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Effects of calcium on biocontrol activity of yeast antagonists against the postharvest fungal pathogen *Rhizopus stolonifer*

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Calcium chloride (2% w/v) significantly inhibited the growth of the pathogen *Rhizopus stolonifer*, but did not affect the colony-forming units (CFU) of yeasts *Candida guilliermondii* and *Pichia membranefaciens* in potato dextrose broth. The concentration of yeast suspension influenced spore germination and germ tube growth of *R. stolonifer in vitro*, as well as disease incidence and lesion development in fruits. There were significant negative relationships between the suspension concentrations of the yeasts and the growth as well as infectivity of the pathogen. The addition of calcium resulted in lower spore germination rates and slower growth of germ tubes *in vitro*, as well as in lower disease incidences and smaller lesion diameters compared with treatments with yeast antagonists alone. When yeast cell suspensions reached a concentration of 5×10^8 CFU mL^{-1}, growth of the pathogen was completely limited *in vitro*, and no infection was found in peach and nectarine fruits treated with or without calcium.

Keywords: calcium, *Candida guilliermondii*, *Pichia membranefaciens*, postharvest decay, *Rhizopus stolonifer*

Introduction

The fungal decay of fruits and vegetables in postharvest storage greatly limits their economic value. Although fungicide treatments have been the main method for controlling postharvest diseases, public concern about fungicide residues in food and the development of fungicide resistance by pathogens has increased the search for alternative means of controlling disease. Biological control of postharvest decays of fruits and vegetables has emerged recently as a promising alternative to the use of synthetic fungicides (Wilson & Wisniewski, 1989; Wisniewski & Wilson, 1992). Treatment of fruit with microbial agents has been demonstrated to be an efficient method for control of several postharvest decays (Janisiewicz & Roitman, 1988; Chalutz & Wilson, 1990; Wilson *et al*., 1993; Fan & Tian, 2000). Some yeasts and bacteria are reported to reduce effectively various postharvest decays of fruits (Janisiewicz, 1988; Chalutz & Wilson, 1990; McLaughlin *et al*., 1990; Piano *et al*., 1997; Fan & Tian, 2001). In order to enhance biocontrol activity of antagonists against fungal pathogens, certain strategies, such as adding calcium salts, carbohydrates, amino acids and other nitrogen compounds to biocontrol treatments, are proposed (Conway, 1982; Conway *et al*., 1987a,b; Janisiewicz *et al*., 1992). Many researchers have shown that calcium plays an important role in the inhibition of postharvest decay of fruits (Conway & Sams, 1985; Conway *et al*., 1992) and in enhancing the efficacy of postharvest biocontrol agents (Conway *et al*., 1991; Wisniewski *et al*., 1995). Postharvest calcium treatment of apples provided broad-spectrum protection against the postharvest pathogens of *Penicillium expansum* and *Botrytis cinerea* (Saftner *et al*., 1997). The addition of $CaCl_2$ (2% w/v) to the formulation of the yeast biocontrol agent, *Candida oleophila*, enhanced the ability of this yeast to protect apples against postharvest decay (Wisniewski *et al*., 1995). The efficacy of controlling grey mould and blue mould rots in apples was enhanced when *Trichosporon* sp., even at a low concentration of 10^5 CFU mL^{-1}, was applied in the presence of $CaCl_2$ (2% w/v) in an aqueous suspension (Tian *et al*., 2001).

In this paper, the effect of calcium treatment on the growth of the yeast antagonists *C. guilliermondii* and *Pichia membranefaciens* was determined, as well as on spore germination and germ tube growth of the fungal pathogen *Rhizopus stolonifer*. The biocontrol effectiveness of the yeasts in combination with calcium was determined for the control of postharvest decay in peach and nectarine fruits caused by *R. stolonifer*.

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Materials and methods

Pathogen inoculum

An isolate of *R. stolonifer* from infected peach fruits was maintained on potato dextrose agar (PDA) and was used in all tests. The spore suspension was prepared by growing *R. stolonifer* on PDA at 25°C for 10–15 days, flooding the culture with sterile distilled water with 0·05% (v/v) Tween 80, and subsequently adjusting the concentration of the suspension to 5×10^4 spores mL^{-1}.

Antagonists

Candida guilliermondii was obtained from the Institute of Microbiology, Chinese Academy of Sciences, Beijing. *Pichia membranefaciens*, a novel antagonist yeast for controlling postharvest diseases of fruits (Fan & Tian, 2000), was isolated from the wounds of peach fruits as previously described (Wilson *et al*., 1993). The yeast (IMI 382465) was identified by CABI Bioscience Identification Services (International Mycological Institute, Egham, UK). The antagonists were grown in 250 mL conical flasks containing 50 mL of nutrient yeast dextrose broth (NYDB: 8 g of nutrient broth, 5 g of yeast extract, and 10 g of dextrose in 1 L water) on a rotary shaker at 200 rpm for 24 h at 28°C. Yeast cells were harvested by centrifugation at 3580 ***g*** for 10 min, washed twice with 0·05 M phosphate buffer at pH 7·0 and resuspended in either 2% w/v $CaCl_2$ in sterile distilled water (SDW) or SDW alone. Suspensions of yeast cells were adjusted to different concentrations according to the experimental treatments.

Fruits

Peach (*Prunus persica*) and nectarine (*P. persica* var. *nectarina*) fruits were harvested from a commercial orchard at Jinzhou, Dalian, China and transported to Beijing. They were refrigerated at 2°C on the day of harvest and then kept in regular storage at 0°C. At harvest the firmness of peach and nectarine fruits was 6·18 and 6·74 kg cm^{-2}, respectively. At the beginning of the biocontrol experiment, the firmness of fruits was 5·40 kg cm^{-2} for peach and 5·50 kg cm^{-2} for nectarine, after 15 and 50 days storage, respectively, at 0°C.

Effect of calcium on antagonist growth

Aliquots of 50 mL NYDB with or without 2% $CaCl_2$ in 250 mL conical flasks were autoclaved (120°C, 15 min) prior to adding 1 mL suspension (about 5×10^8 CFU mL^{-1}) of *C. guilliermondii* or *P. membranefaciens* to each flask. The number of CFU of the yeasts was determined by dilution-plating at 0, 24 and 48 h after incubation on a rotary shaker at 200 rpm at 25°C. Each treatment was replicated three times and the experiment was repeated twice.

Spore germination and growth *in vitro*

The effects of $CaCl_2$ and different concentrations of *C. guilliermondii* or *P. membranefaciens* on spore germination and germ tube elongation of *R. stolonifer* were assessed in potato dextrose broth (PDB) using the method of Piano *et al.* (1997). The suspension of 5×10^6 spores L^{-1} was prepared as described earlier. Yeast antagonists, *C. guilliermondii* and *P. membranefaciens*, were cultured as described above. The cell suspensions of the antagonists were adjusted to various concentrations of 5×10^8, 5×10^7, 5×10^6, 5×10^5 and 0 CFU mL^{-1} with or without 2% w/v $CaCl_2$. Aliquots of 100 μL of the pathogen suspension were transferred to glass tubes (180 × 16 mm) containing 5 mL PDB, then aliquots (100 μL) of the antagonist suspensions with different concentrations were added to each tube. All tubes were put on a rotary shaker at 50 rpm at 25°C. Approximately 200 spores of the pathogen were measured for germination rate and germ tube length per treatment within each replicate after 13 h incubation. Each treatment was replicated three times and the experiment was repeated twice.

Biocontrol activity of yeast antagonists

Peach and nectarine fruits were disinfected with 2% available chlorine using sodium hypochlorite solution for 2 min, washed with tap water, dried, and a uniform wound 4 mm deep and 3 mm in diameter was made on the side of each fruit. The same concentrations (5×10^8, 5×10^7, 5×10^6, 5×10^5 and 0 CFU mL^{-1}) of *C. guilliermondii* and *P. membranefaciens* with and without 2% (w/v) $CaCl_2$ were prepared as described before for tests *in vitro*. At first, the wounds of peaches were inoculated with 30 μL of *C. guilliermondii* suspensions and the wounds of nectarines were inoculated with 30 μL of *P. membranefaciens* suspensions at different concentrations. After 4 h, 15 μL of 5×10^4 spores mL^{-1} suspension of *R. stolonifer* were added to each wound. Treated fruits were arranged separately from each other on 400 × 300 × 100 mm plastic trays. The tray was placed in a plastic bag with about 95% relative humidity and stored at 25°C. Infection rate and lesion diameter were measured 3–4 days after inoculation depending on treatments. There were 10 fruits per treatment with three replicates arranged in complete randomization. Each test was repeated twice.

Statistical analysis

The calcium treatment data were analysed as a one-factor general linear model procedure (ANOVA). To correct for variance heterogeneity, the CFU data of antagonists were $\log_{10}$ transformed before analysis. The treatment means were separated at the 5% significance level using Duncan's multiple range test.

Table 1 Effect of $CaCl_2$ on the growth (log CFU) of yeast antagonists in nutrient yeast dextrose broth after 24 or 48 h incubation at 25°C[a]

Culture time	*C. guilliermondii* 2% $CaCl_2$	*C. guilliermondii* 0% $CaCl_2$	*P. membranefaciens* 2% $CaCl_2$	*P. membranefaciens* 0% $CaCl_2$
0 h	6·71[b] ± 0·12b[c]	6·84 ± 0·23b	6·87 ± 0·16b	6·89 ± 0·24b
24 h	9·07 ± 0·02a	9·12 ± 0·01a	8·88 ± 0·06a	8·94 ± 0·04a
48 h	9·13 ± 0·02a	9·21 ± 0·01a	8·92 ± 0·16a	8·95 ± 0·18a

[a]Data were pooled from two experiments and transformed into log CFU.
[b]Means in a column followed by the same letter do not differ significantly at $P = 0{\cdot}05$ by Duncan's multiple range test.
[c]± represent standard deviation.

Results

Effect of calcium on growth of antagonists

The data from the tests showed that adding calcium did not significantly influence the number of CFU of *C. guilliermondii* and *P. membranefaciens in vitro*. The CFU of the antagonists in NYDB increased with incubation time, but there was no significant difference of CFU of the yeasts after 24 and 48 h incubation on a rotary shaker at 200 rpm at 25°C (Table 1).

Effect of calcium and concentrations of antagonists on pathogen *in vitro*

Both calcium and the concentration of yeast suspensions affected spore germination and germ tube growth of *R. stolonifer in vitro* (Fig. 1). Germination rate and germ tube length of the pathogen decreased when concentration of yeast cell suspensions increased from 0 to 5×10^8 CFU mL^{-1}. There were significant negative relationships between the suspension concentrations of *C. guilliermondii* and *P. membranefaciens* and germination rate ($R^2 = 0{\cdot}9064$ and 0·9029, respectively) and germ tube length ($R^2 = 0{\cdot}9603$ and 0·9642, respectively) of *R. stolonifer*. However, the addition of calcium to the same concentrations of yeast suspensions significantly decreased spore germination rate and germ tube length of the pathogen compared with treatments with antagonists alone after 13 h incubation at 25°C. The growth of *R. stolonifer* could be completely inhibited at a concentration of 5×10^8 CFU mL^{-1} with or without calcium (Fig. 1).

Effect of calcium and concentrations of antagonists on pathogen *in vivo*

The degree of *R. stolonifer* infection in peach and nectarine fruits decreased with increasing concentration of yeast antagonists. Combining calcium with *C. guilliermondii* significantly reduced disease incidence and inhibited lesion diameter of Rhizopus rot in peaches (Fig. 2a and b) ($P = 0{\cdot}05$). A similar effect was seen with calcium and *P. membranefaciens* for the control of Rhizopus rot in nectarines (Fig. 2c and d). There were significant negative relationships between concentration of the antagonists and disease incidence ($R^2 = 0{\cdot}9764$ in peaches and $R^2 = 0{\cdot}9583$ in nectarines) and lesion development ($R^2 = 0{\cdot}9641$ in peaches and $R^2 = 0{\cdot}9343$ in nectarines) (Fig. 2). Disease incidence in wounded fruit treated with $CaCl_2$ suspensions was reduced 2–4·5 and 2–6 times, respectively, compared with treatments only with suspensions of *C. guilliermondii* and *P. membranefaciens* (Fig. 2). Similarly, lesion diameters of the same fruits were between 2–7 and 2–15 times smaller with calcium treatments (Fig. 2b and d). When yeast cell suspensions of *C. guilliermondii* and *P. membranefaciens* reached a concentration of 5×10^8 CFU mL^{-1}, no infection by *R. stolonifer* was found in peach and nectarine fruits treated, irrespective of the presence of calcium.

Discussion

The results of this study indicated that combining $CaCl_2$ with the yeast suspensions significantly enhanced the biocontrol activity of *C. guilliermondii* in peaches (Fig. 2a and b) and *P. membranefaciens* in nectarines (Fig. 2c and d) to Rhizopus rot. The same effects on biocontrol activity, achieved by the addition of calcium, were also observed by using the yeasts of *Pichia guilliermondii* (Droby *et al.*, 1993) and *Candida* spp. (McLaughlin *et al.*, 1990; Wisniewski *et al.*, 1995) as postharvest biocontrol agents. However, the addition of calcium did not directly increase CFU of the antagonists *in vitro* (Table 1), but obviously inhibited spore germination and germ tube growth of *R. stolonifer* in PDB (Fig. 1). This result further supports the results of Wisniewski *et al.* (1995), who found that calcium might reduce fungal infection through direct inhibition of spore germination and growth. In addition, the beneficial effect of calcium on the biocontrol activity of the yeasts was more obvious in fruits than in PDB. Although biocontrol efficacy of yeast antagonists mainly depends on the number of yeast cells present in the wound site (Droby *et al.*, 1991), the addition of calcium directly inhibited the number of pathogens and indirectly increased the ability of the yeast to inhibit the growth of pathogens and the resistance of fruit to pathogens (Tian *et al.*, 2001).

The precise mechanism by which calcium reduces fungal infection is not yet understood, but the role of calcium in resistance may be one of interference with the activity

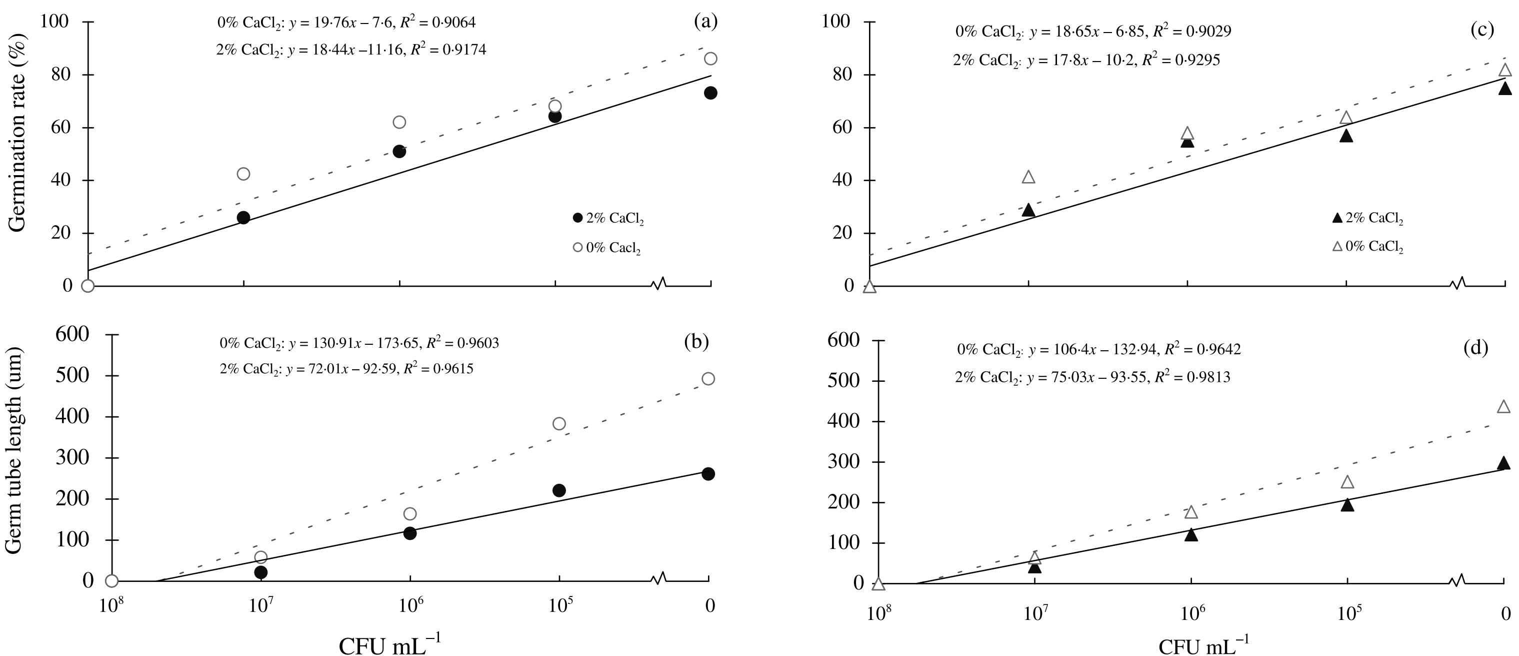

Figure 1 Effect of calcium and different concentrations of *Candida guilliermondii* (a, b) and *Pichia membranefaciens* (c, d) cell suspensions on the germination rate and germ tube length of *Rhizopus stolonifer* spores in potato dextrose broth after 13 h incubation at 25°C.

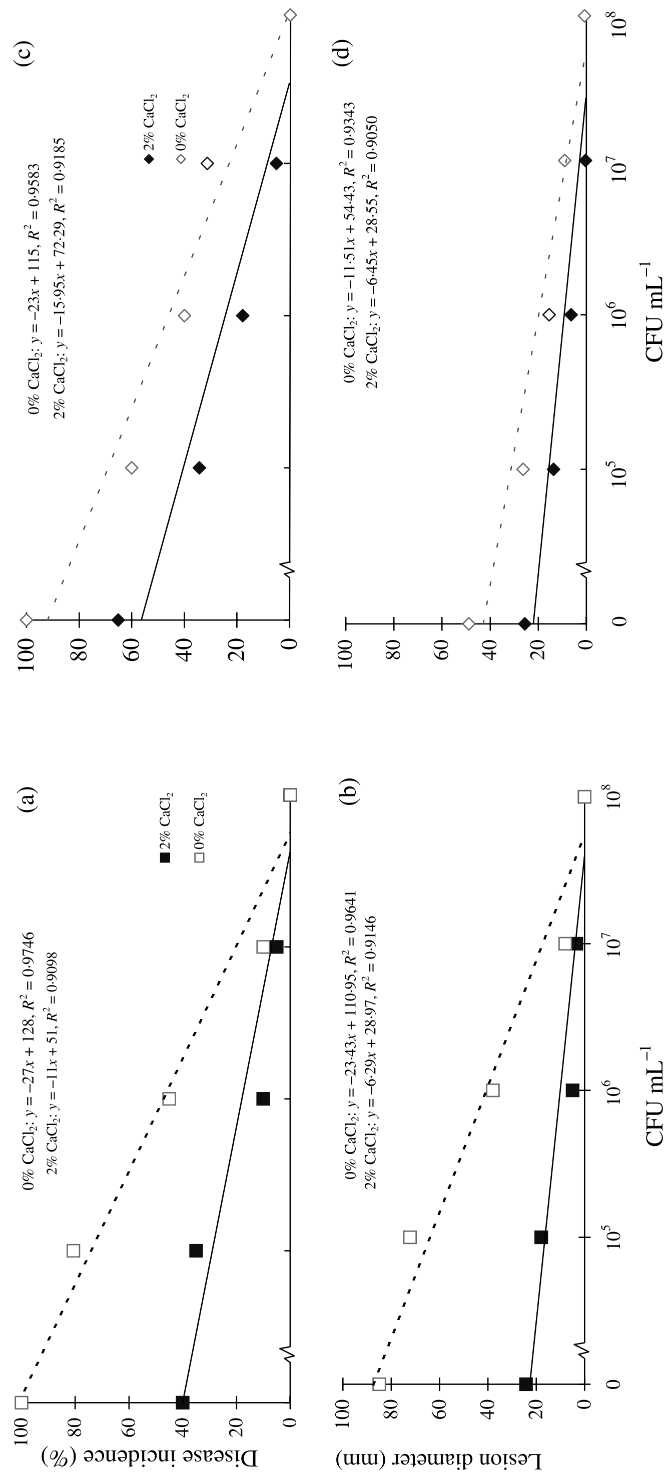

Figure 2 Inhibition effect of calcium and cell concentrations of *Candida guilliermondii* (a, b) and *Pichia membranefaciens* (c, d) on disease incidence and lesion development of *Rhizopus stolonifer* in peach (a, b) and nectarine (c, d) fruits stored at 25°C for 3 days.

of pectolytic enzymes (Conway *et al*., 1992) and may be partially attributable to a decrease in maceration of cell walls by polygalacturonase (PG) due to the improved structural integrity caused by an increase in calcium content (Conway *et al*., 1988). Pathogen–antagonist interactions inside the wound, such as competition for space and nutrients and the production of lytic enzymes on attachment of the antagonist to the mycelium, are believed to be the main mechanisms of inhibiting diseases by fungal pathogens (Chalutz *et al*., 1988; Arras, 1996). Competition for nutrients has been frequently cited as a mechanism of biocontrol by antagonistic yeasts such as *Pichia*, *Candida* and *Cryptococcus* spp. (Roberts, 1990; Arras, 1996; Elad, 1996).

Previous studies have indicated that an increase in the calcium content of the cell walls of apples reduces the activity of polygalacturonase extracted from *P. expansum* culture (Conway *et al*., 1988; Wisniewski *et al*., 1995; Saftner *et al*., 1997). Postharvest calcium treatment significantly limited decays in peaches by *Monilinia fructicola* (Conway *et al*., 1987a,b) and in apples by *Botrytis cinerea* (Lein *et al*., 1997). In a previous study, it was found that biocontrol efficacy of yeast *Trichosporon* sp. to control grey mould and blue mould rots of apple fruits was enhanced in the presence of $CaCl_2$ 2% (Tian *et al*., 2001). Calcium has been considered to increase biocontrol efficacy of antagonists. It may also replace the current requirement for addition of low concentrations of fungicides to ensure consistent performance of yeast biocontrol agents under large-scale and commercial conditions (Droby *et al*., 1993). Therefore, calcium may have important implications for the future use of yeast on a commercial scale for the control of postharvest disease of fruits.

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