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Biocontrol of Mold Growth in High-Moisture Wheat Stored under Airtight Conditions by *Pichia anomala*, *Pichia guilliermondii*, and *Saccharomyces cerevisiae*

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Pichia anomala **inhibits the growth of** ***Penicillium roqueforti*** **and** ***Aspergillus candidus*** **on agar. In this investigation, antagonistic activity on agar against 17 mold species was determined. The abilities of** ***Pichia anomala*****,** ***Pichia guilliermondii*****, and** ***Saccharomyces cerevisiae*** **to inhibit the growth of the mold** ***Penicillium roqueforti*** **in nonsterile high-moisture wheat were compared by adding 10^3** ***Penicillium roqueforti*** **spores and different amounts of yeast cells per gram of wheat. Inoculated grain was packed in glass tubes, incubated at 25°C with a restricted air supply, and the numbers of yeast and mold CFU were determined on selective media after 7 and 14 days.** ***Pichia anomala*** **reduced growth on agar plates for all of the mold species tested in a dose-dependent manner.** ***Aspergillus fumigatus*** **and** ***Eurotium amstelodami*** **were the most sensitive, while** ***Penicillium italicum*** **and** ***Penicillium digitatum*** **were the most resistant.** ***Pichia anomala*** **had the strongest antagonistic activity in wheat, with 10^5 and 10^6 CFU/g completely inhibiting the growth of** ***Penicillium roqueforti*****. Inhibition was least pronounced at the optimum temperature (21°C) and water activity (0.95) for the growth of** ***Penicillium roqueforti*****.** ***Pichia guilliermondii*** **slightly reduced the growth of** ***Penicillium roqueforti*** **in wheat inoculated with 10^5 and 10^6 yeast CFU/g.** ***S. cerevisiae*** **inhibited mold growth only weakly at the highest inoculum level.** ***Pichia anomala*** **grew from 10^3 to 10^7 CFU/g of wheat in 1 week. To reach the same level,** ***Pichia guilliermondii*** **had to be inoculated at 10^4 CFU while** ***S. cerevisiae*** **required an inoculum of 10^5 CFU to reach 10^7 CFU/g of wheat.**

Antagonistic fungi can inhibit the growth of some spoilage or toxin-producing molds during storage of fruits and vegetables (5, 20). Mold growth in grains is commonly prevented by drying; however, drying procedures are costly, especially in a temperate climate. Feed grains can be stored undried in airtight silos where the growth of microorganisms is prevented by the low levels of oxygen and high levels of carbon dioxide caused by the respiration of epiphytic microflora and grain (11). However, air exchange between the silo and the external atmosphere as a result of imperfect sealing, daily temperature fluctuations, and removal of grain makes it difficult to maintain these conditions. The most common spoilage fungi on grains are *Aspergillus* and *Penicillium* species and xerophilic *Eurotium* species (11). *Penicillium roqueforti* is an especially important spoilage microorganism in airtight storage since it produces several mycotoxins, e.g., roquefortin C (6), and can grow at low partial pressures of oxygen and at low temperatures (11).

A way of potentially reducing spoilage during airtight storage further is to introduce a microorganism antagonistic to spoilage molds. The yeast *Pichia guilliermondii*, previously called *Debaryomyces hansenii*, controls a range of postharvest spoilage fungi, such as *Penicillium digitatum* on grapefruit (3), *Botrytis cinerea* and *Penicillium expansum* on apples (20), and *Aspergillus flavus* on soybeans (14). Its adverse effects on *Penicillium digitatum* and *B. cinerea* have been ascribed to competition for nutrients and the secretion of cell wall-degrading enzymes (2, 3, 20). These kinds of control mechanisms are acceptable for use in the biological control of molds in foods and feeds since they do not involve the secretion of potentially hazardous antibiotics. *Pichia guilliermondii* was selected as the active agent in a biofungicide of postharvest diseases under commercial development partly because of its above-mentioned modes of action (7). The antagonistic efficiency of *Pichia guilliermondii* in laboratory experiments has been successfully reproduced in pilot plant tests in which it has been able to compete with standard chemical treatments in preventing fruit decay due to postharvest diseases (7).

The yeast *Pichia anomala* is frequently found in airtight-stored cereals (11). Earlier, we isolated *Pichia anomala* from airtight-stored wheat and demonstrated that it shows antagonistic activity on agar plates against the spoilage molds *Penicillium roqueforti* and *Aspergillus candidus* (1). Furthermore, the degree of inhibition of spoilage fungi was related to the initial concentration of *Pichia anomala* cells. Inhibition was most pronounced at temperatures suboptimal for the growth and sporulation of *Penicillium roqueforti* and *A. candidus*, i.e., below 15°C and above 30°C (1).

Here we report the effects of *Pichia anomala* on the growth of a number of spoilage molds on agar plates. In addition, we studied the inhibition of *Penicillium roqueforti* by *Pichia anomala* on wheat kernels. *Pichia anomala* cells and *Penicillium roqueforti* spores were added to nonsterile high-moisture wheat and incubated with a restricted air supply. The ability of *Pichia anomala* to control mold growth on wheat under these conditions was compared with those of *Pichia guilliermondii* and *Saccharomyces cerevisiae. Pichia guilliermondii* was chosen for this comparison because of its earlier documented ability to act as a biocontrol agent. *S. cerevisiae* is commonly used for baking and brewing and is generally regarded as a safe organism. The influence of the water activities (a_ws) of grains and incubation temperatures on the inhibition of *Penicillium roqueforti* by *Pichia anomala* was further evaluated by using a reduced factorial experimental design.

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MATERIALS AND METHODS

Fungi studied. *Penicillium roqueforti* Thom (J5), a gift from P. Häggblom, and *Pichia anomala* (Hansen) Kurtzman (J121) were originally isolated from airtight-stored grains. *S. cerevisiae* (baker's yeast strain P 1:6; Svenska Jästfabriks AB, Sollentuna, Sweden) was a gift from Björn Lindman. *Pichia guilliermondii* NRRL Y-18314 was originally isolated in Florida from the surfaces of lemons untreated with fungicides (19). Other fungi used were *A. flavus* Link (J7), *A. fumigatus* Fres. (J9), *Cladosporium cladosporioides* (Fres.) de Vries (J41), *B. cinerea* Pers. (J57), *Eurotium amstelodami* Mangin (J6), *Fusarium poae* (Peck) Wollenweber (J24), *F. sporotrichoides* Sherb. (J26), *Monascus ruber* v. Tieghem (J39), *Mucor hiemalis* Wehmer f. *hiemalis* (J12), *Penicillium digitatum* Sacc. (J66), *Penicillium expansum* Link (J2), *Penicillium glabrum* (Wehmer) Westling (J3), *Penicillium italicum* Wehmer (J89), *Rhizopus stolonifer* (Ehrenb.) Lind. (J45), and *Talaromyces flavus* (Klöcker) Stolk & Samson (J37). Fungi came from the fungal collection of the Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden. *Pichia anomala* was identified by the ID 32 C test (Biomerieux, Marcy l'Etoile, France) and morphological studies (9, 10). Identification was confirmed at the Centraalbureau voor Schimmelcultures, Delft, The Netherlands. Molds were identified according to the method of Samson and van Reenen-Hoekstra (17). Fungal cultures were maintained on slants of malt extract agar (MEA; Oxoid, Basingstoke, United Kingdom) at 4°C.

Inocula. Yeast suspensions were prepared by inoculating 100 ml of a yeast extract-malt extract-sucrose broth (0.2 g of yeast extract [BBL, Meyland, France], 1.5 g of malt extract (Oxoid), 1.0 g of sucrose [BDH, Poole, England] in 1.0 liter of distilled water) with a loopful of cells from a culture stored on MEA at 4°C. After incubation on a rotary shaker (100 rpm) at 30°C for 24 h, the number of yeast cells was enumerated with a hemocytometer. The spore suspension of *Penicillium roqueforti* was prepared by collecting spores from 5-day-old colonies (grown on MEA at 25°C) in peptone-water (2 g of peptone per liter of distilled water) with 0.015% Tween 80 added to assist in the dispersal of conidia. The spore concentration was enumerated with a hemocytometer.

Inhibition experiments. (i) Inhibition of different molds with *Pichia anomala* on agar plates. Yeast and mold spore suspensions were prepared as described above. The agar medium in which inhibition experiments were performed was MEA or MEA supplemented with 20% sucrose to reduce a_w. The latter substrate with reduced a_w favors the growth and anamorph formation of xerophilic *E. amstelodami* (17). A top agar was prepared by mixing 6 ml of agar and 1 ml of yeast suspension with either 10^4 or 10^8 cells per ml. The agar-yeast suspension was poured into petri dishes that contained 15 to 20 ml of the same agar medium. Once the top agar had set, 10-μl portions of a mold suspension with 10^5 mold spores per ml were inoculated at three spots on each plate, with three replicates for each mold species. Molds were inoculated as spots to make it possible to measure the radial extension rates of colonies. Plates were incubated at 20°C for 7 days, except for those with *E. amstelodami* or *Monascus ruber*, which were incubated at 30°C for 7 days, and those with *T. flavus*, which were incubated at 25°C for 7 days. Plates not inoculated with any of the three yeasts used in this study served as controls. The degree of inhibition was determined after mixing the agar from each petri dish with peptone-water (0.005% Tween 80, 1 g of peptone in 1.0 liter of distilled water) to obtain a 10-fold dilution. The mixture was then homogenized for 1 min with an Ultra-Turrax Disp25 (Inter Med, Roskilde, Denmark) at 13,500 rpm. Samples (0.1 ml) of 10-fold dilutions of homogenates were surface spread on MEA plates supplemented with 10 ppm of cycloheximide (C-6255; Sigma Chemical Co., St. Louis, Mo.) (MEAC), and CFU were counted after 5 days of incubation at 25°C.

(ii) Inhibition of *Penicillium roqueforti* in wheat by *Pichia anomala*, *Pichia guilliermondii*, and *S. cerevisiae*. Nonsterile winter wheat (cultivar Kosack) stored at a water content of 8% at 20°C was moistened with tap water to obtain a_w of 0.95 (21% water). To equilibrate the moisture content, grains were stored for more than 48 h at 6°C with frequent mixing. a_w was measured at 25°C with a Novasina TH-2/RTD-33/BS equipped with a thermoconstanter (Defensor AG, Pfäffikon, Switzerland).

Wheat grains with a_ws of 0.95 were inoculated with *Penicillium roqueforti* spore suspension to about 3×10^3 mold spores per g. Spores were applied by adding the suspension as drops onto grains and mixing to obtain an even spore distribution on grains. Different yeasts were similarly inoculated to reach 0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 cells per g of grain in separate tests. Thick-walled glass tubes (27 ml) were filled with approximately 17-g portions of inoculated grain and sealed with butyl rubber stoppers. To simulate air leakage, a needle (0.6-mm diameter) was inserted through each butyl rubber stopper. Tubes were incubated at 25°C.

On days 7 and 14, two tubes from each treatment were opened, and the contents were diluted 10-fold with peptone-water, soaked for 1 h, and homogenized for 2 min at normal speed in a Stomacher 400 (Colworth, United Kingdom). To avoid growth inhibition by antagonistic yeast on agar plates intended for mold CFU counting and to provide a selective substrate for *Penicillium roqueforti*, MEAC with 100 ppm of chloramphenicol (Sigma Chemical Co.) and 0.5% acetic acid (MEACC/HAc) was used for surface plating of 0.1-ml aliquots. Chloramphenicol is a bacteriostatic broad-spectrum antibiotic, cycloheximide inhibits growth of *Pichia anomala* (1), and acetic acid selects for *Penicillium roqueforti* (4). To check whether a combination of cycloheximide and acetic acid inhibited the growth of *Penicillium roqueforti*, triplicate samples from seven different spore suspensions were surface spread on MEA and MEACC/HAc, both supplemented with chloramphenicol. These experiments were repeated three times.

Influence of a_w and temperature on the inhibition of *Penicillium roqueforti* in wheat by *Pichia anomala*. The effects of a_w, temperature, and incubation time on the inhibition of *Penicillium roqueforti* by *Pichia anomala* were evaluated by using factorial experimental design. Nonsterile wheat was moistened as described above to a_ws of 0.90, 0.95, and 0.98 (i.e., 18, 21, and 28% water). Moist grains were inoculated with a suspension of *Pichia anomala* cells as described above to 10^5 CFU/g and with *Penicillium roqueforti* spore suspension to 4×10^3 CFU/g. Thick-walled glass tubes were filled with wheat as described above and incubated at 10, 20, and 30°C, and samples were taken after 1, 3, and 5 weeks. The effects of the selected variables on the inhibition of the number of CFU of *Penicillium roqueforti* were evaluated by using a reduced factorial design at two levels with extra axis points and replicated centerpoints, i.e., a central composite design. These experiments were repeated three times. This experimental design allowed evaluation of simple effects, two-factor interactions, and nonlinear response at the second-degree polynomial level. The effects of different factors were estimated by multiple linear regression and validated by internal cross validation, i.e., response data from one experiment were removed and predicted by the model developed from N-1 experiments. This procedure was repeated until the responses of all experiments had been predicted. Deviations between the predicted and measured response values were used to calculate the explained variance. Only terms which increased the explained variance were included in the model. All calculations were done with MODDE software (UMETRI AB, Umeå, Sweden).

RESULTS

The average coefficient of variation for log-transformed CFU values was less than 4% in all individual experiments.

Test of substrate. Neither the addition of acetic acid alone nor the addition of cycloheximide and acetic acid combined influenced the numbers of CFU of *Penicillium roqueforti* compared with the number detected on MEA with chloramphenicol only (data not shown).

In samples with high numbers of yeast CFU, as determined on MEA supplemented with chloramphenicol, some yeast colonies were also detected on MEACC/HAc. In theory, the detection limit in this inhibition experiment might have been influenced by sparse yeast growth on MEACC/HAc. However, there were never more than 10 yeast colonies per plate, making it unlikely that the growth of *Penicillium roqueforti*, for which the substrate had been designed, had been inhibited. When samples of nonsterile wheat were examined, no mold species other than *Penicillium roqueforti* were detected on MEACC/HAc, confirming the selectivity of this substrate.

Inhibition experiments. (i) Inhibition of different molds with *Pichia anomala* on agar plates. *Pichia anomala* strongly reduced the growth and sporulation of most of the molds tested on agar plates (Table 1). There was no correlation between radial extension rate and sensitivity to *Pichia anomala* (data not shown). The degree of inhibition was dose dependent. *A. fumigatus* and *E. amstelodami* were particularly sensitive, with levels below the detection limit (4×10^3 CFU per plate) at an initial concentration of 10^4 yeast cells per plate. The numbers of the most resistant molds, *Penicillium italicum*, *Penicillium digitatum*, *Penicillium roqueforti*, *F. sporotrichoides*, and *R. stolonifer*, were reduced by less than 1 log unit by 10^4 *Pichia anomala* cells. At 10^8 *Pichia anomala* cells per plate, only *Penicillium italicum* and *Penicillium digitatum* grew. However, visible growth and sporulation of these two molds occurred only at inoculation spots (diameter, <4 mm).

(ii) Inhibition of *Penicillium roqueforti* in wheat by *Pichia anomala*, *Pichia guilliermondii*, and *S. cerevisiae*. Both yeasts and molds showed distinct gradients of growth when inoculated on wheat kernels in tubes with a restricted air supply. In the upper 3 cm of each tube, yeasts (and in some cases, also molds) grew abundantly. In the lower part of each tube, no growth or sporulation was visible.

The inhibitory effect of *Pichia anomala* on the number of

TABLE 1. Inhibition of mold growth and sporulation on MEA plates by *Pichia anomala*[a]

Species	Mean mold population in the presence of *Pichia anomala* ± SD (CFU/plate)[b]		
	0	10^4	10^8
Penicillium expansum	8.7 ± 0.2	4.5 ± 0.1	BD[c]
Penicillium italicum	10.4 ± 0.3	9.8 ± 0.2	6.6 ± 0.1
Penicillium glabrum	9.0 ± 0.0	5.9 ± 0.3	BD
Penicillium digitatum	8.6 ± 0.1	7.8 ± 0.1	5.5 ± 0.1
Penicillium roqueforti	8.4 ± 0.0	7.5 ± 0.2	BD
Paeciliomyces variotii	8.4 ± 0.1	6.0 ± 0.0	BD
A. fumigatus	7.2 ± 0.1	BD	BD
A. flavus	8.3 ± 0.1	5.4 ± 0.1	BD
C. cladosporioides	8.3 ± 0.2	6.2 ± 0.1	BD
F. sporotrichoides	7.8 ± 0.1	7.2 ± 0.1	BD
F. poae	7.6 ± 0.2	5.8 ± 0.1	BD
B. cinerea	5.5 ± 0.1	3.9 ± 0.2	BD
Mucor hiemalis	7.6 ± 0.3	6.5 ± 0.1	BD
R. stolonifer	7.8 ± 0.1	7.3 ± 0.1	BD
E. amstelodami	9.0 ± 0.1	BD	BD
T. flavus	9.0 ± 0.1	6.2 ± 0.1	BD
Monascus ruber	6.5 ± 0.1	5.3 ± 0.2	BD

[a] Plates were incubated at 20°C for 7 days, except for those with *E. amstelodami* or *Monascus ruber* and others with *T. flavus*, which were incubated at 30 and 25°C, respectively. Plates with *E. amstelodami* were supplied with 20% sucrose to reduce a_w.

[b] Inoculum levels of *Pichia anomala* were 0, 10^4, and 10^8 cells per plate. Three replicates were used for each mold species.

[c] BD, below detection (limit, 4×10^3 CFU per plate).

CFU of *Penicillium roqueforti* was already obvious after 7 days of air leakage at 10^2 *Pichia anomala* cells per g of wheat (Fig. 1a). At 10^4 to 10^6 *Pichia anomala* cells per g, the numbers of *Penicillium roqueforti* CFU were reduced below the inoculation level. At the two highest levels of *Pichia anomala* inoculation, a weak fungicidal effect was observed on both day 7 and day 14 (Fig. 1). By 7 days, *Pichia anomala* had reached 10^7 to 10^8 CFU/g in tubes inoculated with 10^3, 10^4, 10^5, and 10^6 *Pichia anomala* CFU/g of wheat (Fig. 2a). Values for inoculation levels of below 10^3 CFU are missing. No significant growth of *Pichia anomala* occurred during the second incubation week, indicating that it had grown rapidly to its maximum cell density (Fig. 2b). To evaluate the inhibitory effect of *Pichia anomala* on *Penicillium roqueforti*, wheat kernels were mixed with 6.3×10^4 CFU of *Pichia anomala* and increasing numbers of *Penicillium roqueforti* spores (0 to 4×10^5 CFU/g of wheat). *Pichia anomala* strongly reduced the numbers of CFU of *Penicillium roqueforti* for 14 days, regardless of the inoculation level (data not shown). *Penicillium roqueforti* was totally inhibited at inoculated concentrations of 10^3 CFU/g and lower during the first week. At higher concentrations, the numbers of *Penicillium roqueforti* CFU increased during the first week but not during the second week. The total increase in *Penicillium roqueforti* CFU after 14 days was about 0.5 log unit at all inoculation concentrations (data not shown). *Penicillium roqueforti* was inoculated at 3×10^3 CFU/g of wheat in the other inhibition experiments in this study.

Pichia guilliermondii also reduced the numbers of CFU of *Penicillium roqueforti* in a dose-dependent manner but not to the same extent as *Pichia anomala* (Fig. 1). An inoculation of 10^5 or 10^6 CFU of *Pichia guilliermondii* per g of wheat was required to reduce the numbers of *Penicillium roqueforti* CFU by more than 1 log unit after 7 days compared with those of the control. On day 14, only treatment with 10^6 *Pichia guilliermondii* CFU/g of wheat was clearly inhibitory. During the first week, *Pichia guilliermondii* inoculated at lower levels did not grow as much as *Pichia anomala*. An inoculation of 10^4 CFU/g was required to obtain 10^7 CFU/g, while higher inoculation levels resulted in almost 10^8 CFU/g of wheat (Fig. 2). The numbers of *Pichia guilliermondii* CFU did not increase during the second week of incubation (Fig. 2b).

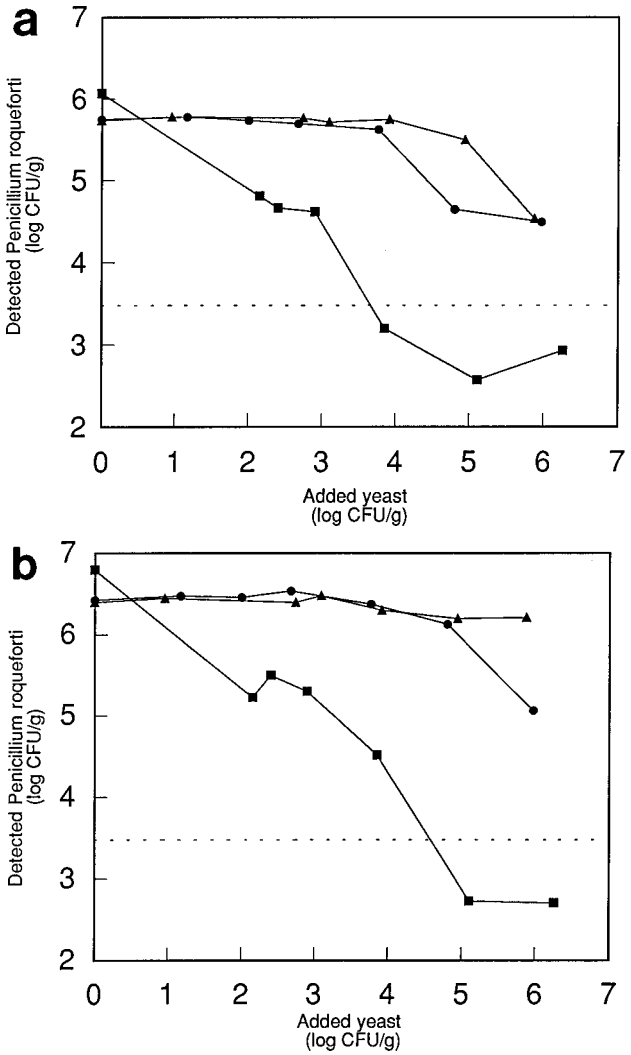

FIG. 1. Effects on *Penicillium roqueforti* growth and sporulation caused by the addition of yeasts *Pichia anomala* (■), *Pichia guilliermondii* (●), and *S. cerevisiae* (▲) to wheat with a_w of 0.95 at 25°C during simulated air leakage for 7 (a) and 14 days (b). In each panel, the dashed line indicates the level of *Penicillium roqueforti* on day 0. Data are given as log mold CFU per gram of wheat ($n = 2$).

S. cerevisiae affected the numbers of CFU of *Penicillium roqueforti* only during the first 7 days and at an initial inoculum concentration of 10^6 CFU/g of wheat (Fig. 1a). After 2 weeks, the numbers of CFU of *Penicillium roqueforti* were no longer affected by the presence of *S. cerevisiae* (Fig. 1b). At 10^5 and 10^6 inoculated CFU/g of wheat, the *S. cerevisiae* numbers reached 10^7 cells after 1 week and 3×10^7 CFU after 2 weeks (Fig. 2). Lower inoculation levels of *S. cerevisiae* resulted in weak growth during the first week and stronger growth during the second week.

Influence of a_w and temperature on the inhibition of *Penicillium roqueforti* in wheat by *Pichia anomala*. *Pichia anomala* had a large inhibitory effect in wheat with a_ws of above 0.95 and had minimal inhibition in wheat with an a_w of 0.90 (Fig. 3 and

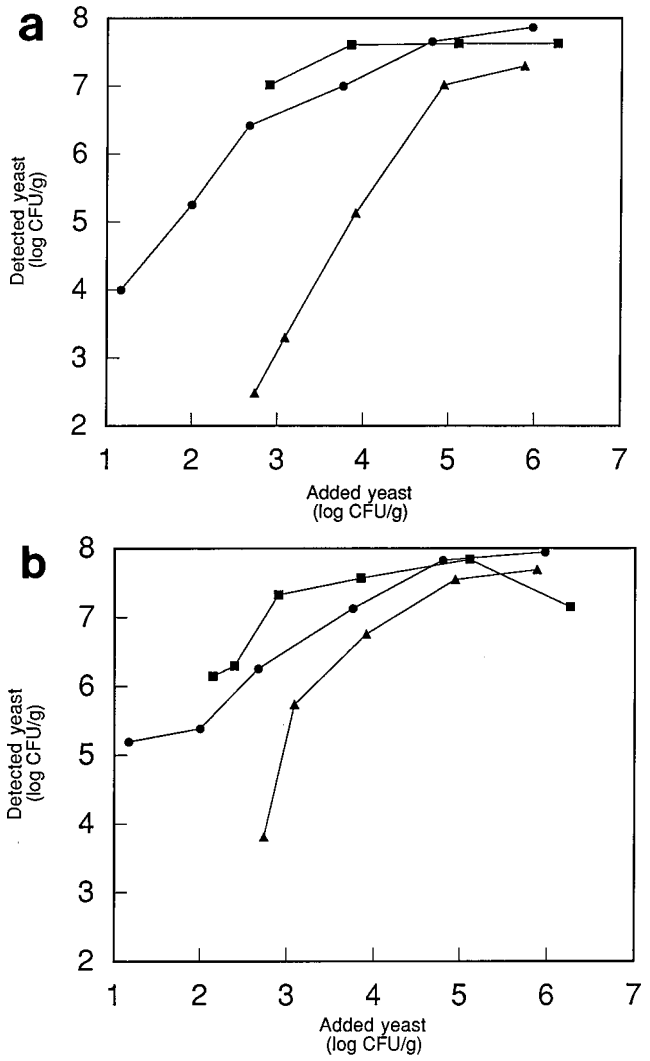

FIG. 2. Growth of yeasts *Pichia anomala* (■), *Pichia guilliermondii* (●), and *S. cerevisiae* (▲) on wheat (a_w, 0.95) challenge inoculated with *Penicillium roqueforti*. Incubation with a restricted air supply at 25°C for 7 (a) and 14 days (b). Data are given as log yeast CFU per gram of wheat ($n = 2$).

4). The optimum temperature for the formation of CFU by *Penicillium roqueforti* grown alone was 21 to 22°C. Regardless of a_w, the inhibition caused by *Pichia anomala* was more pronounced at temperatures either below or above this optimum (Fig. 3 and 4). The degree of variation in model predictions can be exemplified by the standard deviation of the predicted mean at 3 weeks, a_w of 0.96, and 10°C, which was log 4.5 ± 0.45 CFU/g for *Penicillium roqueforti* cocultured with *Pichia anomala*. The corresponding values for *Penicillium roqueforti* grown alone for 3 weeks at a_w of 0.96 and 20°C was 6.9 ± 0.13 CFU/g.

DISCUSSION

The results of this study confirm and extend our earlier findings on the ability of *Pichia anomala* to restrict mold growth and sporulation on agar plates (1). *Pichia anomala* inhibited the CFU numbers of 17 Ascomycotina, Deuteromycotina, and Zygomycotina molds. Four of these molds, *A. fumigatus*, *E. amstelodami*, *Penicillium expansum*, and *T. flavus*,

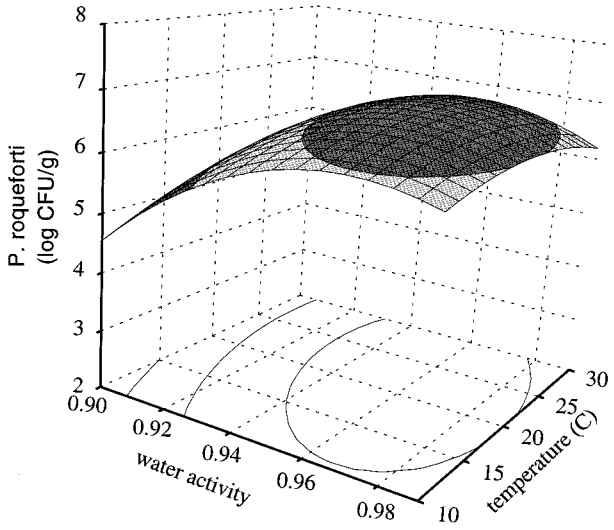

FIG. 3. Numbers of CFU of *Penicillium roqueforti* inoculated at 4×10^3 CFU/g in nonsterile wheat after 3 weeks of incubation with a restricted air supply. The a_w varied between 0.90 and 0.98, and the temperature varied between 10 and 30°C. $R^2 = 0.82$; RSD = 0.33. Experimental design and statistical analysis were done with MODDE software.
RSD = $\sqrt{}$sum of squares of residuals/degrees of freedom of residuals.

were extremely sensitive, whereas *Penicillium italicum* and *Penicillium digitatum* seemed to be comparatively resistant. These two molds are well-known spoilage fungi associated with citrus fruits. *Penicillium roqueforti*, which is of special concern for airtight-stored grains, successfully competed with 10^4 (but not 10^8) *Pichia anomala* cells per plate. One would expect a fast-growing mold to be less sensitive to the presence of yeasts. However, we saw no correlation between radial growth rate and degree of inhibition (data not shown). Also, rapidly sporu-

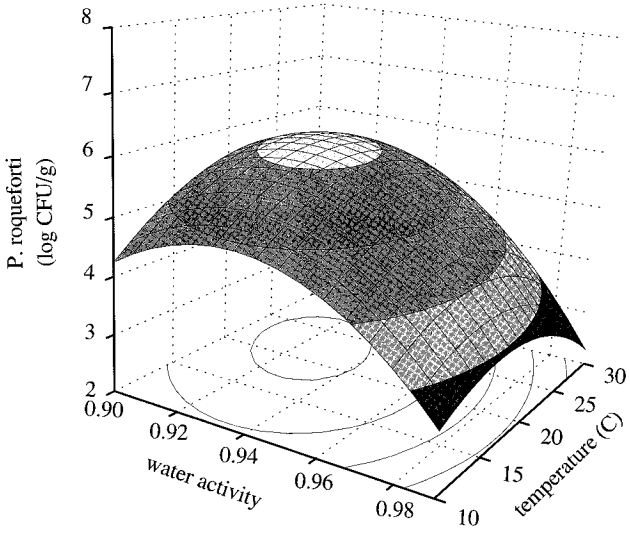

FIG. 4. Numbers of CFU of *Penicillium roqueforti* coinoculated with *Pichia anomala* in nonsterile wheat after 3 weeks. *Penicillium roqueforti* was inoculated at 4×10^3 CFU/g, and *Pichia anomala* was inoculated at 10^5 CFU/g of wheat. Wheat was incubated with a restricted air supply for 3 weeks. The a_w varied between 0.90 and 0.98, and the temperature varied between 10 and 30°C. $R^2 = 0.74$; RSD = 0.80. Experimental design and statistical analysis were done with MODDE software.
RSD = $\sqrt{}$sum of squares of residuals/degrees of freedom of residuals.

lating molds should be better able to overcome inhibition by yeasts (measured in CFU) than more slowly growing ones. Nevertheless, once again, no obvious correlation between mold sporulation and degree of inhibition by *Pichia anomala* was detected.

In this investigation, mold growth was quantified as the number of CFU formed after surface spreading on agar. This is mainly a measure of sporulation and does not give a precise indication of mycelial growth (18). However, we earlier found that the degrees of inhibition by *Pichia anomala* of *Penicillium roqueforti* and *A. candidus* were similar, regardless of whether CFU or hyphal lengths were determined (1).

The strong inhibitory effect of *Pichia anomala* on *Penicillium roqueforti* on agar plates was also evident on moist wheat kernels stored at 25°C, even though this is close to the optimum temperature for this mold (1). High inoculation levels of *Pichia guilliermondii* also restricted the numbers of CFU of *Penicillium roqueforti* on wheat kernels, but not to the same extent as *Pichia anomala*. The inhibitory effects of both *Pichia anomala* and *Pichia guilliermondii* increased with the number of yeast CFU inoculated, although after 7 days, the yeast concentrations in all tubes inoculated with more than 10^3 CFU/g of wheat were roughly similar (Fig. 2a). However, we do not know how fast the different yeasts grew to the level we measured on day 7. The number of yeast CFU may have peaked during the first days of incubation, as has been shown for *Pichia guilliermondii* on soybeans (14). Thus, the different yeast species may have attained their respective highest levels at different times. At its highest inoculation level (10^6 CFU/g), *S. cerevisiae* reduced mold CFU numbers only slightly and then only during the first week. This lack of antagonistic capacity might have been the result of slow growth of *S. cerevisiae*.

Antagonists that show promise under laboratory conditions do not necessarily perform as well in a full-scale trial (8). For example, interactions may vary, depending on substrate type or other environmental conditions (12). Used as a biocontrol agent, a microorganism has to compete with other fungi to establish an active population in the new environment. The ability to rapidly build up a population is assumed to be a prerequisite for successfully competing with spoilage microorganisms or pathogens (16). *S. cerevisiae* did not succeed in meeting this requirement. In our experiments, *Pichia anomala* outcompeted *Penicillium roqueforti* in nonsterile wheat most pronouncedly under conditions suboptimal for mold growth. At the lower a_ws tested, both the growth of yeasts and their ability to inhibit mold proliferation were poor. Biological control is probably most effective in cases in which the degree of adaptation to a specific environment is higher for the biocontrol microorganism than for the potential pathogen (2). However, one isolate of a *Trichoderma* sp. inhibited postharvest diseases for 8.5 months on carrots stored at 2°C, even though the optimal growth temperature for this antagonist is 23°C (5).

Rapid growth of yeast during the first days of interaction with mold seemed to be connected with antagonistic activity in this study. This agrees with the finding that *Pichia guilliermondii* prevented growth of *A. flavus* on soybeans better when the two were applied simultaneously than when the former was applied 3 days before the mold (14). *A. flavus* grew vigorously when inoculated 2 to 3 days after *Pichia guilliermondii*, even though living *Pichia guilliermondii* cells were still abundant. In this case, the *Pichia guilliermondii* population increased during the first 3 days, decreased up to day 5, and then remained almost constant for the rest of the incubation (14). It is possible that *A. flavus* added on day 3 used dead *Pichia guilliermondii* cells as a substrate.

The way in which an antagonist is introduced can affect both the original microbial flora and the antagonistic capacity of the introduced microorganism. Inhibition of *A. flavus* on soybeans by *Pichia guilliermondii* lasted longer when it was applied by dipping rather than by spraying (14). In our investigation, drip application in combination with mixing was used to introduce yeasts. Neither spraying nor dripping is likely to affect the original flora; however, dipping may remove pathogenic microorganisms. Additives supplied to the yeast suspension also can influence inhibition. For example, *Pichia guilliermondii* was more effective against *B. cinerea* and *Penicillium* spp. after the yeast suspension had been supplemented with 2% (wt/vol) $CaCl_2$ (13).

The nontarget microflora is also likely to be affected when an antagonist is introduced in large numbers. The growth of an antagonist might change the environment, making it more suitable for other microorganisms. At the highest a_ws tested in our experiments, *Pichia anomala* seems to have stimulated the growth of lactic acid bacteria, compared with the control experiment inoculated only with *Penicillium roqueforti* (data not shown). Thus, the inhibition of *Penicillium roqueforti* at the highest a_ws tested might be caused by a synergistic effect of *Pichia anomala* and lactic acid bacteria.

In our experimental system, little or no fungal growth was visible in the lower part of each tube. The abundant fungal growth in the upper part would have consumed most of the oxygen leaking in, resulting in high levels of carbon dioxide and low levels of oxygen. In turn, these conditions would tend to reduce growth at the bottom of the tube. Simultaneous production of carbon dioxide and consumption of oxygen by added microorganisms can influence the production of mycotoxins. Ochratoxin production from *Aspergillus ochraeus* has been shown to be totally inhibited by >30% carbon dioxide, regardless of the oxygen concentration (15). At lower levels of carbon dioxide, the inhibitory effect was clear only at oxygen concentrations of lower than 20%.

Although *Pichia anomala* was able to reduce mold growth and sporulation in cereals, we do not know whether mycotoxin production is stimulated, directly or indirectly, by the presence of this antagonistic yeast. Thus, we plan to investigate the effects of *Pichia anomala* on the production of mycotoxins by different storage molds. The effects of *Pichia anomala*-induced changes in the grain atmosphere on inhibition will also be studied.

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REFERENCES

1. **Björnberg, A., and J. Schnürer.** 1993. Inhibition of the growth of grain-storage molds in vitro by the yeast *Pichia anomala* (Hansen) Kurtzman. Can. J. Microbiol. **39:**623–628.
2. **Droby, S., E. Chalutz, L. Cohen, B. Weiss, C. Wilson, and M. Wisniewski.** 1990. Nutrient competition as a mode of action of postharvest biocontrol agents, p. 142–160. *In* Biological control of postharvest diseases of fruits and vegetables, workshop proceedings. Agricultural Research Service document ARS-92. U.S. Department of Agriculture, Washington, D.C.
3. **Droby, S., E. Chalutz, C. L. Wilson, and M. Wisniewski.** 1989. Characterization of the biocontrol activity of *Debaromyces hansenii* in the control of *Penicillium digitatum* on grapefruit. Can. J. Microbiol. **35:**794–800.
4. **Engel, G., and M. Teuber.** 1978. Simple aid for the identification of *Penicillium roqueforti* Thom. Eur. J. Appl. Microbiol. Biotechnol. **6:**107–111.
5. **Funck-Jensen, D., and M. W. Olsen.** 1990. Discussion summary: biological control in closed structures and biospheres, p. 479–483. *In* R. R. Baker and

P. E. Dunn (ed.), New directions in biological control: alternatives for suppressing agricultural pests and diseases. Alan R. Liss, Inc., New York.
6. **Häggblom, P.** 1990. Isolation of roquefortine C from feed grain. Appl. Environ. Microbiol. **56:**2924–2926.
7. **Hofstein, R., S. Droby, E. Chalutz, C. Wilson, and B. Frilender.** 1990. Scaling-up the production for application of an antagonist—from basic research to R & D, p. 197–210. *In* Biological control of postharvest diseases of fruits and vegetables, workshop proceedings. Agricultural Research Service document ARS-92. U.S. Department of Agriculture, Washington, D.C.
8. **Janisiewicz, W. J.** 1987. Postharvest biological control of blue mold on apples. Phytopathology **77:**481–485.
9. **Kreger-van Rij, N. J. W.** (ed.). 1984. The yeasts: a taxonomic study, 3rd ed. Elsevier Science Publishers B.V., Amsterdam.
10. **Kurtzman, C. P.** 1984. Synonomy of the yeast genera Hansenula and Pichia demonstrated through comparison of deoxyribonucleic acid relatedness. Antonie Leeuwenhoek **50:**209–217.
11. **Lacey, J., and N. Magan.** 1991. Fungi in cereal grains: their occurrence and water and temperature relations, p. 77–118. *In* J. Chelkowski (ed.), Cereal grain—mycotoxins, fungi and quality in drying and storage. Elsevier Science Publishers B.V., Amsterdam.
12. **Magan, N., and J. Lacey.** 1985. Interactions between field and storage fungi on wheat grain. Trans. Br. Mycol. Soc. **85:**29–37.
13. **McLaughlin, R. J.** 1990. A review and current status of research on enhancement of biological control of postharvest diseases of fruit by use of calcium salts with yeasts, p. 184–194. *In* Biological control of postharvest diseases of fruits and vegetables, workshop proceedings. Agricultural Research Service document ARS-92. U.S. Department of Agriculture, Washington, D.C.
14. **Paster, N., S. Droby, E. Chalutz, M. Menasherov, R. Nitzan, and C. L. Wilson.** 1993. Evaluation of the potential of the yeast *Pichia guilliermondii* as a biocontrol agent against *Aspergillus flavus* and fungi of stored soya beans. Mycol. Res. **10:**1201–1206.
15. **Paster, N., N. Lisker, and I. Chet.** 1983. Ochratoxin A production by *Aspergillus ochraceus* Wilhelm grown under controlled atmospheres. Appl. Environ. Microbiol. **45:**1136–1139.
16. **Paulitz, T. C.** 1990. Biochemical and ecological aspects of competition in biological control, p. 713–724. *In* R. R. Baker and P. E. Dunn (ed.), New directions in biological control: alternatives for suppressing agricultural pests and diseases. Alan R. Inc., New York.
17. **Samson, R. A., and E. S. van Reenen-Hoekstra.** 1988. Introduction to foodborne fungi, p. 299. Centraalbureau voor Schimmelcultures, Institute of the Royal Netherlands Academy of Arts and Sciences, Baarn, The Netherlands.
18. **Schnürer, J.** 1993. Comparison of methods for estimating the biomass of three food-borne fungi with different growth patterns. Appl. Environ. Microbiol. **59:**552–555.
19. **Wilson, C. L., and E. Chalutz.** 1989. Postharvest biological control of *Penicillium* rots of citrus with antagonistic yeasts and bacteria. Sci. Hortic. (Canterbury) **40:**105–112.
20. **Wisniewski, M., C. Biles, and S. Droby.** 1990. The use of the yeast *Pichia guilliermondii* as a biocontrol agent: characterization of attachment to *Botrytis cinerea*, p. 167–183. *In* Biological control of postharvest diseases of fruits and vegetables, workshop proceedings. Agricultural Research Service document ARS-92. U.S. Department of Agriculture, Washington, D.C.