing end of the chitin chain and release disaccharides. Endochitinases randomly cleave glycosidic linkages at internal sites along the chitin chain and produce low molecular mass oligomers like diacetylchitobioses and chitotrioses (Guthrie *et al*. 2005).

Banana (*Musa acuminata* L. AAA) is a globally important fruit, especially in developing countries and millions of people subsist on bananas as one of their major energy sources. It is a climacteric fruit, characterized by a green storage phase, followed by a burst in ethylene release. Consistent with the ethylene production, numerous biochemical changes occur during ripening of banana, including the synthesis of volatile compounds, alterations in carbohydrate composition, changes in phenolic compounds and breakdown of chlorophyll in peel (Seymour 1993). A number of enzymes associated with banana ripening have been identified and characterized. Some of these enzymes are involved in carbohydrate metabolism and cell wall degradation (Seymour 1993, Da Mota *et al*. 2002), whereas others showed antifungal activity (Peumans *et al*. 2002). Differential screening of cDNA libraries representing banana pulp at different ripening stages yielded several up-regulated genes (endochitinase, β-1, 3-glucanase and methallothionin) as well as down-regulated genes (starch synthase, class III chitinase, and jacalin-related lectin (Clendennen & May 1997). In a recent study, it was found that in naturally ripened banana, the expression of endochitinase gene MaECHI1 gradually increased consistent with the timing of the ripening process (Liu *et al*. 2012). This suggested that this gene was up regulated during ripening which correlated with the previous work performed with this gene expression (Xu *et al*. 2007).

In this study, the chitinase activity and the isoenzyme expression on gels were assessed during the ripening process of banana fruits to see how the pattern of expression and the functionality of the enzyme are associated with the ripening process. Further the antifungal activity of the extracts against the fungus *Botrytis cinerea* was also investigated.

MATERIAL AND METHODS

Plant material

Unripe banana fruits (*Musa acuminata* cv. Giant Cavendish) were obtained from a commercial source (Asda Supermarket) in Aberdeen, UK (imported from Chiquita Brands Inc., Cincinnati, Ohio, USA). Only those fruits normal and healthy in appearance without physical injuries or disease symptoms were selected. Ripening was allowed to proceed naturally under normal laboratory conditions. Fingers from different hands were mixed and randomly taken for the experiments. During ripening, pulp and peel of fruits representing five subsequent ripening stages were collected. Stages of ripening were scored by a peel colour index on a scale from colour number 1 to 5 as PCI 1 to PCI 5 (1 = very green, internal tissue hard, 2 = more green than yellow, 3 = more yellow than green, pulp becoming soft, 4= yellow with green tips and 5 = all yellow, pulp very soft) (Customer Service Department, Chiquita Brands, Inc., Cincinnati, Ohio, USA). Tissues from five individual fruits were pooled to obtain a uniform sample for each ripening stage.

Botrytis cinerea culture

Botrytis cinerea used in this study was isolated from Yorkshire Chinese cabbage (YCC) (*Brassica campestris* sub sp. *pekinensis* var. Kasumi) from a field at Triffitt Nurseries, Allerthorpe, Humberside. The culture was maintained on Potato Dextrose Agar (PDA) plates at 21°C. Routine subculture was done by transferring a plug of medium from a stock plate onto a fresh PDA plate.

Preparation of protein extracts

To extract total protein, frozen tissues at each specific ripening stage were immersed in liquid nitrogen and ground to homogeneity in a mortar. Powdered tissues were further ground in a mortar with the extraction buffer (0.1M sodium citrate buffer with additives, pH 5.0) at the level of 3 $ml.g^{-1}$ fresh weight. The homogenates were transferred to Eppendorf tubes and cellular debris was pelleted by centrifugation at 11,600 g for 15 min. The supernatant fractions were stored at -20°C until required.

Chitinase assay with Carboxymethyl-chitin Remazol Brilliant Violet 5R

The assay developed by Wirth & Wolf (1990) was used. Reaction mixtures contained the extract (1:100 dilution), 0.1M sodium citrate buffer (pH 5.0) and carboxymethyl-chitin remazol brilliant violet 5R (CM-chitin-RBV 5R) solution (2 $mg.ml^{-1}$ in water) (Loewe Biochemical Gmbh Co., Germany). The microcentrifuge tubes

containing 800 µl of reaction mixture (50 µl of extract, 200 µl of substrate and 550 µl of buffer) were incubated at 37°C for 1 h. The reaction was stopped by adding 200 µl of 2 N HCl and then by cooling on ice for 10 min. The tubes were centrifuged (Chillspin, MSE, UK) at 11,600 g for 5 min. The coloured supernatants were gently pipetted in to cuvettes and absorbances were photometrically determined at 550 $nm.h^{-1}$ against a blank. The blank was the reaction mixture without the enzyme extract (with more extra buffer equal to the volume of the extract). The enzyme activity was expressed as the change in absorbance at 550 $nm.h^{-1}$. The assays were done in triplicate for each sample.

Visualization of chitinase isozyme patterns in activity gels

SDS-PAGE was carried out as described by Trudel & Asselin (1989) using glycol chitin impregnated separating gels, with the buffer system of Laemmli (1970). For activity gels, 12% separating gels containing 0.1% (w/v) glycol chitin and 4% stacking gels were used. Glycol chitin was synthesized from glycol chitosan (Sigma) according to Trudel & Asselin (1989). Samples were prepared in non-reducing loading buffer (without β-mercaptoethanol). After electrophoresis at 200V, molecular weight markers were trimmed off from the gel and stained with Coomassie blue with shaking at room temperature. The remainder of the gel was incubated for overnight at 30ºC with reciprocal shaking in 25 mM sodium acetate buffer pH 5.0 (renaturing buffer) containing 1% (v/v) Triton X-100. Following the incubation, gels were stained with freshly prepared 0.01% (w/v) calcofluor white M2R (Sigma, UK) in 0.5 M Tris-HCl buffer (pH 8.9). After 5 min, the brightener solution was removed and gels were destained by incubating for 1 h in distilled water at room temperature. Lytic zones, where chitin had been digested, were detected by inspection of gels in a UV illuminator, where they appeared as dark bands against the fluorescent background of intact glycol chitin (Trudel & Asselin 1989). The destained portion of the gel containing molecular markers was used to calibrate the Calcofluor-stained activity gel.

Assays of antifungal activity

The crude protein extracts were tested *in vitro* for antifungal activity against *B. cinerea*. The effect was tested in two methods,

1. Inhibition of growth of *B. cinerea* on PDA plates:

Two agar plugs (10 mm diameter) bearing *Botrytis* growth were cut from a growing culture and placed firmly (face down) in the centre of two potato dextrose agar (PDA) plates. Wells were cut with a 10 mm diameter cork borer, around the plates approximately 2.5 cm away from the centre. The wells were filled to capacity (200 µl) with filter-sterilised (0.2 µm Acrodisc, Gelman Sciences, UK) crude enzyme extracts prepared from both peel and pulp tissues. Commercially available chitinases from *Serratia marcescens* at 10 $mg.ml^{-1}$ concentration and boiled enzyme extract were used as controls. All plates were incubated at 21ºC and observed daily for any zones of inhibition.

2. Lysis of hyphal tips of *B. cinerea*:

The method used by Zhu & Gooday (1992) was used with some alterations. Petri dishes were poured with 10 ml of PDA (to give a thin layer) and the agar was overlaid with cellophane (British Celanese Co., PT300) which had been boiled in distilled water for 30 min and autoclaved. A spore suspension of *B. cinerea* was stab inoculated on to the centre of the medium and the plates were incubated at 21ºC. After 48 h of incubation, triangular agar blocks containing growing mycelium were cut (still with the Cellophane) and kept them on clean glass slides. Using a micropipette, filter sterilized enzyme extracts (20 µl drops) obtained from banana pulp (PCI 1) were added directly to the growing colony margins by lifting the cellophane with forceps. The extraction buffer was used as the control. The enzyme extract and the buffer were used with 1.5% (w/v) sorbitol solution, osmotically compatible with *B. cinerea*. The agar blocks were observed under the microscope for any swelling and/or bursting of hyphal tips and photographed.

RESULTS

Chitinase assay with carboxymethyl-chitin remazol brilliant violet 5R

Chitinase assay using the substrate CM-chitin-RBV 5R is based on the precipitability of a non-degraded, highly polymerized substrate when acid is added to the reaction mixture. This method is very useful to detect the presence of endochitinase activity in protein extracts. Chitinase activities of extracts from both banana pulp and peel increased progressively and markedly, during natural ripening from PCI 1 to PCI 5, reaching the maximum in ripe fruit (PCI 5). The activities of pulp extracts at all five stages of ripening were slightly higher than that of the peel extracts and the activities obtained at PCI 5 (all yellow) for both pulp and peel extracts were approximately twice that of respective PCI 1 (very green) stage (Fig. 1).

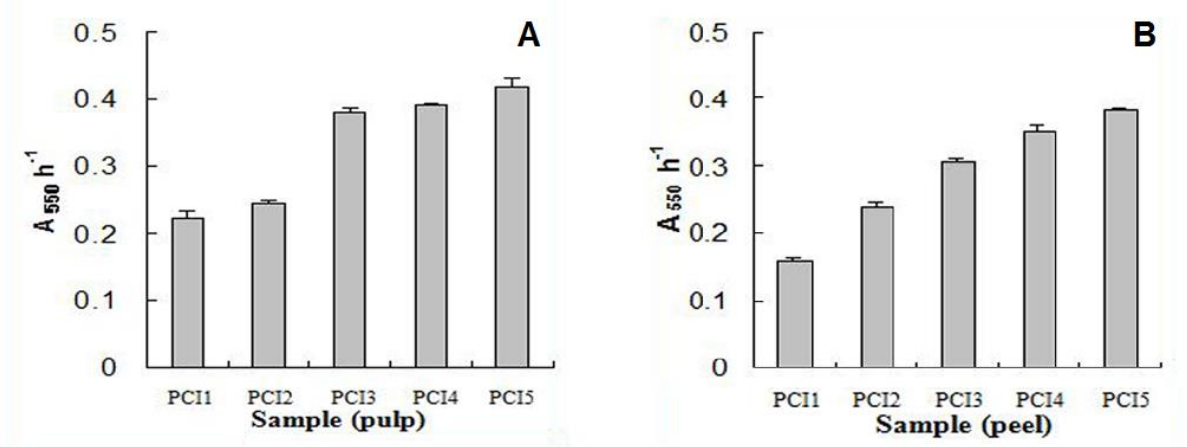

Figure 1. Chitinase activities of banana fruits with CM-chitin RBV 5R at five different stages of ripening (PCI 1 to PCI 5): **A,** Pulp extracts; **B,** Peel extracts.

Visualization of chitinase isozyme patterns in activity gels

To trace major changes in the chitinase expression pattern, crude extracts from peel and pulp of banana were analysed by SDS-PAGE activity gels. Total protein extracts were prepared from equal fresh weights of pulp and peel and equal volumes of both extracts were loaded in wells. The results of the SDS-PAGE analysis clearly demonstrated dramatic changes in the accumulation of chitinase in the pulp and peel during ripening. As shown in figure 2, chitinases of apparent molecular masses of 26 and 32 kDa were the predominant proteins recovered from the extracts. Unlike in the pulp, the 26 kDa chitinase was present in low amounts in peel throughout the ripening process. In pulp, it showed a strong accumulation during the later stages of ripening. The 32 kDa chitinase which was relatively in low concentrations in peel at PCI 1 stage showed a gradual increase with the onset of ripening. But in pulp, the abundance of 32-kDa chitinase progressively increased from PCI 1 to PCI 5.

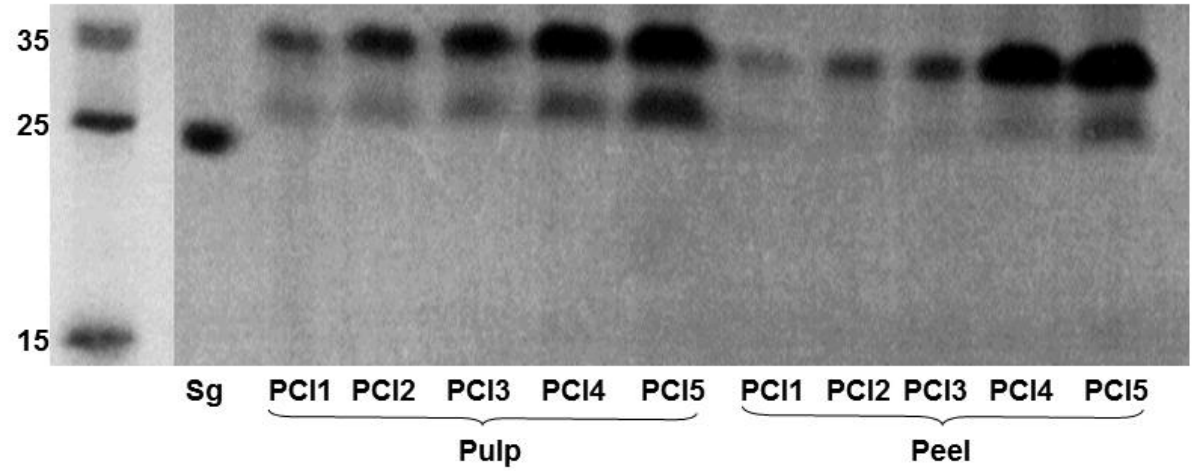

Figure 2. Pattern of chitinase accumulation in banana pulp and peel extracts during five subsequent stages of ripening PCI 1 to PCI 5. Molecular markers are indicated on the left. Sg – chitinase from *S. griseus* as a positive control.

Assays of antifungal activity

1. *Inhibition of growth of B. cinerea on PDA plates*

The fungus *B. cinerea* was screened for its ability to be inhibited by extracts of banana. The fungus was allowed to grow on PDA medium for 2 days at 21ºC before adding the extracts to the wells. It was interesting to note that after overnight incubation of these plates with extracts in wells, there was a detectable inhibition of growth of *B. cinerea*. Further additions of extracts and incubation of plates produced crescents of inhibition of growth on plates (Fig. 3). The crescents of inhibition with pulp extracts as shown in figure 3B were more prominent than that of peel in figure 3A, and this observation showed a pattern similar to the results obtained in dye assay and activity gels. *Botrytis* grew towards the well 6 in plate (A), which contained *S. griseus* chitinases and this showed that growth of *B. cinerea* was not affected by the chitinases of *S. griseus.* With boiled extract, no growth inhibition was observed in well 6 in figure 3B.

2. *Lysis of hyphal tips of B. cinerea*

Examination by light microscopy of the mycelium treated with banana extracts from pulp (PCI 1) showed swelling and subsequent bursting of hyphal tips of *B. cinerea* (Fig. 4A). However, no such lysis was observed in

the control experiment with the sample extraction buffer (Fig. 4B). Although the chitinase activity in pulp tissues increased with ripening from PCI 1 to PCI 5, there was a slight difference in time taken for the fungal tips to burst with each extract. The pulp extract of PCI 1 ripening stage contained relatively low chitinase activity compared with the other pulp extracts, but within 2-3 min of adding the pulp extracts on to the mycelial tips, the bursting initiated with all extracts. But in contrast, the extracts from peel took more time than the pulp to initiate bursting. The number of burst tips was fewer with peel extracts compared to the corresponding pulp and most of the time there were more swollen tips than burst tips with peel extracts.

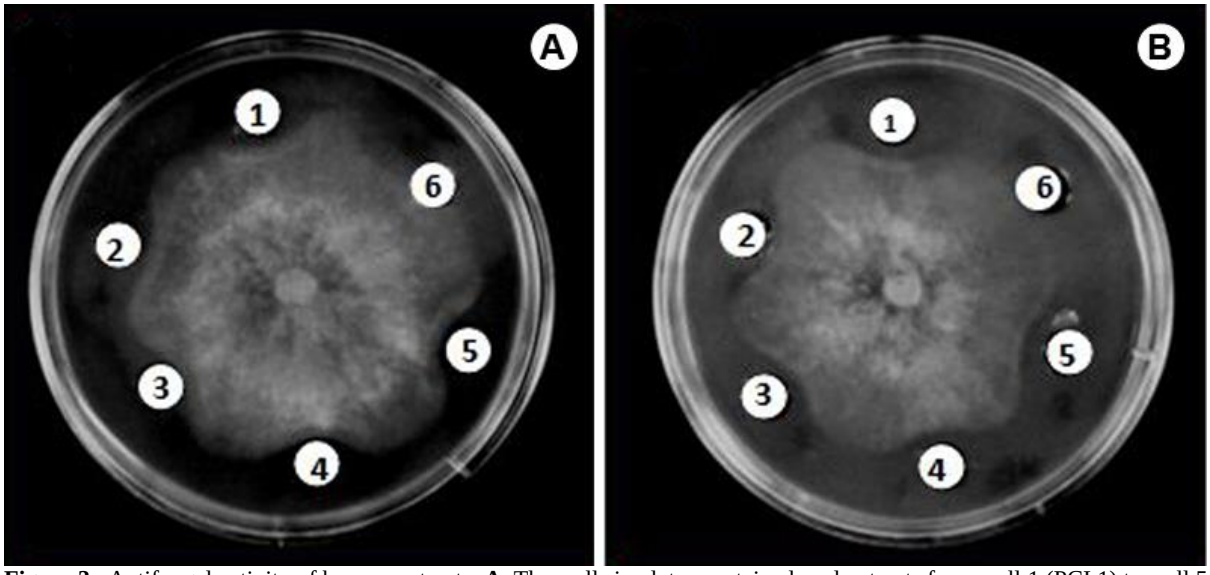

Figure 3. Antifungal activity of banana extracts: **A,** The wells in plates contained peel extracts from well 1 (PCI 1) to well 5 (PCI 5) and well 6 contained chitinases from *S. griseus*; **B,** The wells in plates contained pulp extracts from well 1 (PCI 1) to well 5 (PCI 5) and well 6 contained boiled enzyme extract of PCI 3.

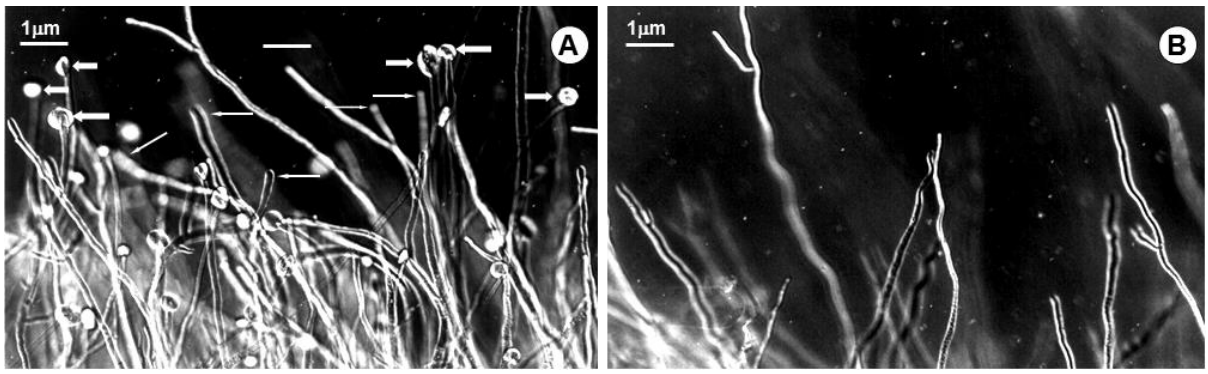

Figure 4. Light micrographs of *B. cinerea* hyphae: **A,** after incubation with crude protein extracts (Note the swollen (narrow arrows) and lysed (wide arrows) hyphal tips with the banana pulp extract); **B,** after incubation with extraction buffer.

DISCUSSION

Ripening of most fruits generally involves the accumulation of sugars and other nutrients and softening and breakdown of cellular structure of the tissues. The eventual result of all these events makes the fruits as an excellent target for pathogens (Robinson *et al*. 1997). These observations have led several researchers to hypothesize that different proteins should accumulate during maturation and ripening of fruits (Derckel *et al*. 1998). It is important to note that the pattern, abundance and the type of proteins reported in a particular study, during ripening of a certain fruit, may not be the same when comparing with another study performed in a different laboratory. As an example, in banana, the stages of ripening can be indicated according to the colour development in peel, from green to yellow. One can divide this whole process into 5, 6 or 7 different stages depending on how the visual colour development in the peel is determined. The whole process can be divided into pre climacteric, early climacteric, climacteric and post climacteric, depending on the respiration rate and ethylene production. Furthermore, fruits can be kept in natural atmosphere to ripen or they can be exposed to ethylene to induce ripening. These aspects may result in differences of conclusions in different studies.

Banana is a climacteric fruit and the onset of ripening is characterized by a large increase in ethylene synthesis. It is believed that ethylene regulates the expression of genes involved in ripening. Several genes including a chitinase gene have been isolated from bananas that were up-and down-regulated during ripening. In

the study by Liu *et al*. (2012), they found that in naturally ripened banana fruit, the expression of endochitinase gene MaECHI1 gradually increased, consistent with the timing of the ripening process. This result suggested that this gene was up-regulated during ripening, which correlated with previous work performed with this gene expression library (Xu *et al*. 2007). Differential screening of cDNA libraries representing banana pulp at different ripening stages also yielded a number of up-regulated (endochitinase, β-1, 3-glucanase, BanTLP, and methallothionin) as well as several down-regulated (starch synthase, class III chitinase, and jacalin-related lectin) genes (Clendennen & May 1997).

In the dye-labelled assay with CM-chitin RBV, chitinase activities were detected in both pulp and peel and they increased during ripening. On SDS-PAGE activity gels, two chitinases of apparent molecular masses of 26 kDa and 31 kDa were detected in both pulp and peel tissues and they showed an accumulation during the subsequent ripening. Similarly, Clendennen *et al*. (1998) identified and characterised an abundant protein of 31 kDa (P31) from pulp of banana. But in contrast to the present study, Clendennen *et al.* (1998) reported that the abundance of P31 decreased as ripening proceeded. Furthermore this P31 was partially purified and polyclonal antiserum was raised against the protein. The P31 antiserum recognized a single 31 kDa polypeptide in banana pulp that was not present in peel, corm meristem or root tissues. From these results, they indicated that P31 was fruit-specific and the physiological role of P31 is not for plant protection, but as a storage protein in banana pulp. Because of the unavailability of any other plant part, such as corm, leaves or roots, it is unknown whether these two abundant chitinases of 26 and 32 kDa assessed in this present study are fruit-specific or present in other parts of the plant. Biochemical studies confirmed that several abundant pulp proteins like BanTLP (Barre *et al*. 2000), β-1,3-glucanase (Peumans *et al*. 2000), and a class I chitinase that is considered as the major banana allergens (Sanchez-Monge *et al*. 1999) are encoded by genes up-regulated during climacteric ripening. In this present study, glucanase activities of peel and pulp extracts were determined using the same protocol of the chitinase assay with CM-chitin RBV solution, except that the substrate for glucanase was 4 mg ml^{-1} carboxymethyl-curdlan remazol brilliant blue (CM-curdlan-RBB) solution. Although there are reports on banana glucanases in pulp of ripe banana fruits (Peumans *et al*. 2000), all attempts made towards determining the glucanase activity with this substrate were unsuccessful. Only the positive control (zymolyase) gave positive results in the assay (results not shown).

The chitinase activity obtained from banana pulp extracts in the present study was tremendous. Having such activity, both pulp and peel extracts were tested against the fungus *B. cinerea*. The fungus *B. cinerea* is an important pathogen of stored and transported fruits, vegetables, ornamental crops and nursery stocks and it occurs geographically wherever the host is present. Interestingly, the growth of fungus on plates was inhibited with both pulp and peel extracts. In addition, inhibition of germination of conidia was observed in *in vitro* experiments (results not shown). Although germination of conidia was inhibited, lysis of tips of germ tubes was not observed with the extracts. But extracts caused lysis of hyphal tips of *Botrytis* grown on agar. In some instances, the fungal tips started to burst within the first minute of appliying the pulp extract to the hyphae and subsequently the cell contents started to flow out. Lysis did not occur at some hyphal tips at the time of observation, but some of them were swollen. Similar observations have been obtained by Mauch *et al*. (1988), when they treated various fungi including *Fusarium solani* f. sp. *phaseoli* (strain W8), *Fusarium solani* f. sp. *pisi* (ATCC 38136) *Alternaria solani* and *Botrytis cinerea* with the crude extracts of pea pods containing chitinase and β-1,3-glucanase activities. In fact, the antifungal activity of chitinases has been found in fruits, such as fig (Li *et al*. 2005), grape (Fernandez-Caballero *et al*. 2009), and papaya (Chen *et al.* 2007), and two homologous chitinases that inhibit *Fusarium oxysporum* were isolated from Gold bananas (Ho & Ng 2007). The work done with banana by Toledo *et al.* (2012) using two-dimensional fluorescence difference gel electrophoresis (2D- DIGE) found that one class III chitinase was in down regulated spots detected in pre-climacteric fruits. In contrast, the two chitinase isoforms were accumulated during ripening and these two up-regulated isoforms revealed in their study could be important in pathogen resistance or in adaptation to postharvest conditions. The strong inhibition of the *in vitro* growth of *B. cinerea* obtained in this present study also suggested that the chitinase-rich banana extracts had an antifungal activity. In addition, the lack of growth inhibitory effect with boiled enzyme extract in well 6 in Figure 3B showed that the inhibition of *Botrytis* growth from wells 1-5 in plate (B) was not due to any contaminating ions or any other molecules present in extracts.

CONCLUSIONS

The results presented in this study demonstrated that banana fruits had increasing chitinase activities during ripening. The chitinases detected were constitutive, and showed antifungal activities under *in vitro* conditions.

Though it was not detected in this study, there are reports showing the accumulation of β-1,3 glucanases during ripening of banana fruits. Therefore induction of these two major PR proteins, which act synergistically against many fungi, will be a better way to enhance disease resistance against pathogens.

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