Nutrient Effects on Biocontrol of *Penicillium roqueforti* by *Pichia anomala* J121 during Airtight Storage of Wheat

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The biocontrol yeast *Pichia anomala* inhibits the growth of a variety of mold species. We examined the mechanism underlying the inhibition of the grain spoilage mold *Penicillium roqueforti* by the biocontrol yeast *P. anomala* J121 during airtight storage. The biocontrol effect in a model grain silo with moist wheat (water activity of 0.96) was enhanced when complex medium, maltose, or glucose was added. Supplementation with additional nitrogen or vitamin sources did not affect the biocontrol activity of the yeast. The addition of complex medium or glucose did not significantly influence the yeast cell numbers in the silos, whether in the presence or absence of *P. roqueforti*. Mold growth was not influenced by the addition of nutrients, if cultivated without yeast. The products of glucose metabolism, mainly ethanol and ethyl acetate, increased after glucose addition to *P. anomala*-inoculated treatments. Our results suggest that neither competition for nutrients nor production of a glucose-repressible cell wall lytic enzyme is the main mode of action of biocontrol by *P. anomala* in this grain system. Instead, the mold-inhibiting effect probably is due to the antifungal action of metabolites, most likely a combination of ethyl acetate and ethanol, derived from glycolysis. The discovery that sugar amendments enhance the biocontrol effect of *P. anomala* suggests novel ways of formulating biocontrol yeasts.

Mold growth yearly destroys large amounts of vegetables, fruits, and cereal grain both pre- and postharvest (2). The growth of molds in food and feed causes reduced nutritional values and production of allergenic spores and hazardous mycotoxins (18). Traditionally, fungicides and chemical preservatives have been used to prevent fungal growth, but concerns for the environment and the health of the consumer along with resistance problems and stricter governmental regulations have generated a demand for alternative control methods. During the last decade many yeasts and other microorganisms that provide a biological control alternative have been described (3, 4, 8, 17, 21). In contrast to filamentous fungi, biocontrol yeasts have the benefit of usually not producing potentially allergenic spores or mycotoxins. Two yeast-based products, Aspire (*Candida oleophila*) and Yield plus (*Cryptococcus albidus*), are commercially available for the biocontrol of molds in apples, citrus, and pome fruits (21). Shemer, a biological control project registered for use in Israel (www.agrogreen.co.il), is based on a newly identified yeast *Metschnikowia fructicola* (17) and is effective against a wide range of pathogens of grape, strawberry, and sweet potato (M. K. Zur et al., Abstr. Int. Workshop Dev. Biocontrol Agents Dis. Commercial Appl. Food Prod. Syst., abstr. O2-7, 2004).

Pichia anomala (formerly *Hansenula anomala* [16]) inhibits the growth of a variety of molds, e.g., *Botrytis cinerea*, *Aspergillus candidus*, *Penicillium roqueforti*, and other plant-pathogenic or wood decay fungi, in environments as diverse as stored apple, grapevine plants, grain in airtight storage, and wood (for a review, see reference 20). *P. anomala* strain J121 can reduce the growth of *P. roqueforti* in vitro, in high-moisture cereal

grain in a test-tube version of a malfunctioning storage system, and in 0.21-m³ pilot scale silos for airtight storage of 160-kg batches of moist grain (3, 7, 22–24). Airtight storage of cereal grain is an energy-saving alternative to hot-air drying. This type of storage is particularly important for regions of the world where cereal grain is harvested at moisture contents that allow the rapid growth of spoilage molds.

Several possible modes of action for biocontrol by yeasts have been suggested, such as competition for space and nutrients, production of cell wall-degrading enzymes, killer toxins, antibiotic metabolites, mycoparasitism, and stimulation of host defense responses (13, 20). For *P. anomala* the production of cell wall lytic enzymes and killer toxins has been suggested to be responsible for antifungal activity (15, 29). Other potential antifungal mechanisms include competition for nutrients, oxygen, and space; ethanol production (20); and formation of ethyl acetate (26), a volatile with antifungal activity (27). Identification of the mode of action of a biocontrol organism is important both for regulatory approval and for optimizing the biocontrol system. The objective of this study was to identify the mode of action of *P. anomala* J121 responsible for the inhibition of mold growth in airtight grain storage silos.

MATERIALS AND METHODS

Fungal isolates. *P. roqueforti* Thom (J5) and *P. anomala* (Hansen) Kurtzman (J121) were originally isolated from stored grain. *P. roqueforti* J5 was maintained on silica gel at 4°C, and *P. anomala* J121 was maintained in glycerol stocks (K₂HPO₄, 0.82 g liter⁻¹; KH₂PO₄, 0.18 g liter⁻¹; sodium citrate, 0.59 g liter⁻¹; MgSO₄ · 7H₂O 0.25 g liter⁻¹; glycerol [87%], 212 g liter⁻¹) at –70°C. All cultures were pregrown on 3% malt extract agar slants (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) at 25°C. For biocontrol assays, *P. anomala* was grown overnight in YPD medium (yeast extract, 10 g liter⁻¹ [Oxoid]; peptone, 20 g liter⁻¹ [Oxoid]; glucose, 20 g liter⁻¹) and diluted to an appropriate concentration in peptone water (2 g liter⁻¹; Oxoid). Spores of *P. roqueforti* were harvested from agar plates and kept in glycerol stocks at –18°C until inoculation.

Cultivation in minisilos. Minisilos were prepared as previously described (22) with some modifications. Commercially available heat-dried nonsterilized wheat

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(Kosack, 10% moisture) was moistened alternatively with water, YPD medium, 2 g of maltose solution liter⁻¹ of rehydrating water, YNB medium (yeast nitrogen base, 6.67 g liter⁻¹) (Difco Laboratories, Detroit, Mich.), or glucose solutions (10, 20, 40, or 60 g liter⁻¹ of rehydrating water) to a water activity (a_w) of approximately 0.96 (~25% moisture). Since the wheat was not sterilized before the experiment, we tested the mold and yeast count and found it to be below the detection limit ($<10^2$ CFU g⁻¹). The wheat was stored at 2°C for 72 h to allow the water content to equilibrate. The moist wheat was inoculated with 10^4 spores of *P. roqueforti* g⁻¹ of grain and 10^5 CFU of *P. anomala* J121 g⁻¹ of grain. Spores and yeast cells were applied as drops onto the grain and mixed by shaking to obtain an even distribution. The inoculated grain was poured into 27-ml test tubes that were sealed with a rubber membrane and perforated with a needle to simulate the air leakage of a full-scale silo. Minisilos inoculated only with mold or yeast were used as controls. The minisilos were incubated at 25°C for 7 days. At least three minisilos were used for every measurement.

Fungal growth determination. The growth of yeast and mold was measured as CFU per gram by using selective growth plates (3). Quantification of mold growth is not as straightforward as that of yeast, but there was a significant correlation between mold CFU and hyphal lengths in previous biocontrol experiments with *P. anomala* and *P. roqueforti* (3). The statistical significance of treatment differences was evaluated with a Student's *t* test.

Determination of ethyl acetate. Ethyl acetate was extracted from the grain by shaking the total minisilo content with 5 ml of 99% decane (Sigma, St. Louis, Mo.) for 5 min (26). Although this extraction method does not differentiate between intracellular and extracellular ethyl acetate, it reflects volatile changes in the atmosphere of the minisilo as ethyl acetate is highly volatile. One milliliter of the extract was filtered through a 0.45- μ m-pore-size Acrodisc syringe filter (Gelman Laboratory, Ann Arbor, Mich.) and 1 μ l was injected into a Hewlett Packard gas chromatograph with a flame ionization detector (250°C; Cheshire, United Kingdom) and a capillary column (HP19091S-833; 250 μ m by 30.0 m). The carrier gas was H₂ at a flow rate of 40 ml min⁻¹. The column temperature was programmed to increase from 60 to 250°C at a rate of 20°C min⁻¹ and was finally held for 2 min at 250°C. Ethyl acetate was identified and quantified by comparison with an external standard.

Consumption of glucose and production of metabolites. Concentrations of glucose and ethanol in grain extracts were measured at appropriate times (see Results). The compounds were extracted by shaking the contents of each minisilo with 5 ml of distilled water. Samples (10- μ l) were analyzed by high-pressure liquid chromatography (Agilent Technologies, Waldbronn, Germany) by using a Hamilton (Hägersten, Sweden) HC-75 column for separation. Analysis was performed at 60°C with 5 mM H₂SO₄ at a flow rate of 0.6 ml min⁻¹ as the mobile phase. The eluate was monitored with an Agilent 1100 refractive index detector. The metabolites were identified and quantified by comparison with chromatograms from authentic reference compounds (glucose [Oxoid] and ethanol [Primalco Oy, Finland]).

Antimold activity of pure ethyl acetate and ethanol. To determine if there is a synergistic antimold effect due to ethanol and ethyl acetate, *P. roqueforti* was grown on agar containing ethanol in a closed environment with vaporized ethyl acetate. A glass cup attached to a microscope slide was placed on the bottom of a plastic petri dish, and 0.6 ml of ethyl acetate (99.8%; Merck) was added to the glass cup. The amount of ethyl acetate in the *in vitro* assay was chosen after preliminary experiments since it is not possible to directly compare headspace concentrations in the minisilos with the plate assay. Water was used as the negative control. Spores of *P. roqueforti* were inoculated in the center of a malt extract agar plate, with or without 1 g of ethanol liter⁻¹ of culture medium. The plate was placed facing down on the petri dish with the ethyl acetate-containing glass slide. The ethanol concentration represents an intermediate concentration detected in the grain (approximately 400 μ g/g of grain). To reduce ethyl acetate evaporation, the plates were wrapped with three layers of parafilm. After 7 days of incubation at 25°C, the mycelial dry weight of the *Penicillium* colony was measured. The colony was placed in distilled water and heated in a microwave oven until the agar melted. The mold colony was then washed in distilled water, placed on a preweighed filter, and dried at 80°C for 24 h. All treatments were done with three replicates.

RESULTS

Effect of nutrient addition on mold and yeast growth. The addition of complex medium (YPD) to a minisilo enhanced the ability of the yeast to inhibit mold growth. In minisilos containing YPD, mold, and yeast, 15 ± 7 *P. roqueforti* CFU g⁻¹ of

FIG. 1. Effects of glucose addition (10 g glucose liter⁻¹ of moistening water) and yeast-mold cocultivation on the growth of *P. anomala* J121 and *P. roqueforti* J5 in a minisilo system. $\circ$, yeast CFU in yeast control without glucose addition; $\square$, yeast CFU in yeast control with glucose addition; $\triangle$, yeast CFU in yeast-mold cocultivation without glucose addition; $\diamond$, yeast CFU in yeast-mold cocultivation with glucose addition; $\bullet$, mold CFU in mold control without glucose addition; $\blacksquare$, mold CFU in mold control with glucose addition; $\blacktriangle$, mold CFU in yeast-mold cocultivation without glucose addition; $\blacklozenge$, mold CFU in yeast-mold cocultivation with glucose addition. Values are the means of three experiments $\pm$ standard deviations. Similar results were observed at all glucose concentrations (10, 20, 40, and 60 g liter⁻¹).

grain were present after 7 days of growth compared to 340 ± 260 CFU g⁻¹ of grain in the mold, yeast, and water control. The addition of YPD to control cultivations with yeast or mold alone did not alter the number of CFU per gram for either organism (data not shown). Adding maltose gave results with respect to mold reduction similar to those obtained with YPD (data not shown). Adding nitrogen and vitamins (YNB) did not alter the number of either yeast or mold CFU per gram.

Glucose effects on the growth dynamics of *P. anomala* J121 and *P. roqueforti* J5 in minisilos were monitored for the 7 days following inoculation. For the first 2 days, the number of mold CFU per gram decreased ~10-fold in all treatments (Fig. 1). *P. roqueforti* growth was observed in treatments without inoculated yeast beginning on day 3, and this growth continued until day 7, reaching a final level of $1.6 \times 10^5 \pm 1.0 \times 10^5$ CFU g⁻¹ of grain. Final *P. roqueforti* CFU levels were not influenced by glucose addition when cultivated without yeasts (Fig. 1). In treatments that contained *P. anomala* but no additional glucose, weak mold growth was observed beginning on day 4 and continued until the end of cultivation, yielding a final value of $1.1 \times 10^3 \pm 0.1 \times 10^3$ CFU g⁻¹ of grain. In contrast, no significant increase in mold CFU was observed, when *P. anomala* and glucose (10 g liter⁻¹) were added to the cultures. Final mold CFU values were significantly different between yeast treatments with or without a glucose supplement of 10 g liter⁻¹ of rehydrating water ($P < 0.05$) (Fig. 1). Higher sugar amendments (20, 40, and 60 g liter⁻¹ of grain moistening solution) resulted in the same inhibition of mold growth ($P < 0.05$; data not shown). Final yeast CFU levels were not significantly affected by glucose addition either in the presence or in the absence of mold (Fig. 1).

Consumption of glucose and production of ethanol. When glucose was added to silos inoculated with yeast, all of the glucose was consumed within 7 days (Fig. 2A). In control silos

FIG. 2. Glucose concentration in minisilos inoculated with *P. anomala* and *P. roqueforti* (A) or with only *P. roqueforti* (B). ♦, no glucose addition; ■, 10 g of glucose liter⁻¹ of grain moistening water; ▲, 20 g of glucose liter⁻¹; ●, 40 g of glucose liter⁻¹; *, 60 g of glucose liter⁻¹. Values are the means of three experiments $\pm$ standard deviations.

that were inoculated only with mold, glucose consumption was very low compared to those inoculated with yeast (Fig. 2B). At the two highest glucose levels, some glucose was consumed during the first 3 days, but virtually no glucose consumption was observed from day 3 to day 7 (Fig. 2B). There is an unusually high standard deviation on day 3.

Ethanol was detected after 7 days in all minisilos whether or not they were inoculated with *P. anomala*. However, in yeast-inoculated minisilos, more ethanol was produced, and the ethanol concentration increased with increasing glucose concentration in the moistening water (Fig. 3). Similar levels of ethanol production also were observed for silos with yeast only and silos inoculated with both yeast and mold. However, at the lowest glucose supplement level (10 g liter⁻¹ of rehydrating water) no clear difference in ethanol concentration was ob-

FIG. 3. Ethanol concentration in water extracts from grain minisilos after 7 days of incubation with different glucose supplements and fungal inocula. ▲, *P. anomala* without *P. roqueforti* inoculation; ■, *P. anomala* and *P. roqueforti* cocultivation; ♦, *P. roqueforti* control without yeast inoculation. Values are the means of three experiments $\pm$ standard deviations.

FIG. 4. Ethyl acetate concentrations in decane extracts from *P. anomala*-inoculated grain minisilos with different glucose amendments after 7 days of incubation. ▲, *P. anomala* without *P. roqueforti* inoculation; ■, *P. anomala* and *P. roqueforti* cocultivation. Values are the means of three experiments $\pm$ standard deviations. No ethyl acetate was detected from treatments without *P. anomala*; i.e., *P. roqueforti* did not produce ethyl acetate even at high levels of glucose supplementation.

served between the two yeast treatments and the silos inoculated with only *P. roqueforti*. When *P. roqueforti* was grown without yeast but with glucose, the ethanol concentration reached the same levels as in treatments with *P. anomala* and 10 g of glucose liter⁻¹.

Ethyl acetate formation. The ethyl acetate concentration in the grain minisilos increased both over time (data not shown) and with the amount of added glucose (Fig. 4). Regardless of the glucose addition, ethyl acetate was never detected in treatments inoculated with only *P. roqueforti*.

Antifungal activity of ethyl acetate and ethanol. The inhibitory action of ethyl acetate and ethanol on fungal growth was evaluated with agar plate assays. The addition of ethanol to the agar (1 g per liter, corresponding to the average amount of ethanol measured in the minisilo grain water) decreased the fungal colony dry weight from 130 ± 39 mg to 65 ± 5.8 mg. When 0.6 ml of ethyl acetate was added to the atmosphere above a plate without ethanol, the mean dry weight of the *P. roqueforti* colony decreased to 7.3 ± 2.8 mg. When ethanol-containing agar was combined with ethyl acetate in the atmosphere, the colony dry weight was further reduced to 4.7 ± 1.5 mg. *P. anomala* itself is able to grow at 10% (wt/vol) ethanol (9) and can survive in 0.2 M ethyl acetate (28).

DISCUSSION

Nutrient competition is frequently suggested as an important inhibition mechanism in biocontrol systems (20). The addition of nutrients to the system may diminish the biocontrol effect if nutrient competition is the mode of action of the biocontrol agent (4, 6, 8). In the present study, the addition of extra nitrogen and vitamins had no effect on the biocontrol ability of *P. anomala*. Thus, competition for nitrogen is not responsible for the biocontrol we observed, even though competition for nitrogen may be the mechanism through which yeast biocontrol of wound-invading pathogens on apples occurs (12).

Surprisingly, the addition of glucose, maltose, or complex

sugar-rich media increased the biocontrol effect. Thus, competition for a carbon source or for an energy source is likely not the major mechanism responsible for the antifungal activity. Exo- β -1,3-glucanase, a cell wall lytic enzyme, is glucose repressible in other strains of *P. anomala* (14). This enzyme is involved in the inhibition of gray mold by *P. anomala* on apple but would be repressed by the glucose added to our system. The addition of glucose without inoculation of *P. anomala* did not alter the growth of the mold, which rules out possible inhibitory actions by the glucose itself or by glucose stimulation of the indigenous microorganisms. Since the final yeast numbers (CFU) were not affected by the addition of glucose, spatial crowding by the yeast presumably is not occurring as a result of sugar addition.

Our results strongly suggest that the enhanced antifungal effect may be due to the products of sugar metabolism. Possible candidate products are ethyl acetate, ethanol, acetate, arabinitol, glycerol, or various combinations of these products (9). Ethanol has well-known antifungal effects (19, 20, 25). Ethanol concentration in the minisilos increased only marginally following the addition of the glucose solution at a concentration of 10 g l⁻¹ of rehydrating water, even though the full inhibitory effect of the glucose addition on mold growth could be observed at this sugar concentration. Furthermore, in minisilos supplemented with 10 g of glucose liter⁻¹ and inoculated with *P. roqueforti* alone, as much ethanol was produced as in the control treatment with *P. anomala* alone. If ethanol alone were the critical inhibitory component, then *Saccharomyces cerevisiae* would be the best yeast for the biocontrol of grain molds, since this yeast is an efficient ethanol producer and is particularly tolerant of high ethanol concentrations (25). However, *S. cerevisiae* has no known biocontrol activity in this minisilo system (23).

Ethyl acetate could result in the enhanced biocontrol activity that follows glucose addition, and this ester demonstrated considerable antifungal activity in our plate assays. We have previously shown a connection between the biocontrol activity and ethyl acetate production in a haploid *P. anomala* strain (11). In the grain minisilos ethyl acetate concentrations were already significantly enhanced after the grain was moistened with water containing 10 g of glucose l⁻¹. The volatile nature of ethyl acetate would enable it to spread easily in the nonhomogeneous environment of the grain silo. Ethyl acetate and ethanol also might have synergistic antifungal effects in grain silos, similar to the effect observed in our agar plate experiments.

Even without glucose supplementation, i.e., at substantially lower ethyl acetate levels, the growth of *P. anomala* reduced the number of mold CFU per gram by about 100-fold relative to growth within silos without yeasts. Thus, in addition to ethyl acetate and perhaps ethanol production, other factors may be involved in the biocontrol activity of *P. anomala* on cereal grain, as is typical of several biocontrol systems (13). In airtight grain storage systems these additional factors might include competition for O₂, inhibitory levels of CO₂, and possibly production of antifungal compounds, e.g., killer toxins (10).

The positive effect of the addition of sugar on the biocontrol activity of *P. anomala* could provide an opportunity to increase the performance of this organism in industrial applications. Sugars can improve the viability of freeze-dried cultures that are important components of the formulation of biocontrol

agents (1, 5). Thus, sugar amendments to a potentially commercialized formulation could have a double effect by increasing both the viability and the biocontrol performance of *P. anomala*.

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