Influence of CaCl_2 on *Penicillium digitatum*, Grapefruit Peel Tissue, and Biocontrol Activity of *Pichia guilliermondii*

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ABSTRACT

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Interactions between CaCl_2 , grapefruit peel tissue, *Penicillium digitatum*, and the yeast antagonist *Pichia guilliermondii* strain US-7 were investigated. Application of 68 or 136 mM CaCl_2 to grapefruit surface wounds reduced the incidence of green mold caused by *Penicillium digitatum* by 43 to 52%. In laboratory tests, a cell suspension (10^7 cells/ml) of *Pichia guilliermondii* containing either 68 or 136 mM CaCl_2 reduced the incidence of green mold from 27 to 3%. In large scale tests, dip application of 136 mM CaCl_2 with US-7 (10^7 cells/ml) significantly decreased the number of wounds infected by *Penicillium digitatum*. CaCl_2 , with or without yeast cells, stimulated ethylene production in grapefruit tissue. Increasing concentrations of CaCl_2 resulted in de-

creased spore germination and germ tube elongation of *Penicillium digitatum*. Pectinolytic activity of crude enzyme preparations of *Penicillium digitatum* was also inhibited by the presence of increasing concentrations of CaCl_2 . US-7 exhibited a strong ability to maintain cytosolic Ca^{2+} homeostasis at levels that did not exceed 1.4 μM when exposed to 150 mM CaCl_2 . On the other hand, strain 114 of *Debaryomyces hansenii*, which failed to give any protection against infection by *Penicillium digitatum*, showed reduced capacity to maintain Ca^{2+} homeostasis. The effect of calcium in reducing infection of grapefruit wounds by *Penicillium digitatum* could be due to direct effects on host tissue (making cell walls more resistant to enzymatic degradation) or the pathogen (interfering with spore germination, growth, and inhibition of fungal pectinolytic enzymes). Alternatively, the ability of US-7 to maintain calcium homeostasis may allow it to grow or assist in its competitive ability in a micro-environment that, because of high levels of calcium ions, is inhibitory to growth of the green mold pathogen.

The application of calcium salts to fruit tissue has been reported to play an important role in reducing physiological disorders, delaying senescence, and inhibiting postharvest decay (4,5,20). Results of these studies indicated that preharvest or postharvest treatment of fruit with calcium solutions resulted in an increase in total calcium content and a concomitant increase in cell wall-bound calcium. The ability of calcium to reduce the development of postharvest diseases of fruit has been attributed mainly to the formation of calcium cross-linkages in the cell wall, resulting in decreased effectiveness of cell wall-macerating enzymes secreted by the pathogen (5). In apple tissue, vacuum or pressure infiltration of CaCl_2 at concentrations of either 8 or 12% resulted in a significant decrease in the development of decay caused by *Penicillium expansum* (4). In contrast to the vacuum- or pressure-infiltrated fruits, there was no decrease in decay severity or increase in the cell wall-bound calcium concentration when apples were dipped in various concentrations of CaCl_2 . During the growing season, however, calcium-rich nutrient regimes applied to apple trees caused an increase in calcium concentration in the fruit and also a reduction in the development of postharvest diseases (5). McGuire and Kelman (12) also demonstrated a relationship between increasing levels of calcium in the cell walls of potato tubers and a reduction in the macerating activity of *Erwinia carotovora*. Volpin and Elad (24) also have reported a similar effect of

calcium in reducing the susceptibility of rose flowers to gray mold caused by *Botrytis cinerea*.

McLaughlin et al. (13) reported that calcium salts improved the efficacy of yeast biocontrol agents against Botrytis and Penicillium rots of apples. Suspensions of yeast cells at concentrations as low as 10^6 to 10^7 CFU/ml effectively inhibited disease development in the presence of different calcium salt solutions. In the absence of the salts, a yeast cell concentration of at least 10 and usually 100 times greater was required to achieve the same level of protection. They further demonstrated that the effect was specifically due to the calcium cations rather than other cations or anions. Salt solutions applied to apple wounds without the antagonist cells failed to reduce the incidence of the rot. The authors suggested that the effect of calcium on the biocontrol activity of the yeast antagonists was due to some interaction with the yeast or its metabolic products at the wound site rather than a direct effect of calcium on the pathogen or the fruit tissue. In contrast, Wisniewski et al. (26) have indicated that calcium chloride reduced germination and germ tube elongation of *B. cinerea* and *Penicillium expansum* in vitro. The inhibition was again demonstrated to be due to the presence of the calcium cation rather than the chloride anion (e.g., MgCl_2 had no effect).

The overall objective of the present study was to determine the factors involved in the ability of CaCl_2 to enhance the biocontrol activity of the yeast antagonist *Pichia guilliermondii* Wickerham isolate US-7 against infection of citrus fruit by *Penicillium digitatum* (Pers.:Fr.) Sacc., the causal agent of green mold. The effect of CaCl_2 on fruit peel tissue and on in vitro spore germination, mycelial growth, and activity of polygalacturonase (PG) of *Penicillium digitatum*, as well as the ability of *Penicillium digitatum* to infect grapefruit surface wounds was investigated. The ability of *Pichia guilliermondii* to tolerate high exogenous concentrations of

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calcium was also monitored and compared with that of *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij isolate 114, which did not exhibit biocontrol activity against infection of *Penicillium digitatum*.

MATERIALS AND METHODS

Fruit material and fungal and antagonist cultures. Grapefruit (*Citrus paradisi* MacFad.) were obtained from local orchards and were either used immediately or stored at 11°C for later use. Cultures of *Penicillium digitatum* were isolated from decayed citrus fruit. Cultures were stored on potato dextrose agar (PDA) slants at 4°C and grown on PDA plates for 1 week at 25°C. Two-week-old cultures were usually used to prepare spore suspensions.

The yeast *Pichia guilliermondii* strain US-7 was previously isolated from the surface of lemon fruit (25). Strain 114 of the yeast *D. hansenii* (ATCC 18858) was obtained from American Type Culture Collection (ATCC; Rockville, MD). The yeast cultures were maintained at 4°C on nutrient yeast dextrose agar medium containing 8 g of nutrient broth, 5 g of yeast extract, 10 g of D-glucose, and 20 g of agar in 1 liter of distilled water or silica gel at -18°C (21). Liquid cultures of yeasts were grown in 100-ml Erlenmeyer flasks containing 25-ml of nutrient yeast dextrose broth on a rotary shaker at 26°C for 48 h. Following the removal of the growth medium by centrifugation at 7,000 rpm ($11,524 \times g$), yeast cells were washed twice in distilled water. Cell pellets were resuspended in sterile distilled water and brought to an initial concentration of 2 to 5×10^9 cell/ml. Cell concentration was adjusted as needed for different experiments.

Effect of CaCl_2 on infection of grapefruit wounds by *Penicillium digitatum* and biocontrol efficacy of the yeast antagonist *Pichia guilliermondii*. Two types of tests were performed to evaluate the effect of CaCl_2 on biocontrol activity of the yeast antagonist *Pichia guilliermondii* against green mold of citrus: controlled laboratory experiments in which yeast cells, with or without salt solution, were applied directly to surface wounds followed by inoculation with a spore suspension of *Penicillium digitatum*, and large scale tests in which wounded grapefruit were dipped into aqueous suspensions containing various combinations of conidia of *Penicillium digitatum*, yeast cells, and calcium salt. In both types of tests, grapefruit were washed with tap water, air-dried, and wounded to a uniform depth of 3 mm at three sites around the stem end using a dissecting needle. In the laboratory assays, 40 μl of yeast cell suspension at concentrations of 10^6 , 10^7 , or 10^8 cells/ml containing CaCl_2 at final concentrations of 68 or 136 mM (1 or 2%, wt/vol) were pipetted into each wound and allowed to dry for about 2 h. Wounds were then inoculated with 20 μl of a spore suspension (10^4 spores/ml) of *Penicillium digitatum*. Wounds treated with either water or yeast cell suspensions alone were used as controls. Fruit were incubated at 25°C under moist conditions, and percent of wound sites infected was determined 7 days after inoculation. Each treatment consisted of 12 fruit for a total of 36 wound sites. The test was conducted three times. Data were analyzed as factorial design.

In the large scale tests, wounded fruit were dipped for 1 min in aqueous suspensions of mixtures of yeast cells (10^7 cells/ml), CaCl_2 (136 mM), and *Penicillium digitatum* conidia (10^4 conidia/ml). Fruit were air-dried, packed in carton boxes, and incubated at 17°C. The number of infected wound sites was determined after 1 week. Each treatment consisted of five boxes with 42 fruit/box (three wounds per fruit, i.e., total of 630 wound sites), and each fruit served as a replicate. The experiment was conducted twice, and the results were analyzed as factorial design.

Effect of CaCl_2 on conidium germination and germ tube length of *Penicillium digitatum*. The effect of CaCl_2 on conidium germination and germ tube elongation of *Penicillium digitatum* was tested in culture. Spore suspensions were prepared from 2-week-old cultures grown on PDA by removing spores from the

surface of the cultures with a sterile bacteriological loop in 5 ml of sterile distilled water. Suspensions were filtered through four layers of cheesecloth to remove fungal mycelia. Spore concentration was determined with a hemacytometer, and the concentration was adjusted to 6×10^6 spores/ml. Aliquots of 100 μl of spore suspension of *Penicillium digitatum* were added to the wells of tissue culture plates (Costar 24-well tissue culture plates, Corning-Costar Corp., Cambridge, MA) containing 900 μl of 20% grapefruit juice amended with different CaCl_2 concentrations (68, 136, and 272 mM; equal to 1, 2, and 4%, respectively). Twenty-microliter aliquots were placed on sterile microscope slides (three drops per slide) and incubated at 25°C for 24 h under dark conditions in sterile petri dishes lined with moist filter paper. Percent of spore germination and germ tube length were determined in three different microscopic fields. A total of 100 spores per replicate were observed. All treatments consisted of three replicates, and experiments were repeated three times.

PG assay. A crude PG preparation was obtained from culture filtrates of *Penicillium digitatum* grown for 1 week in minimal salts medium (22) containing 13.6 g of KH_2PO_4 , 4.0 g of NH_4NO_3 , 2.6 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.23 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.18 mg of $(\text{NH}_4)_2\text{MoO}_4$, 2.0 mg of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 3.1 mg of H_3BO_3 , 5 g of citrus pectin, and 5 g of sodium polypectate in 1 liter of distilled water, pH 4.5. Liquid cultures of *Penicillium digitatum* were prepared by seeding 250-ml Erlenmeyer flasks containing 100 ml of medium with five agar plugs (5 mm in diameter) obtained from the growing margins of a 1-week-old *Penicillium digitatum* culture. Cultures were incubated at 25°C on a rotary shaker (120 rpm) for 7 days. The supernatant was filtered through cheese cloth, centrifuged at 10,000 rpm ($16,464 \times g$) for 15 min, and dialyzed overnight against 4 liters of distilled water at 4°C. The dialyzed solution, which served as a crude enzyme preparation, was kept at -18°C until used.

PG activity was determined spectrophotometrically by using dinitrosalicylic acid (14) to determine the rate of liberation of reducing groups. The reaction mixture contained 0.5 ml of substrate 1% (wt/vol) polygalacturonic acid-Na (Sigma Chemical Co., St. Louis) or citrus pectin (Sigma Chemical Co.) dissolved in 0.1 M acetate buffer, pH 4.2, and 0.5 ml of crude PG preparation. The reactions were terminated after 1 h of incubation at 37°C by the addition of 3 ml of DNS reagent (1% dinitrosalicylic acid, 0.2% phenol, and 0.05% sodium sulfite in 1% NaOH solution). The final volume of the reaction was brought up to 5 ml by the addition of 1 ml of water and then immersed in a boiling water bath for 15 min. The increase in reducing groups was determined with a spectrophotometer at 575 nm after equilibration to 25°C. Reaction mixtures containing boiled enzyme preparation were used as blanks. Different concentrations of CaCl_2 (10, 20, 40, and 100 mM) were added to the reaction mixture to test effects on PG activity.

Ethylene determination. Production of ethylene was determined in grapefruit peel discs treated with CaCl_2 with or without the addition of cells of US-7. Peel discs (13 mm in diameter and 2 to 3 mm thick) were cut from the flavedo part of the fruit peel with a cork borer and immersed for 1 min in the following aqueous solutions: 136 mM CaCl_2 , US-7 cell suspension (10^7 or 10^8 cell/ml), 136 mM CaCl_2 + US-7 (10^7 cell/ml), 136 mM CaCl_2 + US-7 (10^8 cell/ml), or sterile distilled water (control). Discs were then placed on moist filter paper in 50-ml Erlenmeyer flasks. Each treatment consisted of five replicates of 50-ml Erlenmeyer flasks containing four peel discs each. The rate of ethylene production was determined by analyzing the concentration of ethylene that accumulated in the atmosphere of the flasks during a 1-h sealed period as described previously (3). A 2-ml gas sample was withdrawn from the flask with a hypodermic syringe. Ethylene concentration in the samples were determined by flame ionization detection-gas chromatography equipped with a column of acti-

vated alumina (HayeSep T., 100/120 mesh; Alltech Assoc. Inc., Deerfield, IL) held at 32°C with N₂ carrier.

Measurements of cytosolic-free Ca²⁺ ([Ca²⁺]_i) concentrations. [Ca²⁺]_i was measured as described previously (11). Yeast cells were harvested from PDA plates, washed three times with sterile distilled water by centrifugation, and resuspended at 10⁹ cells/ml in a loading solution containing 10 mM dimethylglutaric acid (DMG) at pH 4.5, 50 mM KCl, 100 mM glucose, and 20 µM pentapotassium indo-1. The cells were incubated in the loading solution for 90 min at 30°C in the dark, and then centrifuged and washed three times with buffer D (10 mM DMG, pH 4, and 0.1 mM EDTA) suspended (10⁹ cells/ml) in the same buffer and kept at 0°C in the dark (between 15 min and 1 h) until measurement. Control samples were treated similarly, but without indo-1. Aliquots were removed from the loaded and unloaded cell suspensions, and the cell densities were determined and equilibrated. Samples of 0.2 ml were removed from the loaded cell suspension and added each to a cuvette containing 3 ml of buffer D to which the concentration of CaCl₂ was added. Suspensions were mixed for 5 min in the dark, and the emission spectra at 410 and 480 nm were measured (excitation at 355 nm, slit 2.5 nm) using a Perkin-Elmer fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, CT).

Immediately after measurement, the cell suspension was filtered through a membrane filter (0.2-µm pore size), and the filtrate was collected into another cuvette. An aliquot of the unloaded cell suspension was added to the filtrate to yield the same cell density as in the loaded cell suspension. Emission spectra were measured as above. The values obtained were subtracted from the values obtained with loaded cells. This procedure provided a one-step correction for both cellular autofluorescence and the fluorescence of indo-1 in the solution as a result of a slight leakage of indo-1 from the loaded cells during measurement. Care was taken to maintain all indo-1-containing solutions and cell suspensions in the dark throughout the procedure. Preliminary experiments have established that after 5 min of incubation with the different external calcium concentrations the levels of [Ca²⁺]_i did not change by more than 5% during the following 10 min of incubation (data not shown).

Fig. 1. Effect of CaCl₂ on biocontrol activity of the yeast *Pichia guilliermondii* against *Penicillium digitatum* on grapefruit. Results are expressed as percent infection of surface wounds. Twelve fruit with three wounds each (total of 36 wounds) were used per treatment. Bars indicate standard error of the mean.

Calculation of [Ca²⁺]_i. Values of [Ca²⁺]_i were determined from the ratio of fluorescence intensities at 410 and 480 nm according to Grynkiewicz et al. (10). R_{min} (free dye fluorescence ratio) and R_{max} (Ca²⁺-saturated dye fluorescence ratio) were determined in a calibration solution containing 170 mM KCl, 35 mM NaCl, 25 mM Mg²⁺, 10 mM morpholineethanesulfonic acid (MES)/Tris buffer at pH 6.2, and 0.1 µM pentapotassium indo-1. Ethanol (20%) was included in the calibration solution to correct for the lower polarity of the cytosol compared with the polarity of aqueous calibration solution (18). The fluorescence intensities ratio at 410 and 480 nm of the free dye (R_{min}) was measured in the calibration solution after the addition of NaOH (to adjust the pH to 8) and [ethylenebis(oxyethylenitrilo)]tetraacetic acid (EGTA) (1 mM) and that of the Ca²⁺-saturated dye (R_{max}) was determined after the addition of CaCl₂ (3 mM).

Statistical analyses. Data on the effect of CaCl₂, *Pichia guilliermondii*, and their combination on green mold incidence were analyzed by two-way factorial analysis (Jandel SigmaStat, Statistical Software version 2.0; Jandel Scientific Software, San Rafael, CA). In these experiments, treatments consisted either of 12 (laboratory tests) or 42 (large scale tests) fruit, and each fruit served as one replicate. Disease incidence was calculated as percentage of infected wounds in each fruit. Data obtained from the large scale tests were analyzed by Duncan's multiple range test at *P* = 0.05. The effect of *Pichia guilliermondii*, CaCl₂, or their combination on green mold and ethylene production in fruit peel discs was analyzed by two-way factorial analysis.

RESULTS

Effect of CaCl₂ and *Pichia guilliermondii* on green mold.

Both CaCl₂ and *Pichia guilliermondii* had a significant effect on decay incidence (*P* ≤ 0.001). In laboratory tests, direct application of 68 or 136 mM (1 and 2%, respectively, wt/vol) CaCl₂ to grapefruit wounds reduced infection by 40 to 50% compared with untreated controls (Fig. 1). The application of lower concentrations of CaCl₂ (34 or 68 mM; 0.5 or 1%, respectively) to wounds did not inhibit their infection by *Penicillium digitatum* (data not shown). The application of a mixture of 68 or 136 mM CaCl₂ with a cell suspension of *Pichia guilliermondii* further reduced decay incidence. Percent infection was reduced from 27% to 3 or 0.1% when 10⁷ cells/ml of yeast was supplemented with either 68 or 136 mM CaCl₂, respectively. When *Pichia guilliermondii* was applied at concentration of 10⁶ cells/ml, only the addition of 136 mM CaCl₂ resulted in a significant decrease in decay compared

Fig. 2. Effect of CaCl₂ (136 mM) on biocontrol activity of *Pichia guilliermondii* against *Penicillium digitatum* on grapefruit. CaCl₂ and yeast were applied by dipping. Each treatment consisted of 210 fruit, and each fruit with three wounds each served as one replicate. Columns with different letters are statistically different according to Duncan's multiple range test (*P* = 0.05). Two-way factorial analysis indicated that the effect of either *Pichia guilliermondii* or CaCl₂ was significant at level 0.001 (***). No significance (NS) was obtained in the combination of *Pichia guilliermondii* and CaCl₂.

with the application of yeast alone. However, there was no statistical interaction between CaCl_2 and *Pichia guilliermondii* ($P \leq 0.05$).

A similar effect was obtained in large scale tests when the yeast and CaCl_2 were applied to the wounds by dipping (Fig. 2). Overall levels of infection were higher in these trials compared with laboratory tests. A yeast (10^7 cell/ml) supplemented with 136 mM CaCl_2 was the most effective at reducing infection of surface wounds by the green mold fungus. In fruit treated with both yeast cells and 136 mM CaCl_2 , only 35% of the wounds developed decay. Wounded fruit, dipped in either a suspension of yeast cells or CaCl_2 alone, had 60 or 51% infection, respectively, compared with control fruit, which had 94% of the wounds infected (Fig. 2). Factorial analysis indicated that there was no significant interaction between the yeast and CaCl_2 , but rather that each agent contributed independently to the control of infection.

Effect of CaCl_2 on spore germination and growth of *Penicillium digitatum* in vitro. Spore germination and germ tube elongation of *Penicillium digitatum* were inhibited in vitro in the presence of 136 mM CaCl_2 (Table 1). Pronounced inhibition of spore germination of *Penicillium digitatum* occurred at a concentration of 272 mM (4% wt/vol) of CaCl_2 . At this concentration, only 49% of conidia germinated (Table 1). Germ tube elongation, however, was more sensitive to CaCl_2 . Marked reduction in germ tube length was observed at 136 mM CaCl_2 , while only a small reduction in spore germination was evident at this concentration.

Enhancement of ethylene production in grapefruit peel discs. Ethylene evolution in grapefruit peel discs treated with 136 mM CaCl_2 , yeast, or a mixture of both was recorded at 24 and 48 h after treatment (Table 2). Ethylene evolution measured immediately after the treatments was very low (data not shown). After 24 h, grapefruit peel discs treated with either CaCl_2 or *Pichia guilliermondii* cells (10^7 or 10^8 cells/ml) showed an increase in ethylene production by approximately two- to threefold compared with the water-treated control discs. An additional increase in ethylene

production (five- to sixfold higher than the control) was evident when peel discs were treated with a combination of 136 mM CaCl_2 solution and yeast cells at either concentration. Increased ethylene rates also were evident at 48 h after the treatments; however, the difference in ethylene production between the treated discs and the controls was less pronounced than at 24 h. Yeast cells by themselves could not be identified as a source of ethylene production (data not shown). Factorial analysis indicated that there was no significant interaction between the yeast and CaCl_2 , but rather that each agent affected ethylene production independently (Table 2).

Effect of CaCl_2 on PG activity. PG activity, measured by an increase in the amount of reducing sugars present over time, was markedly affected by the presence of CaCl_2 (Fig. 3). The ability of PG to hydrolyze the substrate was markedly reduced by the addition of increasing concentrations of CaCl_2 (10 to 100 mM) to the reaction mixture containing the crude enzyme preparation and the substrate. When polygalacturonic acid was used as a substrate, all CaCl_2 concentrations tested (10, 20, 40, or 100 mM) exhibited a pronounced inhibitory effect on PG activity (Fig. 3A). In the reaction mixtures containing pectin as a substrate, only the addition of 100 mM CaCl_2 resulted in almost complete inhibition of activity, whereas intermediate inhibition of PG activity was obtained when 10, 20, and 40 mM of CaCl_2 were added to the reaction mixtures (Fig. 3B).

Effects of CaCl_2 on $[\text{Ca}^{2+}]_i$ in yeast. The yeast strains investigated, *Pichia guilliermondii* strain US-7 and *D. hansenii* strain 114, varied in their ability to suppress infection of *Penicillium digitatum* on citrus; strain US-7 showed enhanced efficacy upon

TABLE 1. Effect of CaCl_2 concentration on spore germination and germ tube length of *Penicillium digitatum* in the presence of 68 to 272 mM CaCl_2

CaCl_2 (mM)	Germination ^y (%)	Germ tube length ^y (μm)
0	100 a ^z	70 a
68	100 a	73 a
136	86 b	38 b
272	49 c	27 c

^y Germination and germ tube length were measured following 24 h of incubation at 25°C in the dark. A total of 100 spores were measured for each treatment.

^z Numbers in a column followed by different letters are significantly different ($P \geq 0.05$) as determined by Duncan's multiple range test.

TABLE 2. Effect of CaCl_2 and *Pichia guilliermondii* strain US-7 on production of ethylene by grapefruit peel discs 24 and 48 h after treatment

Treatment	Ethylene production ($\mu\text{l/g}$ fresh wt of peel tissue/h $\pm$ SD ^y)	
	24 h	48 h
Water control	1.2 $\pm$ 0.01	4.8 $\pm$ 0.10
CaCl_2 (136 mM)	2.3 $\pm$ 0.12	8.0 $\pm$ 0.07
US-7 (10^7 cells/ml)	3.1 $\pm$ 0.09	5.0 $\pm$ 0.10
US-7 (10^7 cells/ml) + CaCl_2 (136 mM)	6.0 $\pm$ 0.14	6.2 $\pm$ 0.12
US-7 (10^8 cells/ml)	2.7 $\pm$ 0.05	5.3 $\pm$ 0.15
US-7 (10^8 cells/ml) + CaCl_2 (136 mM)	6.4 $\pm$ 0.17	7.5 $\pm$ 0.1
Two-way factor analysis of variance		
US-7 (A)	***	NS
CaCl_2 (B)	***	**
A $\times$ B	NS	NS

^y Standard deviation of the replicates ($n = 5$) in each treatment.

^z **, *** indicates significant effects at 0.01 or 0.001, respectively. NS = not significant.

Fig. 3. Effect of CaCl_2 on pectinolytic activity of *Penicillium digitatum*. Enzymatic activity assay on A, Na-polygalacturonic acid and B, citrus pectin. Each data point represents an average of results obtained from three experiments. Bars indicate standard error of the mean.

the addition of CaCl_2 (Fig. 1). On the other hand, *D. hansenii* strain 114 failed to give any protection of the wounds following its application with or without the addition of CaCl_2 (data not shown). Strain US-7 maintained cytosolic Ca^{2+} homeostasis at levels that did not exceed 1,400 nM when exposed to calcium concentrations of up to 150 mM (Fig. 4). On the other hand, strain 114 showed a reduced capacity to maintain Ca^{2+} homeostasis when exposed to high Ca^{2+} concentrations. The addition of 100 mM CaCl_2 to the medium induced an increase in Ca^{2+} to 3,000 nM in *D. hansenii* (Fig. 4).

DISCUSSION

The results of the present study demonstrate that CaCl_2 affects the infection of grapefruit surface wounds by the green mold fungus and improves biocontrol activity of the yeast antagonist *Pichia guilliermondii*. Factorial analysis indicated that there was no significant interaction between the yeast and CaCl_2 . This indicates that increased disease control achieved by combining the yeast and CaCl_2 is due to the additive effects of each agent alone rather than any synergism. Improved control of postharvest decay by combinations of calcium salts and *Pichia guilliermondii* is in full agreement with earlier findings reported by McLaughlin et al. (13), who showed that the addition of calcium salts to cell suspensions of yeast antagonists resulted in better control of *B. cinerea* and *Penicillium expansum* on apple. It was hypothesized that the presence of calcium salts in the cell suspension of yeast biocontrol agents increased their effectiveness because of the osmotolerant nature of the yeast strains. The addition of salt solutions, with the lowest osmotic potential to apple wounds in the absence of yeast cells, however, failed to protect the wounds against infection of *B. cinerea* or *Penicillium expansum* infections (13). These results indicate that mechanisms other than osmotolerance may be involved in improving inhibition of infection by yeast antagonists. The beneficial effect of combining calcium with biological control agents may be a result of several different interactions taking place between calcium ions and the host, the pathogen, or the yeast.

Recently, Wisniewski et al. (26) demonstrated that increasing concentrations of CaCl_2 directly affected spore germination, germ tube length, and pectinolytic activity of both *Penicillium expansum* and *B. cinerea*. The application of CaCl_2 with the yeast antagonist *Candida oleophila* enhanced the biocontrol activity of the yeast against both pathogens on apple.

The present study has demonstrated that the application of 68 or 136 mM CaCl_2 alone reduces infection of grapefruit wounds by 42 to 46%. The mechanism(s) by which calcium reduces infection of citrus wounds is not yet fully understood. In an attempt to study the direct effect of calcium on citrus fruit tissue, we have shown

that calcium stimulates ethylene production in excised grapefruit peel. Such an increase in ethylene production in grapefruit peel discs may signal the activation of defense reactions against pathogens. The involvement of ethylene in the induction of physiological and compositional changes in citrus fruit tissue and increased resistance of fruit to infection has been reported previously (1,8,22). The exposure of orange fruit to exogenous ethylene increased both the activity of phenylalanine ammonia lyase, a key enzyme in the phenyl propanoid pathway, and the total phenolics in the rind (8). Induced resistance to *Ceratocystis fimbriata* by ethylene has been reported in sweet potato roots and was accompanied by an increase in the activity of peroxidase and polyphenol oxidase (23).

Calcium ions may reduce fungal infection by direct inhibition of fungal growth and inhibition of cell wall-degrading enzymes produced by the pathogen. We observed inhibition of spore germination and germ tube elongation of *Penicillium digitatum* in culture, as well as inhibition of PG activity. These data indicate additional mechanisms by which calcium may affect the green mold of grapefruit. Although these results are based on in vitro assays, it is likely that similar results could occur in the fruit if free calcium ions are present. The mechanisms by which CaCl_2 inhibits fungal spore germination and germ tube elongation are not known.

In eukaryotic cells, Ca^{2+} plays a central role in signal transduction pathways and in the regulation of metabolic processes (2). High levels of $[\text{Ca}^{2+}]_i$ are toxic to animal cells (2). In contrast to mammalian cells, yeast cells can grow in media containing up to 200 mM calcium. In the present study, we examined the hypothesis that yeast with a high biocontrol activity is less sensitive to high Ca^{2+} concentrations than a yeast strain with lower biocontrol activity. Sensitivity to high external Ca^{2+} concentrations is usually caused by the failure of cellular Ca^{2+} transporters to maintain low levels of $[\text{Ca}^{2+}]_i$. Therefore, we have determined the $[\text{Ca}^{2+}]_i$ levels in cells of two yeast strains that vary in their biocontrol efficacy. The experiments were done by using a method that enables the determination of $[\text{Ca}^{2+}]_i$ in yeast cells exposed to very high concentrations of Ca^{2+} (11).

The presence of the pathogen in high concentration of exogenous calcium may lead to increased concentration of Ca^{2+} in the cytosol. Maintenance of low basal concentrations of free cytosolic Ca^{2+} , in the submicromolar range, is essential for normal cell functions (2,19). While little information is available on the role of Ca^{2+} in fungi, results of experiments with yeasts have shown that mutants that have defective intracellular Ca^{2+} transport systems, or defective vacuolar H^+ -ATPase that produces the proton motive force necessary for the activity of the vacuolar $\text{Ca}^{2+}/\text{H}^+$ exchanger (15) could not grow in high Ca^{2+} concentrations (6,9,11,16). High levels of free cytosolic Ca^{2+} have been measured in some of these mutants (11,17). These findings indicate that in yeast cells, similar to mammalian cells, high concentrations of free cytosolic Ca^{2+} inhibit proliferation.

Our data indicate that the effect of calcium in reducing the infection of grapefruit wounds by green mold fungus could be mediated through the response of the host tissue to calcium and possibly to direct inhibitory effects of calcium on the fungus. Improvement of biological control activity of the yeast antagonist in the presence of calcium may be mediated indirectly by the capacity of this yeast to maintain the cytosolic-free Ca^{2+} concentration at low levels when exposed to high concentrations of Ca^{2+} . This could give the yeast an advantage over the fungal pathogen in tolerating high concentrations of Ca^{2+} .

Biocontrol activity of yeast antagonists depends on the presence of a relatively large number of antagonist cells at the wound site (7). This requirement is considered a possible limiting factor in the commercialization of this technology. Thus, factors that improve biocontrol activity such calcium may be of great value in the implementation of this technology.

Fig. 4. Cytosolic-free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in cells of the yeast *Pichia guilliermondii* strain US-7 and the yeast *Debaryomyces hansenii* strain 114 as a function of Ca^{2+} concentration in the surrounding medium. Bars indicate standard error of the mean.

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