Biocontrol of Postharvest Diseases of Jujube Fruit by *Cryptococcus laurentii* Combined with a Low Dosage of Fungicides Under Different Storage Conditions

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ABSTRACT

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An antagonistic yeast, *Cryptococcus laurentii*, alone or in combination with a low dose of imazalil (25 µg a.i./ml) or kresoxim-methyl (50 µg a.i./ml), was investigated for its ability to control *Alternaria alternata* and *Monilinia fructicola* on jujube fruit under different storage conditions. Biocontrol activity of *C. laurentii* against *A. alternata* and *M. fructicola* at 20°C was enhanced significantly by combination with either fungicide. An integrated strategy for control of *A. alternata* and *M. fructicola* was developed that included a combination of *C. laurentii*, a low dose of fungicide, and controlled-atmosphere (CA) storage. Fruit treated with *C. laurentii* plus imazalil at 25 µg a.i./ml or kresoxim-methyl at 50 µg a.i./ml and stored in CA storage with 10% O₂ + 0% CO₂ at 0°C showed a lower disease incidence caused by both pathogens than fruit stored in air at 0°C. Application of imazalil (25 µg a.i./ml) or kresoxim-methyl (50 µg a.i./ml) did not inhibit the growth of *C. laurentii* in wounds of jujube fruit stored in air at 20 and 0°C or in CA at 0°C. The yeast grew rapidly in the wounds of jujube fruit under all storage conditions. The ability of *C. laurentii* to effectively control postharvest diseases of jujube fruit, the resistance of *C. laurentii* to low rates of fungicides, and the adaptability of this yeast to CA storage indicate that *C. laurentii* has great commercial potential as a biological control product.

Additional keywords: mode of action

Jujube (*Zizyphus jujuba* cv. Dongzao) fruit is susceptible to postharvest diseases caused by various pathogenic fungi. *Alternaria alternata* (Fr.:Fr.) Keissl. is the most important pathogen and causes latent infections and serious postharvest losses (28). Jujube fruit also can be infected by *Monilinia fructicola* (G. Wint.) Honey, *Penicillium expansum* Link, and *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. through wounds that occur during harvest or packing (28). These decays are significantly reduced when fruit are stored in a controlled atmosphere (CA) at -1°C (28).

Synthetic chemical fungicides are the primary means to control postharvest diseases of jujube fruit (29), but concerns about public health, the environment, and the development of resistant pathogens have increased the search for alternative methods (33). Biological control using

microbial antagonists is proving to be a reliable method of natural control of many postharvest pathogens (6,9,16,19). Several antagonistic microorganisms have been found to effectively inhibit postharvest diseases (9,11,16,23). At least four products are commercially available for postharvest disease control: Aspire, based on *Candida oleophila* (Ecogen Inc., Langhorn, PA), YieldPlus, based on *Cryptococcus albidus* (Anchor Yeast, Cape Town, South Africa), and Bio-Save 110 and Bio-Save 111, based on the bacterium *Pseudomonas syringae* (EcoScience, Orlando, FL) (7). Antagonists alone do not always provide commercially acceptable control of fruit decays, but their activity can be enhanced by manipulation of the environment, using mixtures of beneficial organisms, physiological and genetic enhancement of the biocontrol mechanisms, and integration of biocontrol with other methods, such as low doses of fungicides and CA storage (2,12,25,26,30).

CA storage has been demonstrated to have fungistatic properties (4,22). In our previous study, combining CA storage with biocontrol agents resulted in better control of postharvest diseases on apple and pear than CA storage or the biocontrol agents alone (30). To our knowledge, there is little information on the efficacy of combinations of antagonists with fungicides against postharvest pathogens in CA storage.

The objective of this study was to evaluate the effectiveness of *Cryptococcus laurentii* (Kuff.) C. E. Skinner, alone and together with a low dose of fungicide, for control of *A. alternata* and *M. fructicola* of jujube fruit. Disease control was determined in fruit stored in air at 20 and 0°C, and in CA storage with 10% O₂ + 0% CO₂ at 0°C. In addition, the effect of fungicides on growth of *C. laurentii* in wounds of jujube fruit under various storage conditions was determined.

MATERIALS AND METHODS

Antagonists. *C. laurentii* was isolated from apple fruit following the method of Wilson and Chalutz (32). *C. laurentii* (IMI number 386832) was identified by the CABI Bioscience Identification Services (International Mycological Institute, Egham, UK). Yeast cells were cultured in nutrient-yeast-dextrose broth (NYDB: 8 g of nutrient broth, 5 g of yeast extract, and 10 g of dextrose in 1,000 ml of water) for 48 h at 25°C. Washed cell suspensions of the yeast were obtained by centrifugation at 6,000 rpm (2,500 × g) for 10 min, re-suspended in sterile distilled water, and centrifuged again. The concentration of the yeast was determined with a hemacytometer and adjusted to 1 × 10⁸ CFU/ml.

Pathogen. *A. alternata* and *M. fructicola* were isolated from infected jujube fruit and maintained on potato dextrose agar (PDA) for 14 days at 20°C. The spores of both pathogens were suspended in 5 ml of sterile distilled water containing 0.05% (vol/vol) Tween 80, and filtered through four layers of sterile cheesecloth to remove any adhering mycelia. Spore concentrations of both *A. alternata* and *M. fructicola* were determined with a hemacytometer and adjusted to 1 × 10⁵ spores/ml with sterile distilled water.

Fruit materials. Jujube fruit were harvested at commercial maturity and sorted based on size and the absence of physical injuries or infections. The firmness of jujube fruit was 55.7 N as determined by a penetrometer (FT-327; UC Fruit Firmness Tester, Milano, Italy), and total soluble solids were 23.6%. Fruit were disinfested with 2% (vol/vol) sodium hypochlorite for 2 min, washed with tap water, air dried prior to use within 2 days, and held at 0°C before use.

Biocontrol assays of yeasts under different storage conditions. Imazalil (Dec-

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cozil 25 EC; Elf Atochem North America, Inc., Philadelphia) and kresoxim-methyl (Stroby 50% DF; BASF Ltd., Ludwigshafen, Germany) were used in this study. Jujube fruit were wounded (3 mm deep and 3 mm wide) with a sterile nail and treated with 25 μ l of the treatment suspensions as follows: *C. laurentii* (10^8 CFU/ml), *C. laurentii* (10^8 CFU/ml) + imazalil (25 μ g a.i./ml), *C. laurentii* (10^8 CFU/ml) + kresoxim-methyl (50 μ g a.i./ml), imazalil (25 μ g a.i./ml), kresoxim-methyl (50 μ g a.i./ml), and sterile distilled water controls. After 4 h, fruit were challenge inoculated with 20 μ l of the conidial suspensions of *A. alternata* or *M. fructicola* at 1×10^5 spores/ml. Treated fruit were placed in plastic boxes (250 by 150 by 50 mm) with high humidity (about 95%) and stored in air at 20 or 0°C, or in CA storage with 10% O₂ + 0% CO₂ at 0°C. Jujube fruit are sensitive to high CO₂ concentrations and, in a preliminary study, we found that CA with 10% O₂ + 0% CO₂ at 0°C was effective to reduce postharvest decays (*data not shown*). Disease incidence and lesion diameter were measured after 5 days at 20°C. Fruit stored at 0°C in air and in CA storage were observed after 40, 50, and 60 days. Each treatment was replicated three times with 15 fruit per replicate and the entire experiment was repeated twice.

Population dynamics of biocontrol yeasts under different storage conditions. Jujube fruit were wounded and treated with 25 μ l of the yeast suspension at 1×10^7 CFU/ml, alone or in combina-

tion with imazalil at 25 μ g a.i./ml or kresoxim-methyl at 50 μ g a.i./ml. Fruit were stored in air at 20 or 0°C, or in CA storage with 10% O₂ + 0% CO₂ at 0°C, as described above. Fruit samples were taken as described by Janisiewicz et al. (13) at different times after treatment by removing the wound tissue with a cork borer. The resulting cylinders (10 mm deep and 10 mm wide) of five jujube fruit were placed in a mortar with 10 ml of sterile distilled water and ground with a pestle. Then, 50 μ l of serial 10-fold dilutions were plated on NYDA medium. Colonies of *C. laurentii* were counted after incubation at 20°C for 72 h and expressed as log₁₀ CFU/wound. Each treatment was replicated three times, and the entire experiment was repeated three times.

Statistical analysis of data. Statistical analyses of the data were performed with Statistical Analysis System (version 6.12; SAS Institute, Cary, NC). Disease incidence, lesion diameter, and yeast population density were analyzed by two-way factorial analysis. Mean separations were performed by Student-Newman-Keuls test. To test whether the treatments produced similar results in each of the experimental repeats, the experimental repeats were included as a factor in the analysis of variance and the interaction between experimental repeat and treatment was tested for significance. A nonsignificant interaction was found in all cases between experimental repeat and treatment, indicating that treatment effects were similar among the experimental repeats. Results

presented were pooled across repeated experiments.

RESULTS

Effect of combination of *C. laurentii* with fungicides at 20°C in air. The results from two experiments demonstrated that the antagonistic yeast *C. laurentii*, as well as low dosages of imazalil (25 μ g a.i./ml) and kresoxim-methyl (50 μ g a.i./ml), were effective against *A. alternata* and *M. fructicola* in jujube fruit at 20°C (Fig. 1). Neither the yeast nor the two fungicide treatments caused any surface injury to the fruit. Better control of both pathogens was achieved by combining *C. laurentii* with each fungicide at a low dosage than with yeast or fungicide alone. After incubation for 5 days at 20°C, fruit treated with *C. laurentii* plus imazalil (25 μ g a.i./ml) and inoculated with *A. alternata* or *M. fructicola* showed 77 and 93% less decay, respectively, than the controls. For both pathogens, lesion diameters also were significantly reduced by *C. laurentii* + imazalil. Similar results for control of *A. alternata* and *M. fructicola* were found in fruit treated with *C. laurentii* + kresoxim-methyl (50 μ g a.i./ml) and stored at 20°C.

Effect of combination of *C. laurentii* with fungicides at 0°C in air and CA storage. *C. laurentii*, alone or in combination with the fungicides imazalil (25 μ g a.i./ml) or kresoxim-methyl (50 μ g a.i./ml), controlled *A. alternata* and *M. fructicola* effectively at 0°C in air and in CA storage (Figs. 2 and 3). Complete control of *A. alternata* was obtained in fruit treated with *C. laurentii* + kresoxim-methyl at 0°C in air and in CA storage at 40, 50, or 60 days. The addition of imazalil to *C. laurentii* also enhanced the efficacy of the yeast against *A. alternata*, but the effect was greater when fruit were stored in CA storage than in air for the same length of time (Fig. 2). Different results were observed for *M. fructicola* when fruit were treated with the yeast-fungicide combination. Complete control of *M. fructicola* was achieved by combining *C. laurentii* with imazalil at 0°C under CA storage. Combining *C. laurentii* with kresoxim-methyl enhanced the activity of the yeast against *M. fructicola*. Incidence of disease caused by *M. fructicola* was lower and lesion diameter was smaller in fruit treated with *C. laurentii* + kresoxim-methyl and kept in CA storage than in fruit stored at 0°C in air for the same period of time (Fig. 3). Incidence of decay increased and lesions enlarged as storage time increased, but the relative activity of the various treatments was consistent at 40, 50, or 60 days. There was no significant difference in the data obtained in repeated experiments.

Influence of fungicides on the growth of *C. laurentii* under different storage conditions. The similar results obtained in repeated experiments indicated that population dynamics of *C. laurentii* in the

Fig. 1. Biocontrol efficacy of *Cryptococcus laurentii* (CL), alone or in combination with a low dose of imazalil (IM) or kresoxim-methyl (KM) fungicide, on decay of jujube fruit caused by *Alternaria alternata* and *Monilinia fructicola* at 20°C. Fruit were wounded and treated with CL, CL+IM, CL+KM, IM, KM, or water (CK) and inoculated with *A. alternata* or *M. fructicola*. The data were based on three replicates of 15 fruit each and the test was repeated twice with similar results. Bars represent standard errors of the treatment means of pooled data.

wounds of jujube fruit were not significantly influenced by the application of imazalil (25 μg a.i./ml) or kresoxim-methyl (50 μg a.i./ml) in air at 20 or 0°C, or in CA storage throughout the experiment (Fig. 4).

C. laurentii multiplied rapidly in the wounds of jujube fruit and reached the highest levels after 2 days at 20°C, regardless of whether the yeast was used alone or combined with one of the two fungicides

(Fig. 4A). Low temperature and CA storage did not significantly affect the growth of *C. laurentii*. Populations of *C. laurentii* increased to almost the same levels in fruit treated with *C. laurentii*, *C. laurentii* + imazalil, and *C. laurentii* + kresoxim-methyl after 56 days of incubation at 0°C in air or in CA storage (Figs. 4B and C).

DISCUSSION

C. laurentii in combination with a low dose of imazalil or kresoxim-methyl resulted in better control of both *A. alternata* and *M. fructicola* on jujube fruit than the yeast or the fungicide treatments alone at 20°C (Fig. 1). Combining fungicides with biocontrol agents proved to be an effective method to improve control of postharvest pathogens while reducing the chemical residues on the fruit in other studies (2,10,25,27). Imazalil commonly is used for postharvest treatment of jujube fruit in China, and kresoxim-methyl is a new fungicide from the strobilurin group of chemicals (18). The general concentration of

Fig. 2. Biocontrol efficacy of *Cryptococcus laurentii* (CL), alone or in combination with a low dose of imazalil (IM) or kresoxim-methyl (KM) fungicide, on decay of jujube fruit caused by *Alternaria alternata* at 0°C **A** and **B**, in air and **C** and **D**, in controlled-atmosphere storage. Fruit were wounded and treated with CL, CL+IM, CL+KM, IM, KM, or water (CK) and inoculated with *A. alternata* and stored for 40, 50, or 60 days. The data were based on three replicates of 15 fruit each and the test was repeated twice with similar results. Bars represent standard errors of the treatment means of pooled data.

Fig. 3. Biocontrol efficacy of *Cryptococcus laurentii* (CL), alone or in combination with a low dose of imazalil (IM) or kresoxim-methyl (KM) fungicide, on decay of jujube fruit caused by *Monilinia fructicola* at 0°C **A** and **B**, in air and **C** and **D**, in CA storage. Fruit were wounded and treated with CL, CL+IM, CL+KM, IM, KM, or water (CK) and inoculated with *M. fructicola* and stored for 40, 50, or 60 days. The data were based on three replicates of 15 fruit each and the test was repeated twice with similar results. Bars represent standard errors of the treatment means of pooled data.

Fig. 4. Influence of imazalil and kresoxim-methyl on population dynamics of *Cryptococcus laurentii* in wounds of jujube fruit in air at **A**, 20°C or **B**, 0°C, or **C**, in controlled-atmosphere storage at 0°C. The data were based on three replicates and the test was repeated three times with similar results. Bars represent standard errors of the treatment means of pooled data.

both fungicides necessary to significantly control postharvest diseases of jujube fruit is more than 300 µg a.i./ml when used alone (*data not shown*).

CA storage at low temperatures is effective for control of various pathological and physiological problems that occur in stored fruit and vegetables (14,20,31). Decay development is retarded because growth, sporulation, and enzyme activity of the pathogen are reduced, and the improved physiological condition of the host enables it to resist decay more effectively (21). However, as the fruit is removed from storage and placed at ambient temperature, decay develops rapidly and renders fruit commercially unacceptable (4). Consequently, CA storage cannot be used as a sole method for decay control. To optimize the benefit of CA, other methods, such as application of biological control agents or a reduced level of fungicides, are needed to completely prevent decay development and extend the shelf life of fruit (3,24,30). In this experiment, we evaluated an integrated control strategy against *A. alternata* and *M. fructicola* decay of jujube fruit with a combination of a yeast, *C. laurentii*, a low dose of fungicide, and CA storage. Significantly better control of both pathogens was obtained when jujube fruit was treated with *C. laurentii* plus a low dose of imazalil or kresoxim-methyl and stored in CA storage than fruit treated with yeast or fungicide alone. Our previous work showed that *C. laurentii* combined with CA at 10% O₂ + 10% CO₂ provided better control of *A. alternata* on sweet cherry fruit than the individual treatments alone (*unpublished data*).

The mode of action of antagonistic yeasts is complex. It may be due to competition for space and nutrients (5,17), direct physical interaction with fungal hyphae, production of cell-wall lytic enzymes (8,34), and induction of host resistance (1,7). The effects of biological control of postharvest decay are likely the result of interactions of different factors. However, competition for nutrients and the ability to multiply rapidly in wounds of fruit may be the primary mechanisms by which *C. laurentii* exerts biocontrol activity (19). Thus, it is important that the colonizing ability of *C. laurentii* is not inhibited by fungicide. Lima et al. (15) reported that *C. laurentii* was not very sensitive to several fungicides commonly applied on fruit and vegetables. The result of our experiment showed that application of either imazalil or kresoxim-methyl at a low dosage did not significantly reduce the population of *C. laurentii* in the wounds of jujube fruit. *C. laurentii* multiplied quickly in fruit wounds in air at 20 and 0°C, and in CA storage at 0°C. It is quite possible that *C. laurentii* rapidly colonized the wound site of jujube fruit, effectively competed with the pathogens for nutrients, and resulted in starvation of the pathogens. Under these

conditions, the pathogens may have been more sensitive to the fungicides, and the biocontrol efficacy of *C. laurentii* was enhanced.

Commercial development of *C. laurentii* as a biocontrol agent to control postharvest diseases of fruit is in progress (3). It is likely that the successful commercial use of biocontrol will depend on the compatibility of antagonists with postharvest practices, such as fungicide, low-temperature, and CA storage (30). The results reported here showed that *C. laurentii* was resistant to low rates of fungicides and was adapted to CA storage. The yeast grew rapidly in the wounds of jujube fruit under all storage conditions, including CA storage at 0°C, regardless of whether the fungicides were used or not. This indicates that *C. laurentii* has great commercial potential as a biological control product.

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