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Physical Interactions between Yeast *Pichia guilliermondii* and Post-Harvest Fruit Pathogen *Penicillium expansum*

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Attachment of yeast cells or bacteria on fungal hyphae have been observed in various antagonisms between microorganisms. Physical interactions between yeast *Pichia guilliermondii* and postharvest fruit pathogen *Penicillium expansum* in culture were studied in detail using light and transmission electron microscope to give better understanding on their mode of antagonism. Both organisms were co-cultured for 24-hr on potato dextrose agar. Light microscopy observations on the co-culture showed that the yeast cells attached firmly on the fungal hyphae. This attachment was inhibited by several substances such as enzymes degrading protein (protease or trypsin), a respiration inhibitor (sodium azide), an acid (hydrochloric acid) or an alkali (sodium hydroxide). Although autoclaved hyphae did not affect the attachment, but boiled enzymes and autoclaved yeast cells totally abolished the attachment. These evidences suggested that the attachment might be an active process mediated by certain protein from live yeast cells. Transmission electron micrographs on the ultrastructure of the co-culture revealed that the hyphae showed abnormalities in their structure and organelles, and a degree of obvious damage. Physical interactions observed in this study could be contributed to the mechanism of antagonism between *P. guilliermondii* and *P. expansum*.

Key words: *Pichia guilliermondii*, *Penicillium expansum*, attachment, transmission electron microscope

INTRODUCTION

Penicillium expansum (Hypomycetes) causes considerable destructions of wide range of fruit after harvest. It is known to produce a mycotoxin called patulin, which has been found in apple juice (Holmes 1994). It is also a pshycrophilic fungus, i.e. being able to grow at low temperatures (Pitt & Hocking 1985), therefore it still poses a particular problem even for fruit stored in cool storages. This fungus is generally controlled by benzimidazole fungicides. However, benomyl resistant strains have been reported in several countries (Wicks 1977; Baraldi *et al*. 2003). Unavailability of the similar report for tropical countries might due to the lack of research on the similar works. Moreover, controversial debates over chemical residues in food products and their danger to human health (Roberts 1994), increasing resistance of pathogens to chemical fungicides (Delp 1980), and withdrawal of several important fungicides (Janisiewicz 1991) has highlited the need for the safer postharvest pathogen control method.

Application of biocontrol for supressing postharvest pathogen of fruit have been reported (Spadaro & Gullino 2004). The potential use of the yeasts, *Kloeckera apiculata* strain 138, *Candida guilliermondii* (McLaughlin *et al*. 1990; McLaughlin & Wilson 1992), *Cryptococcus laurentii* (Robetts 1990a, b), *Candida saitona* (Chan & Tian 2005), and *Rhodotorula glutinis* against *Botrytis cinerea* (Widyastuti 1998, 2005) have also been studied.

The yeast *Pichia guilliermondii* has been reported to be able to suppress the infection of apple fruit caused by *Penicillium expansum* (Widyastuti 2006). However, studies on the mechanism of action of the antagonism are important as a basis for further work to enhance the performance and to develop useful formulations of the agent. Furthermore, to develop combination between the agents with other diseases control measures.

Attachment of cells of an antagonistic yeast to pathogen hyphae has been observed *in vitro* by Wisniewski *et al*. (1991), who suggested that this attachment may contribute to the mode of action of the antagonism. Therefore, the aim of this study was to examine the physical interactions between the yeast cells *P. guilliermondii* and the fungal pathogen, *P. expansum*, using light and transmission electron microscopy (TEM) in order to provide further understanding of the mode of action of this antagonism.

MATERIALS AND METHODS

Yeast and Fungal Pathogen Isolates. Yeast isolate *P. guilliermondii* strain D20 and mold *P. expansum* (isolated from rotted apples) were kindly given by of R.J. Holmes of Institute Horticultural Development, Knoxfield, Victoria. Australia. The yeast cultures were stored on nutrient yeast dextrose agar (NYDA) containing 8 g nutrient broth, 5 g yeast extract, 10 g D-glucose, and 20 g of agar per liter of water. For routine experiments, cells of yeast were grown in NYDB at 20 ^{o}C for 48 h which has the same ingredients with NYDA except for the absence of agar. Yeast cells was separated from the culture medium by centrifugation at 5,000 g for 20 min (Sorvall centrifuge, Du Pont Instruments). Cells were then washed by mixing with sterile distilled water (sdw) and recentrifuge twice and adjusted at a concentration of 10^{8} cfu ml^{-1}

(Spectrofotometer). Fungal pathogen culture was maintained on Potato Dextrose Agar (Oxoid, CM 139) containing 4 g potato, 20 g glucose, and 15 g agar per liter of water. Fungal suspension was prepared in sdw from 10-14-day-old cultures grown on PDA as described by Janisiewicz (1992).

Light Microscopy of the Interaction Between Yeast Cells and Fungal Pathogen. A fungal suspension and yeast suspension were prepared as above. Following the method of Wisniewski *et al*. (1991), a drop of fungal suspension (10^4 spores/ml) was placed on a 5 mm diameter disc of boiled (5 min in boiled water to remove plasticizers) and sterilized (121 ^oC, 15 min) cellophane over PDA in 90 mm Petri dishes. After 24 h, a 20 µl drop of yeast suspension (10^8 cfu ml^{-1}) was added to the margin of the fungal hyphae. There were five replicates per treatment. After incubation at 20 ^oC for 24 hr (Wisniewski *et al*. 1991), pieces of the cellophane was placed on a glass microscope slide and washed with sdw to remove the unattached yeast cells. The cellophane bearing the co-culture was then observed under a light microscope.

Effect of Several Substances and Heat Treatment (Autoclaving) on the Attachment of Yeast Cells on Fungal Hyphae. Disc of cellophane (5 mm diameter) bearing small colonies of the mycelium of fungal pathogen prepared as described above were washed with sdw and then placed in a Petri dish. A drop of yeast suspension (10^8 cfu ml^{-1}) was then added onto the fungal colony as described above, and immediately a drop of solution of the test substance was also added. The test substances used were: enzyme degrading protein (protease and trypsin, 2 mg l^{-1}), inactivated enzymes (protease, trypsin at the same concentration were placed in the small eppendorf and placed in the boiled water for 5 min), acid and alkali which are able to destroy the yeast cells (0.1 M HCl, 0.1 M NaOH), respiration inhibitors (1% Sodium azide, 1% merkaptoethanol, and 1% Tween 20). Following the method of Wisniewski *et al*. (1991) and the report of previous experiments (Widyastuti 2006) cultures were then incubated at 20 ^oC, 24 h. After incubation the cellophane discs were then examined under light microscope as described above. There were five replicates per treatment. The culture were incubated and observed under light microscope as above.

The effect of heat treatment on the attachment between yeast cells and fungal hyphae was examined under light microscope. Either yeast cells or fungal hyphae were autoclaved at 121 ^oC for 15 minutes. A drop of autoclaved yeast cells suspension was placed on a cellophane disc on PDA as described above. Preparations were then incubated and interactions observed under light microscope as above.

Transmission Electron Microscopy of the Interaction of Yeast Cells and Fungal Hyphae. Electron microscopic observation was conducted on Transmission Electron Microscope (TEM, ELMISKOP 102, Siemens, Germany). Pieces of cellophane (5 mm diameter) were sterilized and placed on the surface of the PDA plate. A drop of spores suspension of the pathogen (1×10^5 spores ml^{-1}) were placed over the cellophane and the Petri dish was then incubated for 24 h before the addition of yeast suspension. Discs of cellophane bearing co-culture of yeast cells and the pathogen (24 h) were then prepared for TEM as detailed below.

The specimens on the cellophane were placed in the solution of 5% (v/v) glutaraldehide and 4% (v/v) paraformaldehide in 0.05 M sodium phosphate buffer (pH 6.8). The specimens were kept overnight in this fixative, and were then washed twice for 15 min with the same buffer. Samples were then fixed in 1% (w/v) osmium tetroxide in sdw for 2 h, followed by double washing in 5 ml sdw. The specimen were then dehydrated through a graded acetone series (10, 30, 50, 70, 90% v/v in sdw), and two times in 100% acetone for 15 min each, were kept for 1 h in 100% acetone before being placed in a 50% (v/v) solution of an embedding medium (Spurr 1969) in acetone. The specimens were then left overnight on a gentle rocking shaker at room temperature. The embedding medium was composed of 5 g vinyl cyclohexane dioxide, 3 g diglycidyl ether poplypropylene glycol, 13 g nonenyl succinic anhydride, and 12 drops of 2-di-methylamino ethanol (Emgrid, Australia). The specimens were then put in vial containing pure embedding medium and shaken gently for 3 h. The medium was replaced once and then were shaken for further 3 h. These samples were then covered with new embedding medium and put in an oven (70 ^oC) overnight. The blocks of specimen were then cut using a microtome (Ultra-cut Reichert Jung, Australia). Ultra-thin sections (80 nm) were placed on a copper microscope grid coated by plioloform film and were stained with a preparation of saturated uranyl asetat in alcohol and lead citrate. Grids were then observed and photographed at 80 kV using a TEM (Elmiskop 102, Siemens, Germany).

RESULTS

Interaction Between Yeast Cells and Fungal Pathogen Observed Under a Light Microscope. The fungus might to sporulate on the fruit surface appear to have white mycelium with grayish green colour (Figure 1a). It was evident that the yeast cells abundantly attached to the hyphae of pathogen when those two organisms grown together, even after thorough washing with distilled water (Figure 1c).

Effect of Several Substances and Heat Treatment (Autoclaving) on the Attachment of Yeast Cells on Fungal Hyphae. Attachment of the yeast cells to the fungal hyphae was disrupted by the addition of low concentration of protein degrading enzymes (protease, trypsin), a respiration inhibitors (sodium azide, β-merkapthoethanol), 0.1 M HCl and 0.1 M NaOH (Table 1). Inactivated enzymes (boiled protease and boiled trypsin) did not abolish the attachment.

However, autoclaved yeast cells abolished their attachment to the fungal hyphae. While, autoclaved hyphae did not prevent the attachment of the yeast cells on it.

Transmission Electron Microscopy of the Interaction of Yeast Cells and Fungal Hyphae. TEM observations of ultrathin sections show the hyphae of *Penicillium expansum* grown axenically on a cellophane film over PDA for 48 h (Figure 2a). The cell wall was mostly thick with a rough, fibrillar outer layer. However, the hyphae cultured together with the yeast cells showed a range of abnormalities. In some sections the plasmalema appeared to be irregular and slightly detached from the cell wall. Granular glycogen-like materials (some

deposited in rossete-like structures) were scaterred in the cytoplasm (Figure 2a).

The most destructive effect was observed in the fungal hyphae cultured for 48 h (Figure 2b,c). Most of the hyphae looked empty and had a number of unrecognizable (unidentified) organels (Figure 2b), and very thin or damaged cell walls (Figure 2c). Many cells of yeast had close contact with the hyphal walls (Figure 2b,c). The extra cellular matrix of yeast was also looked intermeshed with that of the hyphae (Figure 2b). In some sections, part of the hyphae died as the outer cell wall materials appeared to have become separated from the hyphae (Figure 2c).

DISCUSSION

It has been suggested that attachment of antagonistic yeast cells to fungal pathogen hyphae may play a role in the antagonism activity of yeast cells against the pathogen (Wisniewski *et al*. 1991). It was obvious in the present study that, when cultured together on agar medium, the yeast cells attach firmly to the hyphae of the pathogen studied, to the extent that after extensive washing of the hyphae with distilled water, a more or less continuous layer of yeast cells remained attached. Moreover, the yeast cells formed a dense aggregation around the hyphae. Nelson *et al*. (1986) found that attachment of *Enterobacter cloacae* to hyphae of *Phytium ultimum* play a role in the biological control of phytium emergence damping-off.

However, in this experiment such attachment was affected by the presence of protein degrading enzymes, respiration inhibitors and acid/alkali treatment. The effect of respiration inhibitors suggests that attachment might be an active process, while the effect of protein degrading enzymes indicates that protein might play a part in this attachment process (Wisniewski *et al*. 1991). Autoclaving the yeast cells also abolish the attachment, but autoclaving the hyphae did not

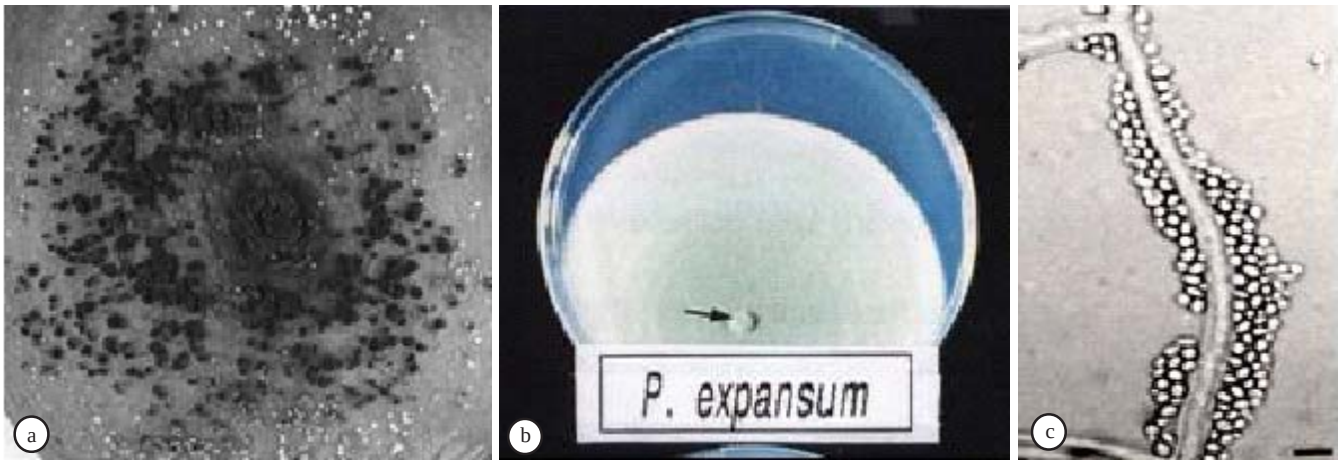

Figure 1. a. *P. expansum* on the surface of apple fruit, b. *P. expansum* on the PDA, c. Attachment of cells *P. guilliermondii* on hyphae of *P. expansum* viewed by a light microscope (Bar = 5 µm).

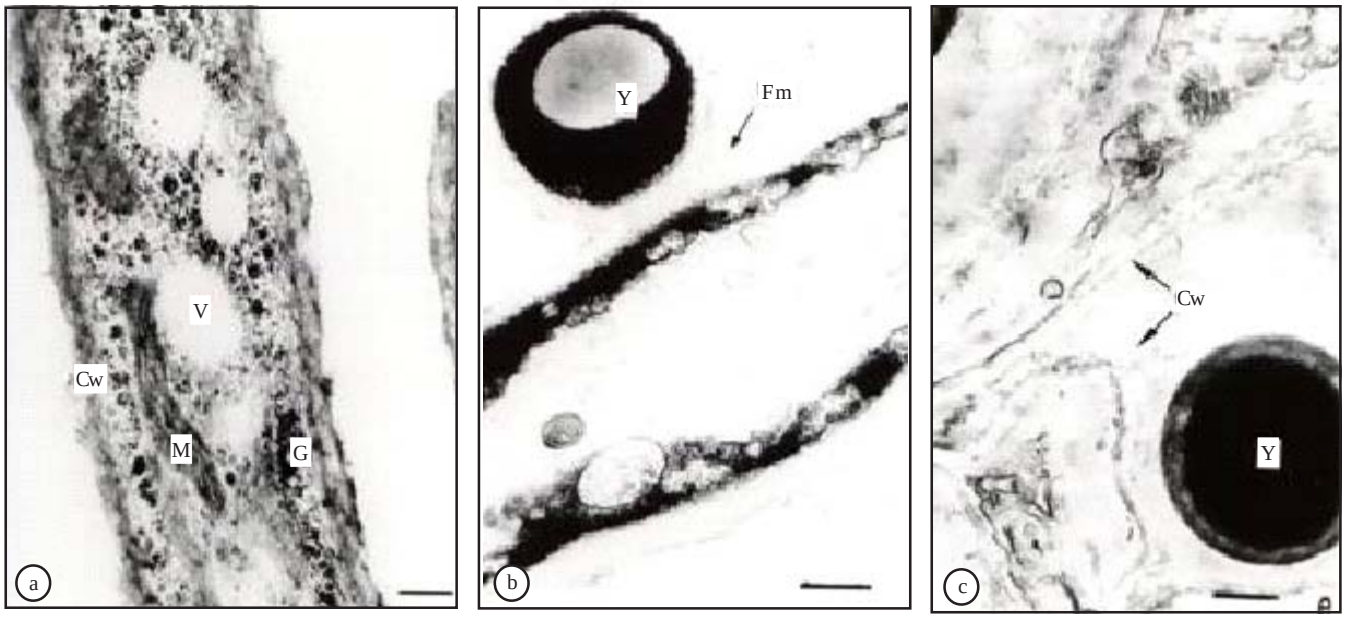

Figure 2. Transmision electron micrograph of a. hyphae of *P. expansum* grown axenically for 48 h, Bar = 0.50 µm, G = glycogen structures, V = vacuola, M = mitochondria, Cw = Cell wall is rough and irregular; b, c. hyphae of *P. expansum* grown with *P. guilliermondii* for 48 h, Fm = fibril materials, Y = yeast cells.

Table 1. Effect of addition of various substances and heat treatment (autoclaving) on attachment of the yeast cells *P. guilliermondii* to hyphae of pathogen *P expansum* as observed under a light microscope after 48 h incubation at room temperature.

Treatment	Attachment
Yeast *P. guilliermodii*	++
Yeast with:	
Protease (2 mg l^{-1})	-
Protease (2 mg l^{-1}, boiled 5 min)	++
Trypsin (2 mg l^{-1})	-
Trypsin (2 mg l^{-1}, boiled 5 min)	++
HCl 0.1 M	-
NaOH 0.1 M	-
Sodium azide (1%)	-
Tween 20 (1% v/v)	+
β-merkaptoethanol	+
Non autoclaved yeast cells	++
Auoclaved yeast cells+ fresh hyphae	-
live yeast cells+autoclaved hyphae	+

-: No attachment, +: Sparce attachment of yeast cells to hyphae following washing, ++: Frequent attchmanet of yeast cells to hyphae following washing, +++: The whole hyphae covered with attached yeast cells following washing.

prevent the attachment. It is possible that heat treatment (autoclaving) disrupted the attachment process mediated by proteins.

The above attachment is likely to be significant in the antagonism of the yeast cells towards the hyphae as observed by Wisniewski *et al*. (1991) on the antagonism between yeast against *Botrytis cinerea* of apple. This antagonism might be mediated by production of toxic substances such as iturins (Gueldner *et al*. 1988), degradative enzymes such as chitinases or glucanases or by competition for certain nutrients or water (Droby *et al*. 1989; Chalutz 1990; Wisniewski *et al*. 1991). Moreover, the intimate association of the cells could facilitate the activity of toxic substances produced by the yeast in a very low concentration, or in response to specific nutrients exuded by the hyphae (eg. a specific carbohydrates in the extra-hyphal matrix). On the other hand, the prolific attachment will most certainly accentuate the effect of competition for exogenous nutrients exerted by the yeast cells as observed in various antagonisms between organisms (Wisniewski *et al*. 1988; Wisniewski *et al*. 1991; Giovanni 1995). Because of the nature of the contact between the yeast cells and the hyphae, it is possible that the yeast cells block the diffusion of nutrient from the substrates (eg. broken cells of wounds in harvested fruit) into the hyphae (Wisniewski *et al*. 1991), or even the binding of hydrolytic enzymes from the hyphae into the substrate (Tronsmo *et al*. 1976), thus interfering with the pathogenicity of the fungus.

The ultra structural observations using TEM showed that the yeast cells caused severe destructive damage to the hyphae. The yeast cells often seen eroded the matrix around the hyphae and established direct contact with the hyphal wall. The yeast cells interact with the hyphal matrix of the pathogen and that the fungal hyphae suffer degree of cellular damage as observed by Chan and Tian (2005). This damage could be the direct results of competition for nutrients or water (Wisniewski *et al*. 1991; El-Ghaouth *et al*. 2003) resulting premature senescence of the hyphae, or due to the possible toxic metabolites exerted from cells of the yeast e.g. antifungal compounds (Gueldner *et al*. 1988).

Physical interactions could be contribute to the mechanism of action of the yeast isolate *P. guilliermondii* in suppressing apple rot pathogen *P. expansum*. These possibilities of mode of action need further elucidation, in order to support the legal registration for commercial application of the yeast cells for suppressing the postharvest fruit diseases. Yeast can be incorporated into general postharvest handling practices, such as dipping or spraying treatments. Recently two commercial biofungicide products based on microbial have been registered in United States and South Africa for postharvest use (Janisiewicz & Korsten 2002).

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