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Complete control of *Penicillium expansum* on apple fruit using a combination of antagonistic yeast *Candida oleophila*

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Three *Candida oleophila* strains (L06, L07 smooth, and L07 rough) were evaluated *in vivo* and *in vitro* as biocontrol agents against *Penicillium expansum* on postharvest 'Golden Delicious' apples (*Malus domestica* Borkh.) in Chihuahua, Mexico. The *in vivo* and *in vitro* activity of exo-β-1,3-glucanase was measured as a possible biocontrol mode of action for *C. oleophila*. Mean disease incidence caused by *P. expansum* was 0.3% for apples treated with fludioxonil + ciprodinil, which were used as a positive control, and 1% for fruits treated with a combination of the three *C. oleophila* strains; the effects of these treatments were significantly equivalent. Disease incidence in control apples was 39% and was significantly different from the other treatments. The *in vivo* exo-β-1,3-glucanase activity began at 24 h and peaked at 72 and 96 h for all treatments. Strain L06 had the highest activity (7.96 nKat) and a specific activity of 2.92 nKat μg^{-1}. *Candida albicans* had the lowest activity (2.83 nKat) and a specific activity of 0.67 nKat μg^{-1}. The highest *in vitro* activity was for *C. albicans* (85.03 nKat) and the lowest for strain L06 (78.2 nKat). Significant differences in both *in vivo* and *in vitro* enzymatic activity were observed between strain L06 and *C. albicans*. Polynomial regression analysis (R^2 = 0.96 *in vitro* and 0.68 *in vivo*) indicated that increased enzymatic activity was associated with reduced fruit disease incidence. The production of exo-β-1,3-glucanase by *C. oleophila* is a possible mode of action for the efficient biocontrol of *P. expansum* on postharvest apples.

Key words: Biocontrol, *Malus domestica*, yeasts.

INTRODUCTION

Penicillium expansum Link, the causal agent of blue mold, is one of the most common fungi affecting postharvest apples (Jurick et al., 2011). This phytopathogenic fungus causes fruit losses ranging from 5% to 20% in developed countries (Cappellini and Ceponis, 1984) and up to 50% in developing countries (El-Ghaouth, 1997). Synthetic fungicides are still commonly used to control rots caused by blue mold on apples although most *Penicillium* species have developed resistance to most fungicides used to control them. An alternative to chemical control is using microorganisms that can control phytopathogenic fungi. The development of alternative methods, such as biological control with microorganisms, has increased in recent years (Wang et al., 2009). Blum et al. (2004) used *Cryptococcus laurentii* isolated from apple fruit as a biological control agent in laboratory tests against *P. expansum*, *Glomerella cingulata,* and *Pezicula malicorticis*. They compared this yeast with synthetic fungicides, such as thiabendazole and iprodione, and found no differences among them and confirmed that *C. laurentii* is an efficient biological control agent.

Yeasts are a natural microbiotic component of the apple's surface. They are able to grow and reproduce rapidly on the surface of the fruit at low and high temperatures (4 and 37 ºC) and therefore have an advantage over other microorganisms used on postharvest apples (Guerrero-Prieto et al., 2004; 2011). Yeasts have been identified as potential antagonists for postharvest disease control because of characteristics that allow them to colonize the surfaces of either healthy or wounded fruits (Guerrero-Prieto et al., 2004; Droby et al., 2009; Sharma et al., 2009; Gholamnejad et al., 2010; Guerrero-Prieto et al., 2011). Knowledge and understanding of yeast mode of action are prerequisites to develop successful biological control strategies. Various yeasts have acted as biocontrol agents of postharvest pathogens (Ippolito et al., 2000; Kurtzman and Droby, 2002) through various modes of action, including lytic enzyme production, induction of resistance, antibiosis, and nutrient and space competition (Droby et al., 2009; Sharma et al., 2009; Guerrero-Prieto et al., 2011; Rivera-Avalos et al., 2012). *Candida*

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oleophila has been reported as a biocontrol agent for *P. expansum* and *Botrytis cinerea* on apples (Wisniewski et al., 1995; El-Neshawy and Wilson, 1997; Guerrero-Prieto et al., 2011). The cell wall of these fungi is composed primarily of β-glucans and other polymers, such as chitin and proteins, which together form a structural network that protects the cell (Schirmböck et al., 1994; Adams, 2004; Latgé, 2007). β-1,3-glucanases play an important role in partially degrading the cell wall of these fungi (Amey et al., 2003; King et al., 2011). Exo-β-1,3-glucanases (glucan 1,3-β-D-glucosidases, E. C. 3.2.1.58) sequentially hydrolyze glycosidic bonds in the non-reducing end of 1,3-β-D-glucans and produce glucose as a final product. Endo-β-1,3-glucanases (glucan endo-1,3-β-D-glucosidases, E.C. 3.2.1.39) randomly hydrolyze the inter-chain bonds and form small oligosaccharide chains as a final product (Vijayendra and Kashiwagi, 2009).

Several studies suggest that the production of lytic enzymes is a mode of action whereby yeasts degrade the cell wall and inhibit the growth of *P. expansum* (Oelofse et al., 2009). The objectives of the present study were to evaluate three strains of *C. oleophila* as biocontrol agents and determine their *in vivo* and *in vitro* activity of exo-β-1, 3-glucanase against *P. expansum* on postharvest apple (*Malus domestica* Borkh.) fruit.

MATERIALS AND METHODS

Experiments were performed at the Centro de Investigación en Alimentación y Desarrollo, A.C. (CIAD, A.C.) in Cuauhtemoc, Chihuahua, Mexico. The original *C. oleophila* strains (L06, L07 smooth, and L07 rough) were obtained from the epiphytic flora of 'Golden Delicious' apples and belong to the CIAD's strain collection "Microorganismos de la zona templada" (Temperate Zone Microorganism Collection) in Cuauhtemoc. The *P. expansum* strain was originally obtained from Oregon State University Mid-Columbia Agricultural Research Station, Hood River, Oregon, USA.

Physical and other characteristics of 'Golden Delicious' apples were as follows: fruit were harvested from a commercial orchard and cold stored ("La Nortenita" orchards and cold storage facility, Cuauhtemoc, Chihuahua. Mexico). Fruit, 21 per treatment, with no visible physical damage and an advanced degree of physiological maturity (postharvest apples) were randomly selected for the experiments. The fruit surface was disinfected with 1% sodium hypochlorite solution for 1 min before being used in the experiments.

Growth of microorganisms and inoculum preparation were as follows: *C. oleophila* strains were grown in NYDA medium (8 g nutrient broth, 5 g yeast extract, 10 g dextrose, and 15 g agar in 1 L distilled water) at 25 °C for 48 h. Strains were then inoculated in physiological saline solution and adjusted to a 1×10^7 (CFU mL^{-1}) concentration. To obtain an enzymatic extract *in vitro*, yeasts were grown in NYDA, transferred to physiological saline solution, and the concentration adjusted to 1×10^7. The *P. expansum* Link strain was grown in PDA (potato dextrose agar) for 7 d, transferred to physiological saline solution, and the concentration adjusted to 1×10^4 conidia mL^{-1}. Concentrations were adjusted with a Neubauer chamber.

Evaluation of biocontrol efficiency was performed by punching two wells, each with a depth of 10 mm and a diameter of 3 mm, into each apple that was inoculated with 30 µL suspension of each of the *C. oleophila* strains or their combination at a concentration of 1×10^7 CFU mL^{-1}. Thirty minutes after the first inoculation, 30 µL of *P. expansum* conidia suspension at a concentration of 1×10^9 CFU mL^{-1} was applied to the same well. The positive control consisted of apples in which the corresponding well was inoculated with only *P. expansum*. The negative control consisted of apples that were inoculated with only sterile water. A treatment with the chemical synthetic fungicide fludioxonil (4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1H-pyrrole-3-carbonitrile) + cyprodinil (4-cyclopropyl-6-methyl-N-phenylpyrimidin-2-amine) at a dose of 1 g L^{-1} was also included. Apples were stored at 4 °C. The diameter of the damaged area was evaluated on a weekly basis for 1 mo and used as an indicator of the damage caused by *P. expansum*. Disease incidence in the positive control was considered as 100% disease incidence, and disease incidence caused by the other treatments was calculated in relation to this value. Experiments were repeated three times and data were pooled into a single ANOVA.

Obtaining enzymatic extract *in vivo* and *in vitro*

Enzymatic extract for *in vivo* experiments was obtained from apples washed with 1% sodium hypochlorite solution for 1 min. Wells with a diameter of 3 mm and depth of 5 mm were punched in each apple. Each well was inoculated with 35 μL suspension of each *C. oleophila* isolate or their combination. *Candida albicans* was used as a positive control. Thirty minutes after yeast inoculation, the *P. expansum* conidia solution was inoculated in the same well of each apple. The negative control consisted of a treatment with only sterile distilled water. Each treatment was replicated three times. Inoculated fruits were placed in plastic bags to reach a high relative humidity (RH) and then stored at 24 ºC for 7 d. Tissue, 0.25 g, was obtained from each well at 0, 6, 12, 24, 48, 72, and 96 h after inoculation. A 50 mM solution of sodium acetate, 500 μL, (pH 5.0, 4 ºC) was added to each tissue sample and centrifuged for 15 min at 10,000 g. Apple fruit tissue was then discarded and the supernatant precipitated by adding 70% acetone solution at -20 ºC. The supernatant was centrifuged at 12.000 g for 30 min, and the precipitate was washed twice with 70% acetone solution. The precipitate was suspended in 2 mL of 50 mM sodium acetate buffer solution (pH 5.0). This solution was used to determine

exo-β-1,3-glucanase activity (Ippolito et al., 2000). Total protein was determined by Bradford's method (Bradford, 1976). Experiments were performed five times and data were then pooled and analyzed by ANOVA.

The enzymatic extract for the *in vitro* experiments was obtained from each one of the various isolates of *C. oleophila* and *C. albicans* that were grown in 250 mL NYDA (8 g nutrient broth, 5 g yeast extract, and 10 g dextrose in 1 L distilled water) at 25 ºC for 3 d. A raw extract, 5 mL, was collected from each isolate and solubilized with 500 µL of 50 mM sodium acetate buffer at 4 °C. This solution was centrifuged for 15 min at 10,000 g, and the supernatant precipitated with 70% acetone at -20 °C. The solution was centrifuged at 12,000 g for 30 min and washed twice with 70% acetone. The precipitate was suspended in 2 mL of sodium acetate buffer solution; this suspension was used to measure exo-β-1,3-glucanase enzymatic activity. When enzymatic activity was observed at the incubation times (24, 48, 72, and 96 h), 2-mL aliquots were drawn. Procedures to obtain the extract and determine exo-β-1,3-glucanase activity were performed as previously described for enzymatic extracts *in vivo* (Ippolito et al., 2000). Experiments were repeated five times, and data were then pooled to be analyzed by ANOVA.

Determining exo-β-1,3-glucanase activity

Exo-β-1,3-glucanase activity in the enzymatic extracts from *in vitro* and *in vivo* samples was measured by incubating them separately for 2 h at 40 ºC with a 3% laminarin solution from *Laminaria digitata* as the substrate. Enzymatic reactions were run at pH 5.0 and stopped by heating the sample for 10 min at 90 ºC. To each sample, 372 μL of 3,5-dinitrosalicylic acid was added. The amount of glucose formed from laminarin was measured by spectrophotometry at 492 nm. The final specific enzymatic activity was reported as nKat μg^{-1} of total protein; 1 nKat is defined as the enzymatic activity that catalyzes production of 1 nmol s^{-1} glucose.

From the total *in vivo* exo-β-1,3-glucanase activity for each treatment, the natural activity of this enzyme on the apple fruit was determined and subtracted from total activity. The enzymatic activity was also measured in apple fruit pulp with no treatment. Exo-β-1,3-glucanase activity was measured for treatments with L06, L07 smooth, L07 rough, and their combination. The enzymatic activity of *C. albicans* was measured as a positive control.

Statistical analysis

Data were pooled and analyzed by ANOVA with the Statistix, version 9.0 software program (Analytical Software, Tallahassee, Florida, USA). Means were compared by Tukey's test (α = 0.05). Relationships between fruit damage and exo-β-1,3-glucanase activity were evaluated by polynomial regression analysis.

RESULTS

Control of *P. expansum in vivo*

Apple fruit damage caused by *P. expansum* was greater when the pathogen was inoculated alone. The diameter of the damaged area on apples was reduced by adding each of the three *C. oleophila* isolates and by treating them with the chemical synthetic fungicide fludioxonil+cyprodinil. Apple fruit damage was reduced by the L06 and L07 rough strains and the combination of three *C. oleophila* strains; this damage was similar to synthetic fungicide used alone (Table 1).

Exo-β-1,3-glucanase activity *in vivo*

The three *C. oleophila* strains (L06, L07 smooth, L07 rough) and their combination showed exo-β-1,3-glucanase activity when challenged by *P. expansum* on apples. Isolate L06 showed the highest enzymatic activity (7.96 nKat) and *C. albicans* the lowest (2.83 nKat) (Table 2). Exo-β-1, 3-glucanase activity was first detected at 24 h. The highest enzymatic activity was detected at 72 h for L06 and L07 rough and at 96 h for L07 smooth, the combination of strains, and *C. albicans* (Figure 1).

Exo-β-1,3-glucanase activity *in vitro*

The *C. oleophila* isolates displayed exo-β-1,3-glucanase activity when grown in NYDA. *Candida albicans* had the highest enzymatic activity (85.03 nKat) and strain L06 the

Table 1. *In vivo Penicillium expansum* disease incidence on postharvest 'Golden Delicious' apples when challenged by three *Candida oleophila* strains their combination, and fludioxonil+cyprodinil.

Treatments	Disease incidence	Disease reduction
	%	
P. expansum	39a	-
L07 smooth	11b	72
L06	7bc	82
L07 rough	7bc	82
Strain combination	1bc	97
Fludioxonil + cyprodinil	0.3c	99
Negative control	0.3c	99

Means with different letters are significantly different according to Tukey's test (P < 0.05).

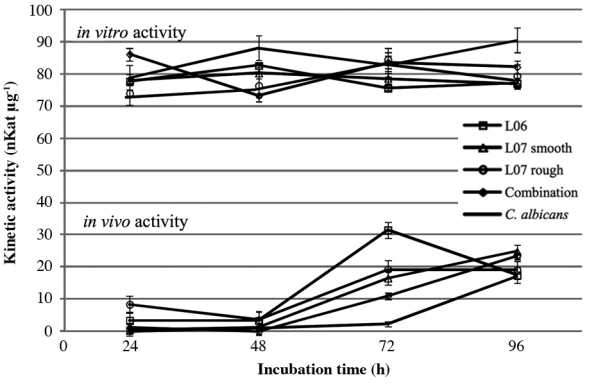

Figure 1. *In vitro* and *in vivo* kinetic activity of exo-ß-1,3-glucanase for three *Candida oleophila* strains.

lowest (78.23 nKat) (Table 2). The enzymatic activity for these two strains measured *in vitro* differed from the *in vivo* measurements. The highest enzymatic activity was observed at 24 and 72 h for *C. albicans*, at 24 and 48 h for the combination of the three *C. oleophila* isolates, and at 72 h for the L07 rough isolate (Figure 1).

A relationship was observed between exo-β-1,3-glucanase enzymatic activity (both *in vivo* and *in vitro*) and apple fruit damage. A polynomial relationship was obtained, that is, enzymatic activity increased as apple fruit damage decreased. Data fitness was higher for *in vitro* enzymatic activity ($R^2 = 0.99$) than *in vivo* activity ($R^2 = 0.68$) (Figure 2).

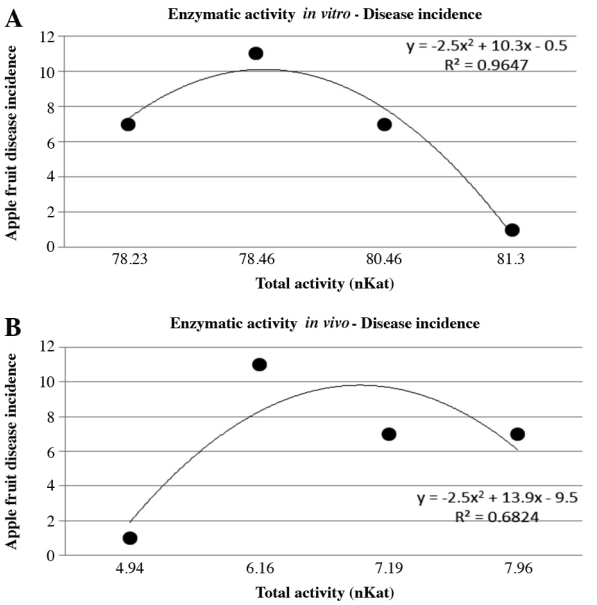

Figure 2. Polynomial relationship between enzymatic activity and apple fruit disease incidence caused by *Penicillium expansum in vitro* (A) and *in vivo* (B).

Table 2. Total protein and exo-β-1,3-glucanase activity for three *Candida oleophila* strains when challenged by *Penicillium expansum* on postharvest 'Golden Delicious' apples.

	in vivo		*in vitro*	
Strains	Total protein	Total activity	Specific activity	Total activity
	μg mL^{-1}	nKat	nKat μg^{-1}	nKat
L06	2.73c	7.96a	2.92	78.23b
L07 rough	2.73c	7.19ab	2.63	80.46ab
L07 smooth	2.43d	6.16ab	2.53	78.46b
Strain combination	3.10b	4.94bc	1.59	81.30ab
C. albicans	4.21a	2.83c	0.67	85.03a

Means followed by different letters within a column are significantly different according to Tukey's test ($P < 0.05$).

DISCUSSION

Damage caused by *P. expansum* on apples was efficiently controlled by strains L06 and L07 rough, either combined or separately, and by the synthetic fungicide used alone. Thus, the effects of the evaluated *C. oleophila* strains in controlling postharvest apple fruit damage caused by *P. expansum* were indistinguishable from those of the evaluated synthetic fungicide. These results indicate that the evaluated *C. oleophila* strains are a viable alternative for *P. expansum* biocontrol. *Candida oleophila* has been reported as an exo-β-1,3-glucanase producer (Bar-Shimon et al., 2004). This enzyme is capable of degrading the cell wall and decreasing spore germination and mycelium growth of phytopathogenic fungi (Lin et al., 2007). Our results clearly demonstrate exo-β-1,3-glucanase activity in *C. oleophila* strains evaluated in both *in vivo* and *in vitro*. We observed numerical differences in enzymatic activity between both growth conditions; *in vitro* activity was slightly more than ten times higher than *in vivo* activity. *In vitro* activity of L06 and L07 rough strains, either alone or combined, was the same. *In vitro* activity for *C. albicans* was the highest, while the lowest was in the L06 strain. Bar-Shimon et al. (2004) reported that *in vitro* exo-β-1,3-glucanase activity of *C. oleophila* was higher during the first developmental stages. In contrast, we observed a variable enzymatic activity during the evaluated period of time. Enzymatic activity of the L06 strain was higher *in vivo*; it was higher for this strain used alone than when it was combined with the other two strains. The observed differences for *in vitro* and *in vivo* enzymatic activity in the evaluated *C. oleophila* strains suggest that they have a higher exo-β-1,3-glucanase activity when directly challenged by *P. expansum*. Observed responses for the evaluated *C. oleophila* strains (particularly strain L06) suggest that they could be very useful in the biological control of *P. expansum*. Strain L06 displayed the highest enzymatic activity *in vivo* and when challenged by the fungus. A similar response for *C. oleophila* was reported by Bar-Shimon et al. (2004), who observed higher enzymatic activity for yeast grown in culture media enriched with *P. digitatum* cell wall fragments plus glucose. β-1,3-glucanase activity by other yeasts on fruit was reported by Fan et al. (2002).

The results of the present study clearly indicate that the three evaluated *C. oleophila* strains produce lytic enzymes with exo-β-1,3-glucanase activity. Enzymatic activity in both *in vivo* and *in vitro* was best fitted to a polynomial relationship in which increased enzymatic activity resulted in reduced apple fruit damage. Exo-β-1,3-glucanase produced by the evaluated *C. oleophila* strains could play an important role as a mode of action for the biocontrol of *P. expansum*. The L06 and L07 rough isolates have a high potential for development as biocontrol agents against *P. expansum* on postharvest apples and may provide a viable alternative for biocontrol under postharvest conditions.

CONCLUSIONS

Penicillium expansum disease reduction on postharvest apple fruit was complete when using the combination of

the three *Candida oleophila* yeasts as well as when using synthetic fungicide. One of the possible modes of action used by yeasts was the production of exo-β-1,3-glucanase.

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