

Control of Storage Decay of Apples with *Sporobolomyces roseus*

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ABSTRACT

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The biocontrol potential of an antagonist occurring naturally on pome fruit surfaces against postharvest diseases of apple (*Malus × domestica*) was investigated. Pink yeast, *Sporobolomyces roseus* (isolate FS-43-238), isolated from pear fruit reduced blue mold (*Penicillium expansum*) from 100 to 0% and gray mold (*Botrytis cinerea*) from 78 to 0% on wounded fruit drop-inoculated with suspensions containing 7.9×10^6 and 6.3×10^5 cfu/ml yeast cells, respectively, and then challenged with the pathogen at 10^4 conidia per milliliter. The reduction in the percentage of infected wounds and in average lesion diameter followed a similar pattern and was effected by the antagonist and pathogen concentrations. On wounded apples dipped in the pathogen or antagonist-pathogen suspensions and stored for 3 mo at 1 C, the incidence of rots was reduced from 33 to 0% for *P. expansum* and from 92 to 4% for *B. cinerea* at yeast concentrations of 7.9 and 5.3×10^6 cfu/ml, respectively. In spray application, where *S. roseus* was mixed with both pathogens (each at 10^4 conidia per milliliter), less than 1% of fruit developed lesions in the antagonist-pathogen treatment, compared to 15% in the control and 9% in the thiabendazole treatment, after 6 mo in storage at 1 C. Wounds were readily colonized by *S. roseus*, and the populations increased from 4.3 to 6.1 log cfu/ml in drop-application experiments after 48 hr at 18 C and from 4.1 to 6.4 log cfu/ml in spray-application experiments after 3 mo at 1 C. In addition to its effectiveness at relatively low concentrations, *S. roseus* shows promise for commercial development because it is ubiquitous in nature, occurs commonly on fruits, and does not grow at 36 C.

Additional keywords: postharvest biocontrol

Biocontrol of postharvest diseases of fruits has become a promising and attractive alternative method to synthetic fungicides during the last decade (17,18,36). This comes at a time when the problems with pesticide use, such as declining effectiveness and hazards to human health and the environment, are more evident and are widely discussed in professional and public forums (3,6,27,33).

Research on the biocontrol of postharvest fruit diseases was invigorated by the findings that *Bacillus subtilis* (Ehrenberg) Cohn isolated from the soil strongly inhibited the incidence of brown rot caused by *Monilinia fructicola* (G. Wint.) Honey on stone fruits (29,30). Further progress was made when bacterial and yeast antagonists from natural microflora on apple (*Malus × domestica*

Borkh.) were shown to control blue mold caused by *Penicillium expansum* Link and gray mold caused by *Botrytis cinerea* Pers.:Fr. on pome fruits (15,16,20,21,31). Subsequently, natural antagonists against various postharvest diseases were used on citrus, stone fruits, grapes, and other fruits (2,26,35). In a few countries, private industry is testing the commercial potential of some of these biological agents.

While screening microorganisms isolated from pome fruits for antagonism against postharvest diseases of pome fruits, we found that a pink yeast, *Sporobolomyces roseus* Kluver & Niel, isolated from pear fruit strongly inhibit blue mold and gray mold on apple and pear (19). *Sporobolomyces* spp. occur commonly in nature in the temperate zone and can be isolated frequently from plant surfaces (8), the atmosphere (14), and seawater (24). *S. roseus* has been reported to be a component of resident microflora on many plants and plant parts including apple flowers; mature, ripe, and overripe apples; mature grapes; and soft fruits such as raspberries and

blackberries (5,7). Occurrence of this yeast frequently increases toward the end of the growing season and is probably associated with the aging of the plant tissue and the incidental increase of nutrients on plant surfaces (5,23). The great colonizing potential of plant surfaces by *S. roseus* was demonstrated in elegant research on wheat leaves (9-12). The yeast effectively removes aphid honeydew from the leaves, which decreases the food base of the pathogen *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur and reduces disease (12). It has been shown that antagonism between *S. roseus* and other necrotrophic pathogens is based primarily on nutrient competition (1,10). Biocontrol potential of *S. roseus* has also been demonstrated against *Septoria nodorum* Berk. on wheat leaves (12), *Cladosporium herbarum* (Pers.:Fr.) Link on larch (25), *Cladosporium cladosporioides* (Fresen.) De Vries on antirrhinum leaves (4), and *Alternaria porri* (Ellis) Cif. on onion leaves (34). The objective of this research was to investigate the biocontrol potential of *S. roseus* against two major postharvest pathogens of pome fruits, *P. expansum* and *B. cinerea*.

MATERIALS AND METHODS

Pathogens. *P. expansum* and *B. cinerea* were isolated from decayed apples after several months in storage. These isolates were the most aggressive ones in our collection and produced the largest lesions on inoculated apples. The fungi were maintained on potato-dextrose agar (PDA) with periodic transfers through apple. The inocula consisted of aqueous conidial suspensions of 10^3 , 10^4 , or 10^5 conidia per milliliter for the drop-inoculation experiment. In the spray- and dip-application experiments, concentrated stock suspensions were added to achieve a final concentration of 10^4 conidia per milliliter. The suspensions were prepared from 10- and 14-day-old cultures of *P. expansum* and *B. cinerea*, respectively, as previously described (16).

Antagonist. The antagonistic yeast, isolate FS-43-238, was isolated from a pear surface according to a previously

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described procedure (15). The organism was identified as *Sporobolomyces roseus* Kluyver & Niel by Centraalbureau Voon Schimmelcultures, Netherlands. The yeast was maintained on nutrient yeast dextrose agar (NYDA) at 5 C.

For the drop-inoculation experiment, yeast cells were obtained from cultures grown in 250-ml Erlenmeyer flasks containing 50 ml of nutrient yeast dextrose broth (NYDB) on a rotary shaker at 150 rpm for 36 hr at 24 C. Cells were harvested by centrifugation at 7,000 rpm for 10 min. The pellet was resuspended in water, and the cell concentrations were adjusted to 0.8, 1.7, 2.8, 6.3, or 79×10^5 cfu/ml with a spectrophotometer according to a standard curve.

For the spray-application experiment, the yeast was grown as previously described, except that 500-ml Erlenmeyer flasks with 100 ml of NYDB medium were used. After centrifugation of 1.5 L of the culture, the pellet was resuspended in 300 ml of water and used to make 1 L of an aqueous stock suspension of the antagonist. Appropriate volumes of the stock suspension were added to a 12-L container on a modified fruit packing line to make 12 L of the antagonist suspension at 7.9×10^6 cfu/ml. Concentrated stock suspensions of both *P. expansum* and *B. cinerea* were added to the 12-L container to achieve a final concentration of 1×10^4 conidia per milliliter of each pathogen. The antioxidant diphenylamine (DPA) (15%) was also added to this container to achieve a final concentration of 0.2%.

For dip application, the yeast suspensions were prepared as for the spray application, except that the concentrations were 2.6, 5.3, or 7.9×10^6 cfu/ml, and separate applications were made with *P. expansum* and *B. cinerea*.

Fruit. Golden Delicious apples were obtained from commercial orchards in Kearneysville, West Virginia, that used standard cultural practices. Fruit used for the drop or dip experiments were held in storage at 1 C. Those for the spray-application experiments were used immediately after harvest. At the time of the drop-, spray-, and dip-application experiments, apple firmnesses were 66.7, 72.1, and 64.9 N; soluble solids concentrations were 11.0, 10.4, and 11.5%; and maturity stages according to the starch-iodine test were 7, 7, and 6 on a 1-9 scale, respectively (28).

Drop inoculation. Immediately before treatment, fruit were wounded with a sharp instrument and two blocks of tissue $3 \times 3 \times 3$ mm, 2 cm apart, were removed along the stem-calyx axis. The fruit were placed on fruit trays in plastic containers, and the wounds were inoculated with 25 μ l of antagonist suspension containing 0, 0.8, 1.7, 2.8, 6.3, or 79×10^5 cfu/ml. Within 30 min, the wounds of the fruit treated with each concentration of the antagonist were inoculated with 20

μ l of 1×10^3 , 1×10^4 , or 1×10^5 conidia per milliliter suspension of *P. expansum* or *B. cinerea*. Inoculated apples were stored at 22 C for 7 days. The diameters

of the lesions developing from the wounds were measured perpendicular to the stem-calyx axis. There were three replications of three fruit (six wounds)

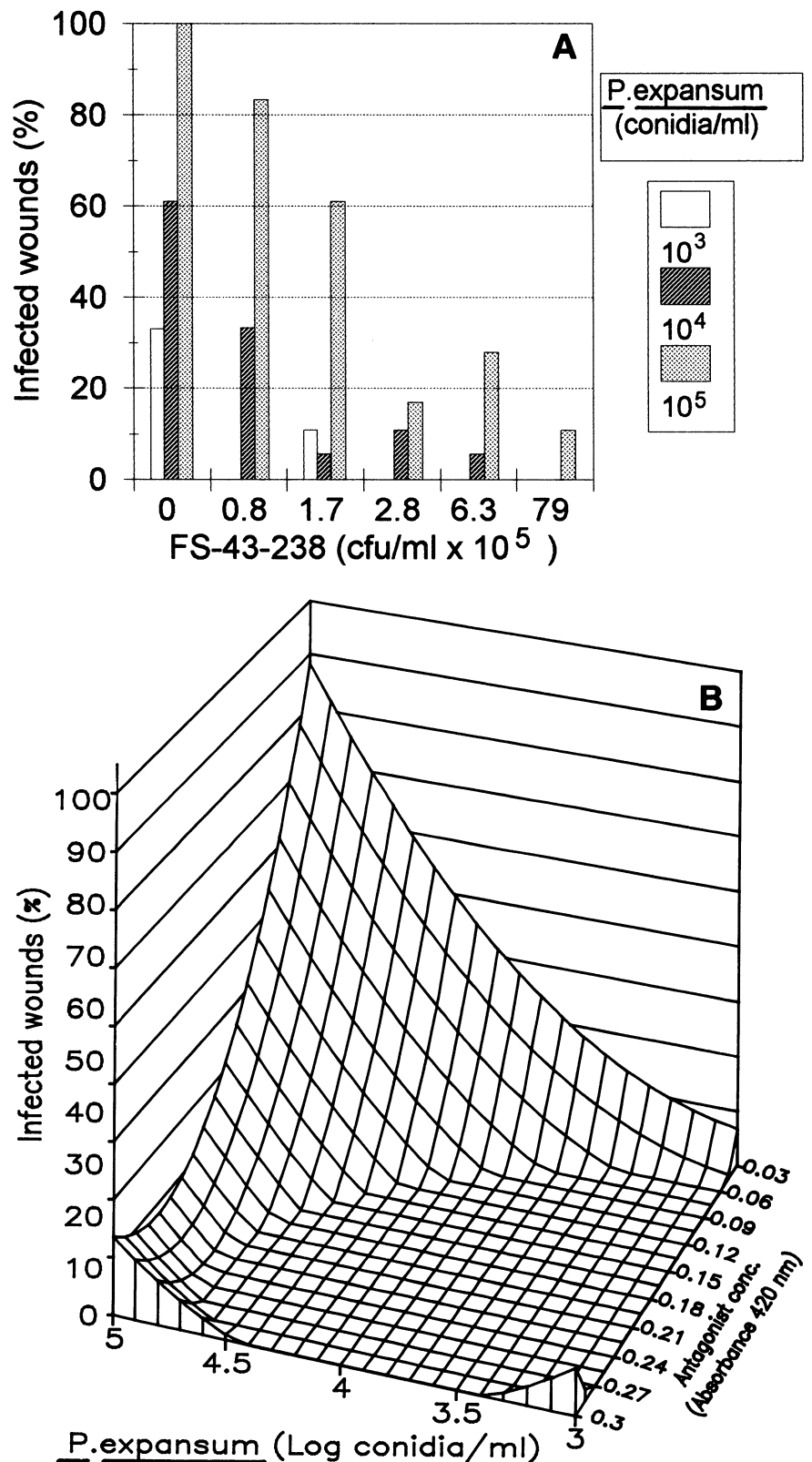


Fig. 1. Effect of the antagonist *Sporobolomyces roseus* (isolate FS-43-238) on (A) observed and (B) predicted percentage of wounds infected with blue mold on Golden Delicious apple. Fruit were wounded, drop-inoculated with various concentrations of *S. roseus*, challenged with various concentrations of conidia of *Penicillium expansum*, and incubated for 7 days at 22 C. $Y = 1.404 - 0.888 \times concp - 1.622 \times conca - 1.360 \times concp \times conca + 17.434 \times conca \times conca + 0.166 \times concp \times concp$, $R^2 = 0.92$, where *concp* = concentration of *P. expansum*, *conca* = concentration of *S. roseus*. Absorbance 0.3 = 7.9×10^6 cfu/ml.

per treatment. Treatments were arranged in a completely randomized block design with each block consisting of three plastic containers of fruit inoculated with the

six concentrations of antagonist and three concentrations of a pathogen in all possible combinations. Prior to this experiment, similar tests were conducted

in which fruit were treated with various concentrations of the antagonist plus one concentration each of the pathogen, with similar results.

Spray-application test. Golden Delicious apples were wounded with two 16-penny nails (4 mm diameter) placed 2 cm apart and protruding 3 mm from a wooden block. Two wounds were made on each apple midway along the calyx-stem end axis. The wounded apples were treated on a modified packing line with a roller conveyer and a spray box with six nozzles arranged in a two-row staggered pattern which covered all fruit with spray. A container below the spray box was equipped with a recirculating centrifugal submersible pump which pumped the suspension back to the nozzles. The application flow rate was 1.3 L/min/nozzle, and the speed of the conveyer was approximately 3 m/min. The treatments were mixtures of: 1) antagonist (FS-43-238) + pathogens + DPA, 2) pathogens + DPA, or 3) pathogens + DPA + thiabendazole at 0.03% a.i. (Mertect 340-F, 42.28%). After treatment application, the fruit were put into polyethylene liners and placed in 1-bu boxes. There were 20 fruit per liner and three liners for three removal dates per box. Each liner contained a replicate, and there were five replicates per treatment. The boxes were placed in 1 C storage in a completely randomized block design. The fruit samples were removed from storage at 3-mo intervals, and the percentage of wounds that developed rots was determined. The experiment was repeated once.

Dip-application test. Golden Delicious apples were wounded as in the spray-application test and dipped for 2 min in a 15-L container with a 10-L suspension of *P. expansum* or *B. cinerea* at 1×10^4 conidia per milliliter and the antagonist at 0, 2.6, 5.3, or 7.9×10^6 cfu/ml. Treated fruit were placed on fruit tray packs and put into 1-bu boxes with polyethylene liners. Fruit were stored at 1 C for 3 mo, and the percentage of wounds that developed rots was then determined. There were 15 fruit per replicate, and each treatment was replicated three times. Treatments were arranged in a randomized complete-block design with each box consisting of a single block. Tests with *P. expansum* and *B. cinerea* were conducted at different times and treated as separate experiments. The experiments were repeated once.

Survival of the antagonist. In the recovery tests, yeast was applied to fruit at the same time as for biocontrol tests, and the fruit were handled identically. Golden Delicious apples were wounded as described in the drop-inoculation tests, but only one wound was made per fruit. Each wound was inoculated with 25 μ l of an aqueous suspension of the antagonist at a concentration of 7.9×10^6 cfu/ml. The fruit were placed on fruit

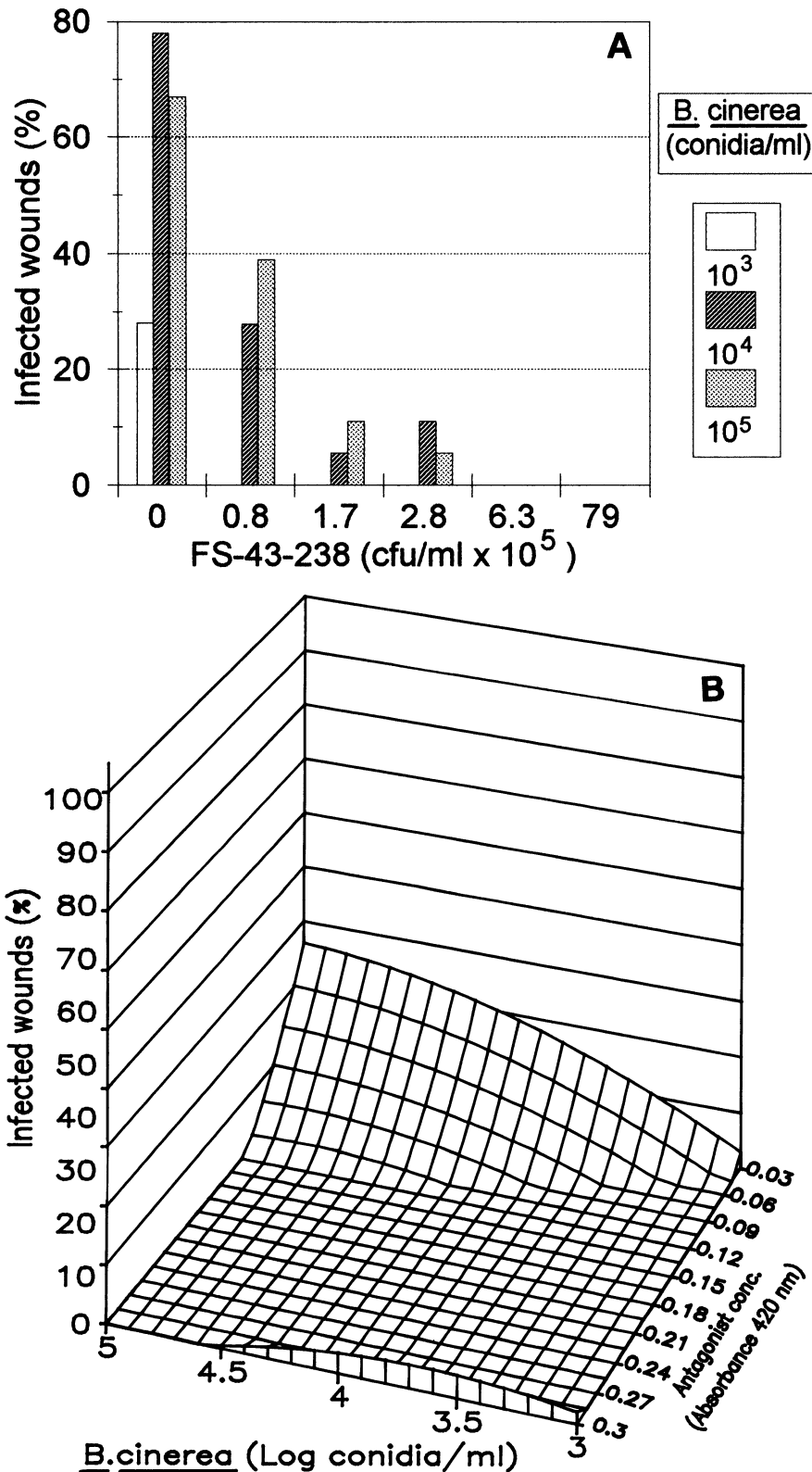


Fig. 2. Effect of the antagonist *Sporobolomyces roseus* (isolate FS-43-238) on (A) observed and (B) predicted percentage of wounds infected with gray mold on Golden Delicious apple. Fruit were wounded, drop-inoculated with various concentrations of *S. roseus*, challenged with various concentrations of conidia of *Botrytis cinerea*, and incubated for 7 days at 22 C. $Y = -1.088 + 0.566 \times concp - 2.069 \times conca - 0.616 \times concp \times conca + 11.766 \times conca \times conca - 0.052 \times concp \times concp$, $R^2 = 0.95$, where *concp* = concentration of *B. cinerea*, *conca* = concentration of *S. roseus*. Absorbance 0.3 = 7.9×10^6 cfu/ml.

trays in plastic boxes and incubated at 18 or 1 C. Fruit samples were withdrawn periodically; and the wounded areas were removed, ground in 0.05 M phosphate buffer (13), and plated on NYDA medium with a spiroplater. The inoculated culture dishes were incubated at 24 C for 48 hr, and the colonies were counted. There were three single-fruit replicates per sampling date. A similar recovery procedure was used when yeast was applied by spray on the packing line. Fruit were wounded with nails and inoculated with 7.9×10^6 cfu/ml of the yeast. NYDA medium was amended with streptomycin at 25 mg/L to inhibit bacterial contaminants. In the spray application there were five single-fruit replicates per recovery date.

Data analysis. Analysis of variance was performed on data from rot development with the General Linear Model (GLM) procedure of Statistical Analysis System (SAS) based on type III sums of squares of balanced linear model and completely randomized block design (32). Means from dip and spray applications and antagonist recovery experiments were separated by LSD at $P = 0.05$.

RESULTS

S. roseus reduced or completely controlled rot development at wounds on Golden Delicious fruit drop-inoculated with *P. expansum* (Fig. 1) or *B. cinerea* (Fig. 2). Lesion diameter ($P = 0.0009$) and percentage of wounds infected ($P = 0.0001$) with *P. expansum* were reduced by increasing concentrations of the antagonist. A similar effect was observed for *B. cinerea* ($P = 0.0623$ and $P = 0.109$ for lesion diameter and percentage of wounds infected, respectively). The concentration of the pathogen affected lesion diameter ($P = 0.0001$) and percentage of wounds infected ($P = 0.0036$) on apples inoculated with *P. expansum* or *B. cinerea* (both significant at $P = 0.0001$). No lesions developed on Golden Delicious apples protected with *S. roseus* at 7.9×10^6 cfu/ml and inoculated with *P. expansum* at 10^3 or 10^4 conidia per milliliter (Fig. 1), or with *B. cinerea* at 10^3 , 10^4 , or 10^5 conidia per milliliter (Fig. 2). Complete control of *B. cinerea* was achieved on fruit protected by the antagonist at 6.3×10^5 cfu/ml and challenged with any of the three concentrations of the pathogen.

The greatest reduction in the percentage of wounds infected in the dip experiment was achieved by the two highest concentrations of the antagonist on apples inoculated with *P. expansum* or *B. cinerea* (Fig. 3). The two high concentrations of the antagonist were equally ($P = 0.05$) effective against both pathogens.

In the spray-application experiment, the yeast reduced the percentage of wounds infected on fruit removed from

storage after 3 and 6 mo (Table 1). There was no difference between the antagonist and fungicide treatments on fruit removed after 3 mo; however, fungicide-treated fruit had a higher incidence of rots on fruit stored 6 mo.

Populations of *S. roseus* in drop-inoculated wounds of Golden Delicious apples increased from 2.3×10^4 to 1.9×10^6 cfu per wound within the first 48 hr after application on fruit stored at 18 C (Fig. 4). The populations then declined but were still greater after 19 days than at the beginning of the experiment. On fruit stored at 1 C, populations increased by one order of magnitude after 13 days in storage and stabilized at this level over the next 22 days. Populations of the antagonist in wounds on sprayed apples increased from 1.3×10^4 cfu per wound to 2.3×10^6 cfu per wound after 3 mo in storage at 1 C.

DISCUSSION

Control of blue mold and gray mold with *S. roseus* was consistent in all experiments with various methods of application. This contrasts with earlier reports on control of leaf diseases with *S. roseus*, where the control was often impressive but not consistent or adequate for commercial development (9–11). Major limitations were the low availability of nutrients on the leaf surface and fluctuating environmental conditions (11). Environment was not a problem in our system, where the storage conditions were stable. Wounds, the major points of entry for *P. expansum* and *B. cinerea*, were rich in nutrients and readily colonized by *S. roseus*. In our early work with bacterial antagonists, we showed that the outcome of biocontrol of *P. expansum* on apples is determined during the first 48 hr under ambient conditions, and that rapid wound colonization during this period is critical, especially for

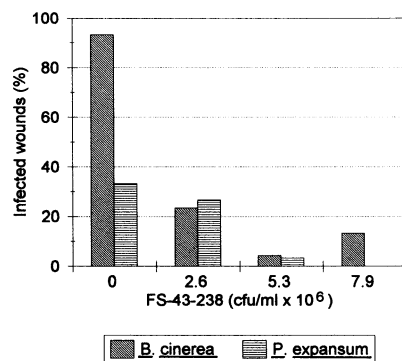


Fig. 3. Effect of the antagonist *Sporobolomyces roseus* (isolate FS-43-238) on the percentage of wounds infected on Golden Delicious apple. Fruit were wounded, dipped in a container with suspensions containing various concentrations of the antagonist and *Penicillium expansum* or *Botrytis cinerea* at 1×10^4 conidia per milliliter, and stored for 30 days at 1 C. *P. expansum* LSD (0.05) = 9.69, *B. cinerea* LSD (0.05) = 9.38.

non-antibiotic-producing biocontrol agents (22). In the drop-inoculation experiment at 18 C, during the first 48 hr after treatment, populations of *S. roseus* increased by almost two orders of magnitude. Similar increases occurred in spray treatments after 3 mo in storage at 1 C. It appears that in both instances, the colonization limit was reached and is similar on both types of wounds. Consistent control after various methods of application of *P. expansum* and *B. cinerea* with *S. roseus* at the concentration of 7.9×10^6 cfu/ml proved the reliability of this biocontrol agent. The mechanism of biocontrol has not been fully determined, but it appears that nutrient competition plays a major role in antagonism against both pathogens. Other significant attributes of *S. roseus* as a biocontrol agent of postharvest diseases are: 1) ability to grow at high relative humidity without free water (1); 2) resistance to shear forces of pumps and nozzles during spray application (W. J. Janisiewicz and D. L. Peterson, unpublished); and 3) compatibility with diphenylamine, an antioxidant used for control of superficial scald, a physiological disorder. *S. roseus* does not grow at 36 C, which is

Table 1. Effect of the antagonist *Sporobolomyces roseus* (isolate FS-43-238) and thiabendazole (TBZ) on the percentage of wounds infected on Golden Delicious apple

Treatment ^a	Infected wounds (%)	
	3 mo	6 mo
Control	15.0	15.0
<i>S. roseus</i>	0.5	0.0
TBZ	1.5	8.5
LSD (0.05)	5.9	7.4

^a Fruit were wounded, spray-inoculated on the sorting line with suspension containing either mixture of *Penicillium expansum*, *Botrytis cinerea* (each at 1×10^4 conidia/ml), and diphenylamine (0.2%) alone (control) or in combination with the antagonist or with TBZ at 0.03% a.i., and stored at 1 C. Fruit samples were removed for evaluation after 3 and 6 mo storage.

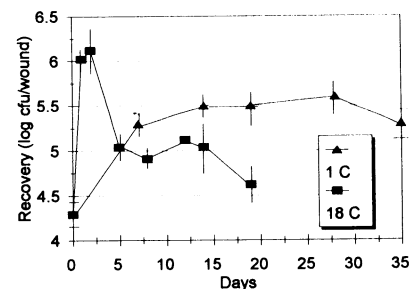


Fig. 4. Recovery of *Sporobolomyces roseus* (isolate FS-43-238) from wounds of Golden Delicious apple drop-inoculated with the antagonist and incubated at 1 or 18 C for up to 19 and 35 days, respectively. The wounds were removed with a cork borer, ground in phosphate buffer, plated on nutrient yeast dextrose agar, and incubated for 48 hr at 22 C. Bars indicate standard error of the means.

below normal body temperature (W. J. Janisiewicz, unpublished). Since it is also ubiquitous in nature and a major component of the phyllosphere and fruit microflora (5,7), once safety testing is complete, it is more likely to be accepted by the public and regulatory agencies for direct application to commodities like fruits and vegetables.

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