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Screening and identification of yeast strains from fruits and vegetables: Potential for biological control of postharvest chilli anthracnose (*Colletotrichum capsici*)

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Abstract

Yeasts antagonistic to *Colletotrichum capsici* were isolated from Thai fruits and vegetables. Four antagonists (R13, R6, ER1, and L2) were found that inhibited *C. capsici* growth with biocontrol efficacies of 93.3%, 83.1%, 76.6%, and 66.4%, respectively. Identification by 26S rDNA, and ITS region sequence together with physiological and morphological characteristics, showed them to be *Pichia guilliermondii*, *Candida musae*, *Issatchenkia orientalis*, and *Candida quercitrusa*, in order of their efficacy. *P. guilliermondii* strain R13 showed efficacy in reducing disease incidence on *C. capsici* infected chilli fruits to as low as 6.5%. Lower disease incidence was observed at lower storage temperature. The application of *P. guilliermondii* is more effective for preserving chilli fruits than conventional preservation with chlorinated water.

Keywords: Biocontrol; Antagonist; Yeast; *Pichia guilliermondii*; *Colletotrichum capsici*; Chilli fruits; Postharvest

1. Introduction

The general strategy of biological control is to use one living organism to control another (Druvefors, 2004). The control agents may be antagonistic microorganisms or even natural plant- and animal-derived compounds (Pal and Gardener, 2006). Recently, biological control has been developed as an alternative to synthetic fungicide treatment and considerable success has been achieved upon utilizing antagonistic microorganisms to control both preharvest and postharvest diseases (Janisiewicz and Korsten, 2002). A variety of microbial antagonists have been reported to control several different pathogens on various fruits and vegetables (Fravel, 2005; Mari and Guizzardi, 1998).

Among these antagonistic organisms, natural yeasts have been efficacious as biological control agents (Irtwange, 2006; Qing and Shiping, 2000). Yeasts possess many properties that make them useful for control purposes. Yeasts generally do not produce allergenic spores or mycotoxins as many mycelial fungi do or antibiotic metabolites likely to be produced by bacterial antagonists (Droby and Chalutz, 1994). Yeasts generally have simple nutritional requirements and are able to colonize dry surfaces for long periods of time, as well as withstand many pesticides used in the postharvest environment (El-Tarabily and Sivasithamparam, 2006; Wilson and Wisniewski, 1989). In addition, yeasts can grow rapidly on inexpensive substrates in fermenters and are therefore easy to produce in large quantities (Druvefors, 2004). Past studies and suggested modes of action of biocontrol yeasts indicated less likelihood of any hazard to consumers (Arras and Arru, 1997; Arras

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et al., 1999). Furthermore, yeast cells contain high amounts of vitamins, minerals, and essential amino acids and there are several reports on the beneficial effects of yeast in foods and feeds (Hussein et al., 1996).

Anthracnose caused by *Colletotrichum capsici* is a major disease of tropical vegetables such as chilli (*Capsicum annuum* L. var. *acuminatum* Fingerh.) (Hadden and Black, 1989; Pakdeevaraporn et al., 2005). This disease appears as ripe fruit-rot and die-back (Mehrotra and Aggarwal, 2003). Ripe fruit-rot is more conspicuous as it causes severe damage to mature fruits in the field as well as during transit and storage. The first outward sign of infection is the appearance of a small, black, circular spot, generally sharply defined or at times diffused. Die-back usually appears after rain or after a prolonged dew period. The attack commences from the growing point of the flower bud, the tops of the affected branches wither and turn brown. The infected plants bear fewer fruits of low quality. Under conditions favorable to disease development, up to 50% of the fruits can be damaged (Smith and Crasson, 1959). Typically, symptoms first appear on mature fruits as small, water-soaked, sunken lesions that rapidly expand. The lesion may increase to 2–3 cm in diameter on large fruits. *C. capsici* produces conidia in an acervulus and the spore dimension is $17–18 \times 3–4$ μm. The appressoria from germinated conidia then penetrate into chilli epidermal cells. Mycelial growth inside host tissues produces a thermostable toxin which causes injury, particularly to the protoplasmic content of the host tissues (Narain and Das, 1970).

Demand for chillies in the world is increasing every year (Food and Agricultural Organization of the United Nations, 2004) and good quality chillies, i.e., absence of diseased appearance or fungal toxins, are prerequisites for import and export (The Chile Pepper Institute, 2004). However, control of anthracnose disease on chilli fruits still relies mainly on the use of synthetic fungicides. This may result in fungicide-resistant pathogens as occurred in copper fungicides and dithiocarbamates (Parry, 1990). In addition, there is also a problem of fungicide contamination in foods and the environment. Thus, there is an increasing need for alternative control strategies. Several studies have shown successful use of yeast antagonists to control plant pathogens in fruits (Lurie et al., 1995; Wilson et al., 1993).

Our objectives were to investigate the possibility of using harmless yeast biocontrol agent(s) of chilli anthracnose in order to reduce the use of chemical agents that may be harmful to humans and the environment. In this study, epiphytic yeast strains from fruits and vegetables were isolated and identified. Their capabilities to control anthracnose disease caused by *C. capsici* were investigated. The most efficacious yeast biocontrol agent was used against *C. capsici* on infecting chilli fruits with positive results which warrant further investigation for postharvest preservation.

2. Materials and methods

2.1. The fungal pathogen

Colletotrichum capsici DOAC 1511 was obtained from the Mycological Laboratory of Department of Agriculture (DOA), Thailand. The fungal pathogen was maintained on potato dextrose agar (PDA) slants at 4 °C.

2.2. Sources of fruits and vegetables

Sources of yeast isolates were 11 species of tropical fruits and vegetatbles, i.e., banana (*Musa sapientum* L.), mango (*Mangifera indica* L.), longan (*Dimocarpus longan* Lour.), pineapple (*Ananus comosus* Merr.), rambutan (*Nephelium lappaceum* L.), rose apple (*Eugenia javanica* Lamk.), sapodilla (*Achras sapota* L.), bird pepper (*Capsicum frutescens* L.), chilli (*C. annuum* L. var. *acuminatum* Fingerh.), sweetbell pepper (*C. annuum* L. var. *grossum*), eggplant (*Solanum melongena* L.), and plate brush (*Solanum torvum* Swartz) harvested from untreated fields in Singhburi Province, in the central region of Thailand. Red chilli fruits (*C. annuum* L. var. *acuminatum* Fingerh.), which had not been treated with any fungicides were used for a biocontrol assay.

2.3. Severity of disease caused by C. capsici

The severity of anthracnose disease on chilli fruits caused by *C. capsici* was studied by using 30 wounded chilli fruits (one wound per fruit) inoculated with *C. capsici* at concentrations of 5×10^2, 5×10^3, 5×10^4, 5×10^5, and 5×10^6 spores ml^{-1}. Control fruits were treated with sterile distilled water. All chilli fruits were put on $17 \times 11 \times 4$ cm plastic trays wrapped with high density polyethylene sleeve. Sterile water was used to maintain high humidity (95% relative humidity (RH)) of the fruit trays. The trays were stored in a ventilated cabinet (Memmert, type UM 600, Schwabach, Germany) in the dark at 28 °C. Lesion diameter (average length of lesion in *x*-axis and *y*-axis) was measured 5 days after inoculation. The experiments were repeated three times and each treatment was conducted in triplicate of 30 wounded control and experimental chilli fruits.

2.4. Yeast isolation

Epiphytic yeasts were isolated from the surfaces of fruits and vegetables according to the method of Assis and Mariano (1999). Ten grams of the sample were suspended in 100 ml sterile distilled water and shaken vigorously for a few minutes. Serial dilutions of the yeast sample suspension were made in sterile distilled water. An aliquot of 0.1 ml of each dilution was plated on yeast malt extract agar (YM agar) adjusted to pH 3.5 with sterile tartaric acid and the cultured plates were incubated at 28 °C for 48 h. All colonies of yeast were examined under the microscope (Accu-

max XSZ-107) and 225 colonies from fruits and vegetables were chosen on the basis of their different visual characteristics. The yeast isolates were re-streaked on YM agar to obtain pure cultures and they were maintained on nutrient yeast dextrose agar (NYDA) slants containing 8 g l^{-1} nutrient broth, 5 g l^{-1} yeast extract, 10 g l^{-1} glucose, and 20 g l^{-1} agar. The cultures were stored at 4 °C until further study on antagonistic activity.

2.5. Screening for yeast antagonists

Screening for yeast antagonists against *C. capsici* was conducted in two steps. Firstly, 225 colonies of yeast colonies isolated from fruit and vegetable samples were streaked on potato dextrose agar (PDA) plates containing *C. capsici* at 5×10^4 spores ml^{-1}. The cultures were incubated at 28 °C for 5 days. Then yeast isolates that resisted mycelial invasion (54 colonies) were chosen for the well test according to the method of He et al. (2003). Yeast isolates were cultured in nutrient yeast dextrose broth (NYDB: NYDA without agar) on a rotary shaker for 48 h. Yeast cells were collected by centrifugation at 3000 rpm for 20 min and washed twice with sterile distilled water (DIW) and resuspended in DIW. To test for their antagonistic activities, the washed yeast cell suspension (20 μl of 5×10^9 cells ml^{-1}) was injected into a well (6 mm in diameter) in the center of a plate containing PDA plus 15% chilli juice and kept for 1–2 h to allow penetration of the cell suspension into the well. Then 20 μl of 5×10^4 spores ml^{-1} of *C. capsici* (optimal concentration as found from experiment in Section 2.3) were applied to the well. All plates were incubated at 28 °C in the dark. The fungal growth was observed for each plate and diameters of fungal colonies (average length of diameter in *x*-axis and *y*-axis) were measured 5 days after incubation. Each treatment (each isolate) was done in triplicate and each experiment was conducted three times. The yeast isolates that completely inhibited fungal growth called yeast antagonist were used for further studies.

2.6. Identification of yeast antagonists by rDNA sequence comparison

Identification of antagonistic yeasts was done using the rDNA sequence comparison technique as described by White et al. (1990) and Mitchell et al. (1992). The reaction mixture contained specific primers for D1/D2 region of the large subunit, the 26S rDNA, that were NL-1 (5′-GCATA TCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGT CCGTGTTTCAAGACGG-3′). In addition, primers of ITS region (Internal Transcribed Spacer region), ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TC CTCCGCTTATTGATATG-3′), were also used to amplify the intervening 5.8S gene for achieving a high separation value (Kurtzman, 1992; Martorell et al., 2005; Suh and Blackwell, 2004). The 26S and 5.8S genes were amplified by the PCR technique, which yielded about 600 bp in each DNA fragment. Aliquots of 10 μl of amplified products were separated electrophoretically on 0.8% (w/v) agarose gel in Tris–borate–EDTA (TBE) buffer at a constant voltage of 100 V for 35 min. The bands were stained with ethidium bromide and photographed under transilluminated UV light. Then the products were purified by using QIAquick (QIAGEN, Germany). Sequencing was carried out using the Automate DNA Sequencer (3100-Avant Genetic Analyzer). The sequences were aligned and compared with the NCBI database by the Internet using Basic Local Alignment Search Tool (NCBI BLAST: Bethesda, MD, USA) (Altschul et al., 1997).

2.7. Morphological and physiological characteristics

Morphological characteristics of yeast antagonists were examined by observing cell and colony patterns according to the methods described by Kurtzman and Fell (1998). Yeast cells from different isolates were cultured in liquid medium (5% malt extract broth) for 2–3 days at 25 °C. The resulting yeast cells were then observed for shape and spore, i.e., whether they existed singly, paired, or aggregated in large clumps. The average length and width of 20 cells from each isolate were measured. Colony morphologies of each yeast isolate were examined in cultures grown in solid medium (5% malt extract agar) at 25 °C. The features of yeast cultures on the plates, i.e., color, texture were recorded 3–7 days after incubation. Physiological characteristics of yeast antagonists were determined by detecting their ability to ferment certain sugars semi-anaerobically and to assimilate a variety of carbon compounds as major source carbon in aerobic condition. These tests were repeated three times for each strain. The results on morphological and physiological characteristics were used in the confirmation of yeast identification by the rDNA sequencing technique.

2.8. Biocontrol assay by in vivo test

Screening of yeast isolates for biocontrol efficacy was also done by using *in vivo* tests (Dan et al., 2003). Chilli fruits (no wound or scar on the surface) from untreated field were selected for the experiments. They were surface-sterilized with 0.5% NaCl for 5 min and then washed with tap water. After air drying, chilli fruits were treated with 70% ethanol. Each fruit was wounded by using a sterile cork-borer (6 mm in diameter and 1 mm in depth), one wound per fruit.

The four strains of yeast isolates with high biocontrol efficacies, selected based on data from the *in vitro* tests, were cultured in NYDB. The cells were collected by centrifugation at 3000 rpm for 20 min. Yeast cells were washed twice with DIW and resuspended in DIW. Then 20 μl of cell suspension of each yeast strain at concentration of 5×10^9 cells ml^{-1} was applied to the wound of 30 surface-sterillized chilli fruits. After 1–2 h to allow the yeast cells to penetrate into the wound, 20 μl of *C. capsici* at a

concentration of 5×10^4 spores ml^{-1} was added to the wound. Chilli fruits were put on plastic trays wrapped with polyethylene sleeve and stored in a ventilated cabinet (95% RH) in the dark at 28 °C. Percentage of disease incidence and severity of disease, as indicated by increased wound diameter (lesion diameter) were recorded 5 days after storage. The ability to reduce disease incidence of these yeast strains was compared. For control, wounded chilli fruits were inoculated with *C. capsici* only. There were three replicates of 30 wounded chilli fruits per treatment. The whole experiment was conducted three times.

2.9. Effects of yeast cell concentration and storage temperature on biocontrol efficacy

Thirty wounded chilli fruits were used for each experiment on the biocontrol efficacy of various concentrations of the four antagonistic yeast strains. The wounds were inoculated with 20 µl of each antagonistic yeast isolate at concentrations of 5×10^6, 5×10^7, 5×10^8, and 5×10^9 cells ml^{-1} as counted by a hemocytometer. After air drying for 1–2 h, 20 µl of *C. capsici* at concentrations of 5×10^4, 5×10^5, and 5×10^6 spores ml^{-1} were added to each wound. The chilli fruits were placed on plastic trays wrapped with polyethylene sleeve (95% RH) and stored in a ventilated cabinet in the dark at 28 °C for 5 days before the measurement of the disease incidence and severity.

To determine the effects of storage temperature on biocontrol efficacy, 30 wounded chilli fruits were inoculated with 20 µl of 5×10^8 cells ml^{-1} of yeast cell suspension. After 1–2 h, 20 µl of 5×10^4 spores ml^{-1} of *C. capsici* were applied as described above. Disease incidence was recorded 5 days after incubation at 18, 23, 28, and 33 °C.

As positive and negative controls for 0 and 100% infection in all of the *in vivo* tests, 20 µl of sterile distilled water and a spore suspension of *C. capsici* (20 µl of 5×10^4, 5×10^5, and 5×10^6 spores ml^{-1}) were added to the wounds. Each treatment was done in triplicate of 30 wounded chilli fruits and the entire experiment was repeated three times. The data were transformed into a percentage of biocontrol efficacy (BC) and disease incidence (DI) according to the formula: $\%BC = [(T - A)/T] \times 100$, where T is the number of infected wounds inoculated with *C. capsici* only, and A is the number of infected wounds inoculated with the antagonist(s) and the pathogen. The percentage of disease incidence, $\%DI = (A/T) \times 100$.

2.10. Postharvest disease control

Intact chilli fruits (no visible wounds or scars on the surface) from untreated orchards were cleaned with tap water. They were tested for preservation by the four antagonistic yeast strains compared to that by water treatment and chemical treatment. Each group of 30 fruit samples were thoroughly sprayed with 300 ml of 5×10^8 cells ml^{-1} of each yeast cell suspension in distilled water. The other two groups were sprayed with 300 ml of distilled water, and chlorinated water (200 ppm chlorine), respectively. Fruits were air dried, and placed on plastic trays wrapped with polyethylene sleeve. All samples were kept in the incubator at 28 °C for 1–2 h followed by storage at 10 °C (95% RH) in the dark. The percentage of disease incidence in each group was recorded at different time intervals, i.e., 15, 30, and 45 days. Each treatment was performed in triplicate of 30 wounded chilli fruits and the entire experiment was conducted three times.

2.11. Statistical analysis

Data analysis for disease incidence, yeast biocontrol efficacy, and lesion diameter were done by using the general linear model (GLM) procedure of SPSS software (version 10.0 for Windows, SPSS Inc., Chicago, IL, USA). The least significant difference (LSD) test at $P < 0.05$ was used for mean separation.

3. Results

3.1. Screening for yeast antagonists

A preliminary experiment showed that *C. capsici* at concentrations from 5×10^2 to 5×10^4 spores ml^{-1} caused lesion diameters from 6.7 to 15.8 mm. The spore concentrations of more than 5×10^4 spores ml^{-1} did not produce significant increase in lesion diameter (data not shown). Thus the fungal concentration of 5×10^4 spores ml^{-1} was used in the subsequent experiments.

Primary screening of 225 yeast cultures isolated from 11 species of fruits and vegetables by their abilities to resist fungal invasion on plate culture technique resulted in only 54 positive isolates. All of these isolates were obtained from the 11 species together. Secondary screening of these isolates for biocontrol efficacy against *C. capsici* growth using the well test (Section 2.5) showed that only four yeast strains were effective in inhibition of *C. capsici* growth, i.e., no mycelial growth on the PDA plates as observed under the microscope (data not shown). In these four yeast strains, two were isolated from rambutan, these are assigned R13 and R6 and other two strains from red eggplant and longan, ER1 and L2, respectively. These four yeast strains were tested further for confirmation of their potential in controlling chilli anthracnose caused by *C. capsici*.

3.2. Identification of the yeast antagonists

Yeast strains R13, R6, ER1, and L2 with the ability to control *C. capsici* growth in the *in vitro* tests were found to be different in colony characteristics and cell morphologies as shown in Table 1. Each strain was analyzed for 26S (D1/D2 region). The sequence analysis of large subunit (26S) ribosomal DNA gene of yeast strains R6, ER1, and L2 showed high identity with those of *Candida musae*, *Issatchenkia orientalis*, and *Candida quercitrusa*, i.e., 97%,

Table 1
Identification of the four strains of yeast isolates by the rDNA sequencing and morphological characteristics (colony, cell, and spore morphologies)

Yeast isolate	Identity[a] (%)	rDNA[b] region	Species	Colony morphology[b]	Cell morphology	Spore morphology
R13	98	ITS1/ITS4	*Pichia guilliermondii*	Tannish-white, butyrous	Ovoidal (1.5–4.8) × (2.0–15.0) μm, singles, pairs, short chains	Hat-shaped, 1–4 spores per ascus
R6	97	D1/D2	*Candida musae*	White, creamy	Ovoidal (3.0–5.0) × (4.0–8.0) μm, singles, small groups	No spore
ER1	99	D1/D2	*Issatchenkia orientalis*	Light cream, butyrous	Ovoidal to elongate, (1.3–6.0) × (3.3–14.0) μm, singles, pairs	Spherical, 1 spore per ascus
L2	99	D1/D2	*Candida quercitrusa*	White, butyrous	Ovoidal, (2.0–5.0) × (4.5–7.0) μm, pseudohyphae	No spore

[a] The percentage identity among DNA fragments was calculated with BLAST program and the sequences were compared with those from NCBI database.
[b] Region of the rDNA gene used for identification.

99%, and 99%, respectively (Table 1). However, identification of R13 by analysis of large subunit 26S rDNA showed 99% identity with *P. guilliermondii*, *Candida fukuyamaensis*, and *Candida xestobii* (not shown in Table 1). Thus the small subunit of 5.8S rDNA (TSI1/TSI4 region) was further used for more effective identification (Kurtzman, 1992; Martorell et al., 2005). The results showed 98% identity of R13 to *P. guilliermondii* (Table 1). The results of physiological characteristics of yeast strains according to fermentation and assimilation tests (Table 2) agreed with the data of the rDNA analysis (Table 1). The different patterns of carbon fermentation and assimilation of R13, R6, ER1, and L2 as shown in Table 2 indicated that R13 could utilize more carbon sources than others, while ER1 used very few carbon sources in this system.

3.3. Comparison of in vivo biocontrol efficacy of the four antagonistic yeast strains

The biocontrol efficacy of the four antagonistic yeast strains was confirmed by using the *in vivo* test, or by their demonstrated ability to reduce disease incidence in chilli fruits. The results in Table 3 show that strain R13 had the highest biocontrol efficacy of 93.3%, or disease incidence of only 6.7%. Yeast strains R6, ER1, and L2 showed biocontrol efficacies of 83.1%, 76.6%, and 66.4%, respectively. Moreover, the four yeast antagonists were significantly effective ($P < 0.05$) in reducing the diameter of lesion caused by *C. capsici*, i.e., from 15.4 mm in *C. capsici* infection to 6.7, 7.8, 9.1, and 10.3 mm upon treatment with R13, R6, ER1, and L2, respectively (Table 3).

3.4. Effects of antagonistic yeast concentration on disease control

The results of testing different concentrations of the four yeast strains against *C. capsici* at various concentrations show that the yeast strain R13 is more effective than the other three strains (R6, ER1, and L2) in reducing disease incidence at all concentrations tested (Fig. 1). Upon infection with 5×10^6 spores ml^{-1} of *C. capsici*, the disease incidence was reduced from 23.3% to 10.5% as concentrations of R13 cells increased from 5×10^6 to 5×10^8 cells ml^{-1}. However, R6, ER1, and L2 strains reduced the disease incidence to lesser extents, i.e., from 25.8% to 15.6%, 28.2% to 17.9%, and 33.5% to 22.5%, respectively. Control group with only *C. capsici* (5×10^4–5×10^6 spores ml^{-1}) infection showed 100% disease incidence (data not shown).

At lower *C. capsici* concentrations of 5×10^5 and 5×10^4 spores ml^{-1}, disease incidence decreased from 20% to 6.7% and 13.3% to 6.5% as R13 increased from 5×10^6 to 5×10^8 cells ml^{-1} (Fig. 1A). However, increasing the concentrations of the yeast strain R13 from 5×10^8 to 5×10^9 cells ml^{-1} did not result in greater reductions in disease incidences caused by 5×10^6–5×10^9 spores ml^{-1} of *C. capsici*. Similar findings were observed in the yeast strains R6, ER1, and L2 although the extent of disease reduction was smaller (Fig. 1B–D).

3.5. Effects of temperature on disease control by yeast antagonists

Yeast strain R13 was significantly superior to the other three yeast strains in reduction of disease incidence at all temperatures (18–33 °C) tested over a period of 5 days (Fig. 2). The antagonistic effects of the four yeast strains were inversely related to the storage temperature. The biological control was more efficacious at 18 °C compared to that at 23, 28, and 33 °C. The disease incidences in R13 treatment were 1.1%, 3.3%, 6.7%, and 10.0% at 18, 23, 28, and 33 °C, respectively. At 33 °C, the disease incidence in R13 treatment was about 2, 4, and 5 times lower than that of R6, ER1 and L2 treatments. Control with only *C. capsici* inoculation showed disease incidence of 100% at storage temperature of 18–33 °C (data not shown).

3.6. Postharvest disease control by yeast antagonists

Disease incidence in chilli fruits sprayed with R13 cells was significantly less than those treated with the other three yeast strains at all storage times (15, 30, and 45 days) tested (Table 4). Although the disease incidence gradually

Table 2
Comparison of fermentation and assimilation tests (using biochemical reactions) of the four effective yeast strains (R13, R6, ER1, and L2) with *Pichia guilliermondii*, *Candida musae*, *Issatchenkia orientalis*, and *Candida quercitrusa* as listed by Kurtzman and Fell (1998)

Test	Strain R13	*P. guilliermondii*	Strain R6	*C. musae*	Strain ER1	*I. orientalis*	Strain L2	*C. quercitrusa*
Fermentation								
D-Glucose	+	+	+	+	+	+	+	+
D-Galactose	−	v	−	−	−	−	−	v
Sucrose	+	+	−	−	−	−	−	−/s
Maltose	−	−	−	−	−	−	−	−/s
Lactose	−	−	−	−	−	−	−	−
Raffinose	+	+	−	−	−	−	−	−
Trehalose	+	+	+	s	−	−	−	−
Assimilation								
D-Glucose	+	+	+	+	+	+	+	+
D-Galactose	+	+	−	−	−	−	+	+
L-Sorbose	+	v	+	+	−	−	+	+
D-Glucosamine	+	+	+	l	+	+	+	+
Actidione	+	NL	−	NL	−	NL	−	NL
Saccharose	+	+	+	+	−	−	+	+
N-acetyl-D-glucosamine	+	+	+	l	+	+	+	+
DL-Lactate	−	v	−	−	+	+	+	l
L-Arabinose	+	+	−	−	−	−	−	−
Cellobiose	+	+	−	−	−	−	−	−
Raffinose	+	+	−	−	−	−	−	−
Maltose	+	+	+	+	−	−	+	+
Trehalose	+	+	+	+	−	−	+	+
2-Keto-D-gluconate	+	+	+	+	−	−	+	+
α-Methyl-D-glucoside	+	+	−	l	−	−	+	+
D-Glucitol	+	+	+	+	−	−	+	l
D-Xylose	+	+	+	+	−	−	+	v
D-Ribose	+	+	−	−/l	−	−	+	l
Glycerol	+	+	+	+	+	+	+	+
L-Rhamnose	−	v	−	−	−	−	−	−
Palatinose	+	NL	+	NL	−	NL	+	NL
Erythritol	−	−	−	−	−	−	−	−
Melibiose	+	+	−	−	−	−	−	−
D-Glucuronate	−	NL	−	−	−	NL	−	−
Melezitose	+	+	+	+	−	−	+	+
D-Gluconate	+	v	+	l	−	−	+	+
Levulinate	−	NL	−	NL	−	NL	−	NL
D-Mannitol	+	+	+	+	−	−	+	l
Lactose	−	−	−	−	−	−	−	−
myo-Inositol	−	−	−	−	−	−	−	−

+, Positive reaction; −, negative reaction; v, variable reaction; s, positive but slow reaction; −/s, negative or positive but slow reaction; l, latent reaction (rapidly developing a positive reaction after a lag phase); −/l, negative or latent reaction; NL, test results not listed by Kurtzman and Fell (1998).

Table 3
The effectiveness of the four antagonistic yeast strains in reduction of disease (anthracnose) incidence in *Colletotrichum capsici* infected chilli fruits

Yeast isolate	Disease incidence[a] (%)	Biocontrol efficacy[a] (%)	Lesion diameter[b] (mm)
Pichia guilliermondii R13	6.7 ± 0.40a	93.3 ± 0.40a	6.7 ± 0.23a
Candida musae R6	16.9 ± 0.87b	83.1 ± 0.87b	7.8 ± 0.17b
Issatchenkia orientalis ER1	23.4 ± 0.92c	76.6 ± 0.92c	9.1 ± 0.28c
Candida quercitrusa L2	33.6 ± 0.52d	66.4 ± 0.52d	10.3 ± 0.35d
Control	100.0 ± 0.00e	0.0 ± 0.00e	15.4 ± 0.40e

[a] %Disease incidence = $(A/T) \times 100$ and %Biocontrol efficacy = $[(T - A)/T] \times 100$, where T is the number of infected wounds of chilli fruits inoculated with *Colletotrichum capsici* only (control), and A is the number of infected wounds of chilli fruits inoculated with both yeast antagonists and *C. capsici*. The results are presented as mean of three independent experiments ± standard error. Values of each column followed by a different letter indicate significant difference ($P < 0.05$) according to LSD test.

[b] Lesion diameter is the average length of lesion in *x*-axis and *y*-axis.

increased with storage time in all treatments, the R13 treatment was more efficacious than that of chlorinated water at 30 and 45 days of storage. The percentage of disease incidence in chilli fruits sprayed with R13 cell suspension and chlorinated water were 13.3% and 16.7%, 16.7% and 20.0% at 30 and 45 days of storage, respectively (Table

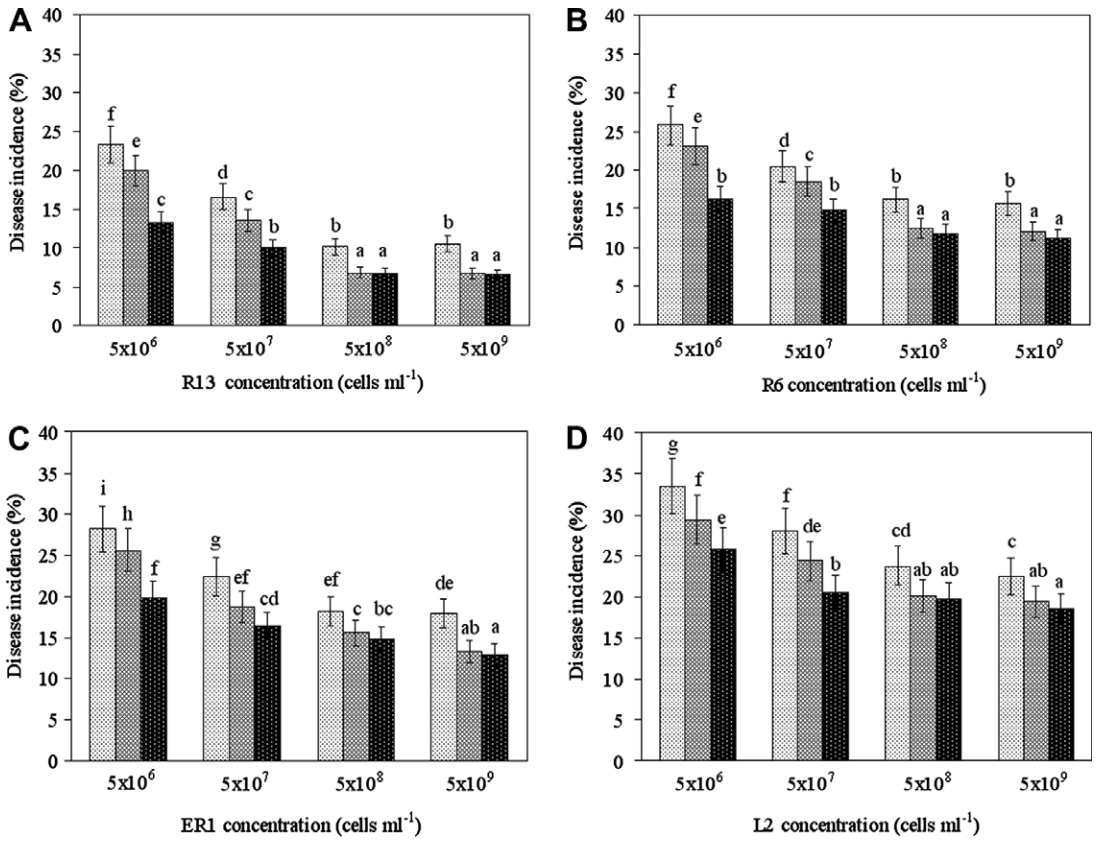

Fig. 1. Effects of yeast concentration on reduction of disease (anthracnose) incidence in chilli fruit. Thirty wounded fruits were inoculated with 20 μl of the yeast antagonists: *Pichia guilliermondii* R13 (A), *Candida musae* R6 (B), *Issatchenkia orientalis* ER1 (C), and *Candida quercitrusa* L2 (D) at concentrations of 5×10^6, 5×10^7, 5×10^8, and 5×10^9 cells ml^{-1}. After air drying, the fruits were inoculated with 20 μl of different concentrations of *Colletotrichum capsici*: 5×10^6 spores ml^{-1} (▒), 5×10^5 spores ml^{-1} (▒), and 5×10^4 spores ml^{-1} (▒). Data were recorded 5 days after storage at 28 °C. Bars represent the means of three independent experiments ± standard errors. Letters on the top of each bar indicate the results of LSD test ($P < 0.05$). Values with shared letters in the same graph are not significantly different. Control with only *C. capsici* infection resulted in 100% disease incidence.

4). In all cases, the disease incidences after yeast and chemical treatments were significantly less than that washed with only distilled water.

4. Discussion

This study demonstrates that four epiphytic yeasts (*P. guilliermondii* strain R13, *C. musae* strain R6, *I. orientalis* strain ER1, and *C. quercitrusa* strain L2) isolated from fruits and vegetables reduced disease incidence in chilli pepper fruit caused by *C. capsici* to varying degrees. *P. guilliermondii* strain R13 had the highest biocontrol efficacy, both *in vivo* and *in vitro* (Figs. 1 and 2; Table 3). The antagonistic activity of the four yeast strains depends on year, initial concentration and concentration of disease (Figs. 1 and 2). The disease incidence of *C. capsici* infected chilli fruits can be reduced to as low as 6.5% by the yeast strain R13, while the other three strains reduced the disease incidence to a lesser extent (Fig. 1A–D). In all four yeast strains, lower disease incidences were observed at the lowest storage temperature tested (Fig. 2). This is not surprising since the most conducive temperature ranges for *C. capsici* conidia germination and disease development have been shown to be at 28–33 °C (Misra and Mahmood, 1960). After harvesting, chilli fruits should therefore be kept at a lower temperature to preserve qualities such as color, texture, and low disease incidence (Agblor and Waterer, 2001). For this reason, a lower temperature range of 18–20 °C has been used in several studies on the efficacy of various yeast strains (Arras et al., 1999; Droby et al., 1997; Lima et al., 1999).

This most efficacious yeast strain R13 has been successfully identified as *P. guilliermondii* by using the smaller subunit, 5.8S rDNA (ITS1/ITS4), but not by 26S rDNA (D1/D2). This 26S rDNA (D1/D2) taxonomy of *P. guilliermondii* and related taxa, however, may not help differentiate species due to the strong possibility that rDNA D1/D2 sequences and other traits may be too conservative to characterize species within the clade (Suh and Blackwell, 2004). Martorell et al. (2005) has suggested that the non-coding internal transcribed spacer regions (ITS) exhibit greater interspecific differences than 26S rRNA genes (Cai et al., 1996; Kurtzman, 1992), thus allowing the differentiation of closely related species.

Several antagonistic yeasts have previously been isolated from fruits and vegetables and efficaciously used as biocontrol agents. *Issatchenkia orientalis* strains 16C2 and 2C2 isolated from grape berry (*Vitis vinifera* L. cv. Negroamaro) were effective in reducing colonization of *Aspergillus*

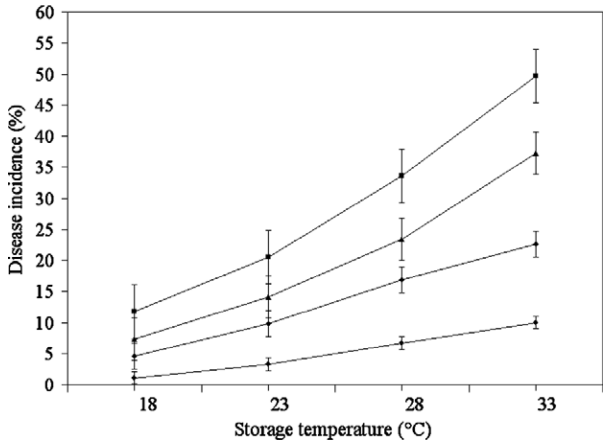

Fig. 2. Effects of incubation temperature on percentage of disease (anthracnose) incidence on chilli fruits. Thirty wounded fruits were inoculated with 20 μl of yeast antagonists: *Pichia guilliermondii* R13 (●), *Candida musae* R6 (♦), *Issatchenkia orientalis* ER1 (▲), and *Candida quercitrusa* L2 (■) at concentration of 5×10^8 cells ml^{-1}. After air drying, the fruits were inoculated with 20 μl of *Colletotrichum capsici* at concentration of 5×10^4 spores ml^{-1}, and then incubated at 18, 23, 28, and 33 °C. Data were recorded 5 days after incubation. Bars represent standard errors of the mean of triplicate experiments. Control with only *C. capsici* infection resulted in 100% disease incidence at all temperatures tested.

carbonarius (Bainer) Thom. and *Aspergillus niger* Tiegh. on grape berry (Bleve et al., 2006). *C. musae* isolate 18 has been shown to have biocontrol efficacy against *Botrytis cinerea* Pers.: Fr. infection in strawberries (*Fragaria x ananassa* Duch. cv. Sweet Charlie) (El-Neshawy and Shetaia, 2003). Several strains of *P. guilliermondii* at concentrations similar to those used in this study have previously been shown to have biocontrol efficacy against infection by various fungi on citrus fruit, grape fruit, apple, pear, table grape and strawberry (Arras et al., 1999; Droby et al., 1997; Lima et al., 1999). However, there is no report on the use of *P. guilliermondii* to control *C. capsici* in chilli fruits. This study presents the first evidence that *P. guilliermondii* strain R13 can reduce disease incidence caused by *C. capsici* to as low as 6.5% (Fig. 1 and Table 3) in conditions similar to other investigations (Arras et al., 1999; Droby et al., 1997; Lima et al., 1999). Strain R13 helps to delay postharvest decay of intact chilli fruits. The disease incidence in R13 treated chilli fruits is about two times lower than those of the untreated (Table 4). The other yeast strains, i.e., R6, ER1, and L2 although can delay the postharvest decay, but to a lesser extent. The results suggest that *P. guilliermondii* strain R13 has a high potential to be a biocontrol agent in postharvest control against *C. capsici* infection of chilli fruits.

Pichia guilliermondii has been shown to be nonpathogenic as tested on guinea pigs and outbred Swiss mice (Arras et al., 1999, 2002). Large-scale production of *P. guilliermondii* was efficiently done by using fermentation with inexpensive industrial waste materials (Janisiewicz and Korsten, 2002). The resulting yeasts maintained their antagonistic activity against spore germination of *Penicillium digitatum* (Pers.: Fr.) Sacc. It also effectively inhibited the decay of several citrus cultivars, both injured and non-injured. The degree of postharvest disease prevention by the yeast was comparable to a commonly used wax containing a low concentration of chemical fungicide. In addition, combining the yeast with 200 ppm thiabendazole (TBZ) resulted in a reduction in the incidence of decay to a level equal to that of the commercial treatment of 2000 ppm TBZ. The efficacy of *P. guilliermondii* strain US-7 was maintained under packinghouse conditions at a cell concentration as low as 10^7 cells ml^{-1}, either by drenching or spraying (Droby et al., 1993). These results suggest the possibility of using *P. guilliermondii* strain R13 as a commercial biocontrol agent for preventing anthracnose disease caused by *C. capsici*.

Postharvest chilli fruits are usually preserved by washing or spraying with chlorinated water at 75–400 ppm chlorine and stored at low temperature (7–10 °C) before shipment (Smith et al., 1998; Suslow, 1997). The present study, however, shows that *P. guilliermondii* strain R13 is more effective in preserving chilli fruits than chlorinated water (200 ppm chlorine) at 10 °C over a period of 30 and 45 days (Table 4). This suggests that *P. guilliermondii* strain R13 should replace the chlorinated water for treatment. The preparation of chlorinated water from chlorine gas used as such or chlorine arising from hypochlorite salts may be harmful to the worker (Dänet, 2005). Moreover, the use of chlorine in fruits and vegetables is banned in some countries due to its reaction with organic matter leading

Table 4
Percentage of disease (anthracnose) incidence in intact chilli fruits sprayed with the four antagonistic yeasts, chlorinated water, and distilled water

Treatment	Disease incidence[a] (%)		
	15 days	30 days	45 days
Pichia guilliermondii R13	3.30 ± 0.69a	13.30 ± 0.29a	16.70 ± 0.48a
Candida musae R6	4.40 ± 0.29ab	14.40 ± 0.40a	18.10 ± 0.35b
Issatchenkia orientalis ER1	5.10 ± 0.23b	17.50 ± 0.26b	21.40 ± 0.66d
Candida quercitrusa L2	5.70 ± 0.52bc	19.40 ± 0.46c	23.70 ± 0.29e
Chlorinated water	3.30 ± 0.46a	16.70 ± 0.42b	20.00 ± 0.46c
Distilled water	6.70 ± 0.41c	23.30 ± 0.40d	26.70 ± 0.34f

[a] The results are presented as mean of three independent experiments ± standard error. Values of each column followed by a different letter indicate significant differences ($P < 0.05$) according to LSD test. Values with shared letter are not significantly different ($P < 0.05$).

to formation of chlorinated compounds: some of their derivatives have been proven to be carcinogenic (Camelo, 2004; Carlsen, 2004). Accumulation of these substances also causes pollution of the environment (Link et al., 1994; Smith, 2001; Stringer and Johnston, 2002). These findings provide a powerful incentive for development of the yeast strain R13 as an alternative to anthracnose disease control by *C. capsici* in postharvest chilli fruit. Nevertheless, more studies including field trials need to be performed.

The mode of action of *P. guilliermondii* against *P. digitatum* and *B. cinerea* infection of fruits has been suggested to be competition for nutrients and secretion of cell wall-degrading enzymes (Droby et al., 1989, 1990; Mari and Guizzardi, 1998; Wisniewski et al., 1990). These control mechanisms are acceptable for use in the biological control of molds in foods and feeds since they do not involve secretion of potentially hazardous antibiotics. Preliminary results suggest similar action of R13 to other *P. guilliermondii* strains. Thus our results should encourage the use of *P. guilliermondii* on a large scale for biocontrol of postharvest chillies.

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