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Development of a Microbial Community of Bacterial and Yeast Antagonists To Control Wound-Invading Postharvest Pathogens of Fruits

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Two antagonists, the bacterium *Pseudomonas syringae* and the pink yeast *Sporobolomyces roseus*, against blue mold (caused by *Penicillium expansum*) on apple controlled this disease more effectively when combined at approximately equal biomass (50:50 of the same turbidity) than in individual applications. Addition of L-asparagine enhanced the biocontrol effectiveness of *P. syringae* but decreased that of *S. roseus* and had no significant effect when the antagonists were combined. Populations of both antagonists increased in apple wounds and were further stimulated by the addition of L-asparagine. The carrying capacity of wounds for *P. syringae* was not affected by *S. roseus*. Populations of *P. syringae* in wounds inoculated individually or in a 50:50 mixture with *S. roseus* reached the same level after 3 days at 22°C. However, populations of *S. roseus* recovered after applications of the mixture were consistently lower than those recovered after individual applications. Similar effects were observed in in vitro tests in which populations of *S. roseus* grown in mixtures with *P. syringae* were consistently lower than those grown alone, while the populations of *P. syringae* were not affected by the presence of *S. roseus*. A total of 36 carbon and 35 nitrogen compounds were tested for utilization by both antagonists. Fourteen nitrogenous compounds were utilized by both *P. syringae* and *S. roseus*, and an additional nine compounds were utilized by *P. syringae*. *S. roseus* and *P. syringae* utilized 17 and 13 carbon sources, respectively; 9 sources were common to both antagonists. Populations of these antagonists in apple wounds appear to form a relatively stable community dominated by *P. syringae*. This domination is probably due to the greater ability of this organism to utilize nitrogen sources, which is the limiting growth factor in carbon-rich apple wounds.

Limitations of synthetic fungicides for the control of plant diseases have become apparent in recent years, and alternative methods of control are urgently needed (3, 21). This need is particularly important for postharvest fungicides, because only a few chemicals are currently registered and the future use of some of these is questionable owing to declining effectiveness or problems with registration under new governmental regulations (17, 21). Recent progress in biological control of postharvest diseases of fruits and vegetables has generated increasing interest in exploring the commercial potential of this technology. Biocontrol of postharvest diseases of pome fruits shows particular promise, since major diseases, such as blue mold caused by *Penicillium expansum* and gray mold caused by *Botrytis cinerea*, can be effectively controlled (7–13, 19). In any biocontrol system, once biocontrol agents have been identified and extensively tested, work should be directed toward improvement of biocontrol. Primary approaches to accomplish this task involve manipulation of the physical, chemical, or biological environment to the antagonist's advantage; manipulation of culture conditions during growth (production) of the antagonist; and improvement of the antagonist strains. Conditions (temperature, humidity, and atmosphere) of fruit storages are stable and amenable to manipulation; however, their parameters are designed to maintain a high quality of fruit during storage and should not be changed to accommodate the antagonists. Thus, from the beginning, antagonists should be selected to be compatible with storage conditions. However, the remaining factors can be manipulated. In biocontrol studies of blue mold on apple, the addition of nutrients such as L-asparagine or L-proline and the nutrient analog 2-deoxy-D-glucose favored the antagonist and enhanced biocontrol (8, 10, 13). Also, the addition of calcium chloride enhanced biocontrol of gray mold on grapes and blue mold on apples after harvest (15, 16). The role of calcium chloride in these studies was not explained, but calcium has been implicated in resistance of fruits and vegetables to various postharvest diseases (2).

Generally, biological control agents have a relatively narrow spectrum of activity compared with fungicides. Initially, mixtures of mutually compatible antagonists were used to broaden the spectrum of diseases controlled on apples after harvest (7). Subsequently, antagonists with a broader spectrum of activity, capable of controlling multiple diseases, have been isolated (9, 11, 12). In biocontrol enhancement studies, we observed that carbohydrates were not the limiting factor in colonization of wounds by the antagonist *Pseudomonas syringae* but that the addition of nitrogenous compounds increased the population of the antagonist, often by more than 1 order of magnitude (13). This suggests that the carrying capacity of the wounds could be greater than the population of the single antagonist would indicate and that addition of another antagonist may further increase the population of the antagonists in the wounds and thus increase biocontrol. The main objective of this research was to explore the possibility of enhancing the

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biocontrol of blue mold of apple by developing a microbial community in apple wounds from antagonists effective against *Penicillium expansum.*

MATERIALS AND METHODS

Pathogen. *Penicillium expansum* Link isolate MD-8 was isolated from decayed apple and maintained on potato dextrose agar with periodic transfer through apple. Conidial suspensions were prepared from a 10-day-old culture grown on potato dextrose agar as described previously (9).

Antagonists. The pink yeast *Sporobolomyces roseus* Kluyver and van Neil (isolate FS-43-238) was isolated from pear fruit in Kearneysville, W.Va. (11). The bacterium, a nonpathogenic strain of *P. syringae* van Hall (isolate L-59-66), was isolated from apple leaves (9). This bacterium has been frequently isolated from pome fruits. Both antagonists were maintained on nutrient yeast dextrose agar (NYDA) slants. For biocontrol experiments, the cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of nutrient yeast dextrose broth (NYDB) on a rotary shaker (150 rpm) at 22°C. After 24 h, the culture was subcultured to the same medium and grown under the same conditions overnight. Then the cultures were harvested by centrifugation and resuspended in water or an 80 mM solution of L-asparagine, and the concentrations were adjusted to optical densities (ODs) of 0.02, 0.05, 0.1, and 0.3 (equivalent to 95, 90, 80, and 50% transmittance at 420 nm, respectively). Use of concentrations of both antagonists at the same optical densities gave much closer equivalents of biomass than did use of equal CFU per milliliter because of the differences in size between bacteria and yeasts (6). For biocontrol factorial experiments, suspensions of the yeast and bacterium at each concentration were mixed in proportions from 0 to 100% in 20% increments and in a proportion of 50:50.

In the biocontrol experiments with L-asparagine, concentrations of the antagonists were adjusted to an OD of 0.3, and bacterium (L-59-66)/yeast (FS-43-238) proportions in the mixtures were 0:100, 50:50, and 100:0, respectively.

Fruit. Golden Delicious apples were harvested from a commercial orchard in Kearneysville, W.Va., where they had been grown under standard management practices, and stored in regular storage at 1 ± 1°C. For the factorial experiments, the fruit maturity was stage 9 in the iodine test on a scale of 1 to 9 (18); flesh firmness, 40 N (9.0 lb); and soluble solids, 11.3%. For the experiments with L-asparagine as an additive, the fruit maturity was stage 9 in the iodine test; flesh firmness, 35.6 N (8.0 lb); and soluble solids, 10.2%.

Biocontrol tests. In the factorial experiments, Golden Delicious apples were wounded midway between the calyx and stem end axis (a block of tissue measuring 3 by 3 by 3 mm was removed) and the wounds were inoculated with 25 μl of the antagonist suspension prepared at various proportions as described above. Within 1 h, the wounds were challenged with 20 μl of *P. expansum* conidial suspension (10^4 conidia per ml). The fruit were placed on tray packs in plastic boxes and incubated at 22°C. The lesion diameter was measured after 7 days. The fruit were arranged in a randomized block design. There were three fruit per replicate and three replicates per treatment. The experiment was repeated.

In the experiments with L-asparagine, two wounds were made on each fruit 2 cm apart along the calyx stem end axis. The wounds were inoculated with the antagonists alone or in an equal mixture (50:50) and challenged with 2.5×10^4 conidia of *Penicillium expansum* per ml as described above. The fruit were arranged in a randomized block design. There were nine fruit per replicate and three replicates per treatment. The experiment was repeated.

Recovery of the antagonists. Apples were wounded as described above with one wound per fruit. The wounds were inoculated with 25 μl of suspension of each antagonist separately or in an equal mixture (50:50). The fruit were placed on tray packs in plastic boxes and incubated at 22°C. Samples of the fruit were removed at daily intervals starting 1 h after application (day 0). The wounded area was removed with a no. 5 cork borer, ground in 0.05 M phosphate buffer (pH 6.8) (3a), and plated on NYDA with a spiral plater as previously described (8). Samples from wounds inoculated with the mixture of the antagonists were plated in duplicate, one on regular NYDA for recovery of the bacterial antagonist and the second on NYDA supplemented with streptomycin sulfate at 25 mg/liter, which inhibited the bacterium but allowed the yeast to grow. The colonies were counted with a laser counter after incubation for 24 and 48 h for the bacterial and yeast cells, respectively. In samples from wounds inoculated with the mixture of antagonists, the 24-h incubation period enabled bacteria to be counted before the yeasts became visible. The fruit were arranged in a completely randomized block design. There were three single fruit replicates per treatment. The experiment was repeated. In the experiments with L-asparagine, there were six single fruit replicates per treatment.

Mixed-culture test in vitro. Cultures of *S. roseus* and *P. syringae* were grown in NYDB overnight. The cultures were harvested by centrifugation and resuspended in water to an OD of 0.125; then 0.2-, 0.4-, 0.6-, 0.8-, and 1.0-ml samples of these suspensions were added individually to 125-ml Erlenmeyer flasks containing 50 ml of NYDB or NYDB with L-asparagine (80 mM). Another set of flasks was inoculated with both organisms in the proportions 0.2:0.8, 0.4:0.6, 0.6:0.4, and 0.8:0.2 per flask (total volume of organisms, 1 ml). The flasks were incubated on the shaker at 150 rpm and 24°C for 36 h. Samples from the flasks were taken at 12-h intervals and were plated with a spiral plater on NYDA or NYDA supplemented with 25 mg of streptomycin per liter for recovering bacterial and yeast cells, respectively. Samples from flasks inoculated with the mixture of the antagonists were plated in duplicate on NYDA and NYDA plus streptomycin. The colonies were counted with a laser counter after incubation for 24 and 48 h for the bacterium and yeast, respectively. The experiment was conducted twice.

Nutritional characteristics. Minimum salt media with no carbon or nitrogen source were used to test the utilization of various nitrogen and carbon sources by the antagonists. The medium used for the yeast contained the following (per liter): K_2HPO_4, 0.87 g; KH_2PO_4, 6.94 g; $MgSO_4$, 2.2 g; NaCl, 0.1 g; $FeSO_4 \cdot 7H_2O$, 1.83 mg; $ZnSO_4 \cdot 7H_2O$, 0.187 mg; $CuSO_4 \cdot 5H_2O$, 0.156 mg; H_3BO_3, 0.04 mg; $MnSO_4$, 0.022 mg; $MoO_3 \cdot H_2O$, 0.1 mg; and vitamins solution as described by van der Walt and van Kerken (22). The medium used for bacterium contained (per liter) 2.78 g of Na_2HPO_4, 2.77 g of KH_2PO_4, 20 ml of trace elements (Hutner's vitamin-free mineral base [4]), 0.01 mg of biotin, 1.0 mg of nicotinic acid, and 0.5 mg of thiamine hydrochloride, plus tetrazolium chloride at 50 mg/liter as a color indicator for oxidation of tested substrates. Utilization of various carbon and nitrogen sources was determined with microtiter plates as described previously (13). The antagonists were suspended in the minimum salt basal media supplemented with NH_4Cl (1 g/liter for the bacterium and 4 g/liter for the yeast) or glucose (3 g/liter for the bacterium and 10 g/liter for the yeast), and individual carbon or nitrogen sources were added, respectively. Growth of the yeast was determined by measuring the increase in turbidity at 405 nm after a 72-h incubation at 26°C, and growth of the bacterium was determined by measuring the increase in turbidity at 590 nm after a 48-h incubation at 26°C.

Data analysis. The model for response surfaces of the effect of the ratio of the antagonists in the mixture on lesion size and percentage of wounds infected was based on estimates of parameters from the general linear models of the Statistical Analysis System, based on type III sums of squares for the balanced linear model and randomized block design (20). Means of lesion diameter and percentage of wounds infected at various antagonist concentrations were separated by using the least significant difference test. The general linear models procedures were also used for analysis of data from experiments which included addition of L-asparagine. The analysis of disease incidence (percentage of wounds infected) was performed on arcsine-transformed data. A Waller-Duncan multiple-range test was performed for separation of means of lesion diameters and percentage of wounds infected on fruit treated with various ratios of the antagonists. The Fisher protected *t* test was used to compare effects of L-asparagine on lesion development. Analysis of covariance was used to determine the homogeneity of slopes over time for each treatment (proportions of antagonists and medium type) in in vitro tests, and the specification of a contrast was used to show differences between antagonists alone and in mixtures.

RESULTS

Biocontrol tests. The ratio of the antagonists significantly affected the severity (lesion size) ($P = 0.0001$) and incidence (percentage of wounds infected) ($P = 0.0039$) of blue mold. The antagonists in the mixture were more effective than either antagonist alone in reducing the severity and incidence of blue mold ($P = 0.05$). The concentration of the antagonists also significantly affected the severity ($P = 0.0005$) and incidence ($P = 0.0338$) of blue mold. As the concentrations of the antagonists increased, the severity and incidence of the rots decreased ($P = 0.05$). The response surfaces in Fig. 1 and 2 indicate the effect of the ratio and concentration of the antagonists on lesion development and percentage of wounds infected.

In experiments with L-asparagine, the ratio of the antagonists significantly affected the severity ($P = 0.0001$) and incidence ($P = 0.0001$) of rots. A 50:50 mixture of antagonists was more effective than the single antagonists in reducing rot severity ($P = 0.05$) and incidence ($P = 0.05$) at both concentrations of the pathogen.

Interaction between antagonist ratio and L-asparagine had a significant effect on rot severity ($P = 0.0001$) and incidence ($P = 0.0001$). L-Asparagine enhanced biocontrol by *P. syringae*, resulting in further reduction in rot severity and incidence on fruit infected at both concentrations of *Penicillium expansum* ($P = 0.05$) (Fig. 3). However, addition of L-asparagine diminished the effectiveness of biocontrol on fruit protected by *S. roseus*, resulting in greater rot severity and incidence than those produced by treatment without L-asparagine ($P = 0.05$).

Populations dynamics. Populations of *P. syringae* and *S. roseus* applied separately increased more than 10-fold 72 h after application and then began to decline (Fig. 4). Popula-

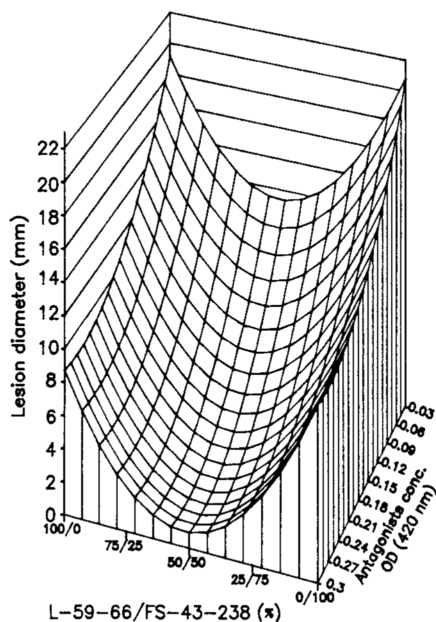

FIG. 1. Effect of the ratio of the antagonists (*P. syringae* L-59-66 and *S. roseus* FS-43-238) in the mixture and antagonist concentration on the predicted severity of blue mold (lesion diameter) on Golden Delicious apples. The two antagonists were mixed at various proportions at each concentration (equal OD value). Fruits were wounded, inoculated with the antagonist mixture, challenged with 10^4 conidia of *Penicillium expansum* per ml, and incubated for 7 days at 22°C. The response surface was generated from the equation $Y = 20.8583 - 88.6187C - 5.163Rt + 0.0406CRt + 183.7213C^2 + 0.7029Rt^2$ with $R^2 = 0.71$, where Y is the lesion diameter, C is the concentration of the antagonists, and Rt is the ratio of the antagonists in the mixture.

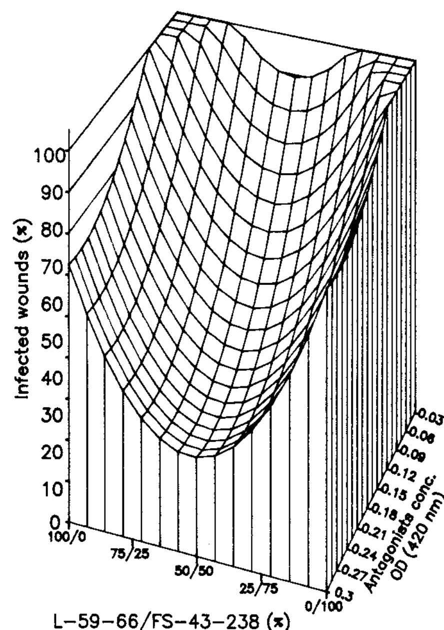

FIG. 2. Effect of the ratio of the antagonists (*P. syringae* L-59-66 and *S. roseus* FS-43-238) in the mixture and antagonist concentration on the predicted incidence of blue mold (infected wounds) on Golden Delicious apples. The two antagonists were mixed at various proportions at each concentration (equal OD value). Fruits were wounded, inoculated with the antagonist mixture, challenged with 10^4 conidia of *Penicillium expansum* per ml, and incubated for 7 days at 22°C. The response surface was generated from the equation $Y = 1.3698 - 5.0517C - 0.276Rt - 0.0429CRt + 10.7553C^2 + 0.0392Rt^2$ with $R^2 = 0.57$, where Y is the percentage of wounds infected, C is the concentration of the antagonists, and Rt is the ratio of the antagonists in the mixture.

tions of *P. syringae* in the mixture (50:50) also increased more than 10-fold and, at the end of the experiment (96 h), were similar to the *P. syringae* populations applied individually. *S. roseus* populations applied in the mixture increased more than fivefold and were consistently lower than the *S. roseus* populations applied individually.

When L-asparagine was added to the antagonist suspensions, populations of *P. syringae* applied individually increased 100-fold and populations of *S. roseus* applied individually increased more than 10-fold 7 days after application to the wounds (Fig. 5). Populations of *P. syringae* applied in the 50:50 mixture increased 100-fold after 3 days, and at the end of the experiment they were similar to populations of *P. syringae* from individual application. *S. roseus* populations in the 50:50 mixture application increased more than 10-fold after 3 days and then exhibited a declining trend toward the end of the experiment and were consistently smaller than the populations of *S. roseus* from individual applications.

Interaction of the antagonists in vitro. The growth of *P. syringae* was not affected by the presence of *S. roseus* at all ratios tested in NYDB with ($P = 0.936$) or without ($P = 0.475$) L-asparagine. In the mixture or alone, in the media with or without L-asparagine, *P. syringae* increased to about 10^{10} CFU/ml after 36 h, regardless of the initial inoculum level (Fig. 6A and B). The growth of *S. roseus*, in contrast, was significantly affected by the presence of *P. syringae* in the medium with ($P = 0.0001$) or without ($P = 0.0002$) L-asparagine at all ratios tested (Fig. 6C and D). In NYDA inoculated with *S. roseus* alone, the yeast population increased to about 10^8 CFU/ml in all flasks after 36 h of growth, regardless of the initial inoculum level. However, in the mixture with the bacterium, the increases were smaller and proportional to the initial inoculum. After 36 h, there were about 10-fold more cells in the flask inoculated with 0.8 ml than with 0.2 ml of *S. roseus*, but the largest population in the mixture was still about 10-fold lower than in flasks inoculated with *S. roseus* alone. The addition of L-asparagine had no effect on the growth of *S. roseus* alone, but it reduced growth in the mixtures. A decline in the populations was observed after 36 h.

Nutrient utilization. Overall, of the 71 carbon and nitrogen sources tested, 32 were utilized by *P. syringae* and 31 were utilized by *S. roseus* (Table 1). Twenty-three of these nutrients were utilized by both antagonists. Of the 35 nitrogen sources tested, *P. syringae* utilized 23 and *S. roseus* utilized 14. All 14 compounds utilized by *S. roseus* were also utilized by *P. syringae* (Fig. 7). Of the 36 carbon sources tested, 13 were utilized by *P. syringae* and 17 were utilized by *S. roseus*. Nine of these carbon sources were utilized by both antagonists.

DISCUSSION

A mixture of the antagonists *P. syringae* and *S. roseus* at approximately equal biomass enhanced biocontrol of *Penicillium expansum* compared with control by treatments with the antagonists applied separately at biomass equivalent to that in the mixture. The effects were similar both in the initial factorial experiments and in later tests, in which L-asparagine was used. Because response surfaces generated from the factorial experiments indicated optimal performance of biocontrol at a mixture of about 50:50, this ratio was used in subsequent tests to compare the effectiveness of the mixture with individual applications of antagonists. The tests were quite severe, since they were performed with mature apples, which are highly susceptible to *Penicillium expansum*. Results were similar in experi-

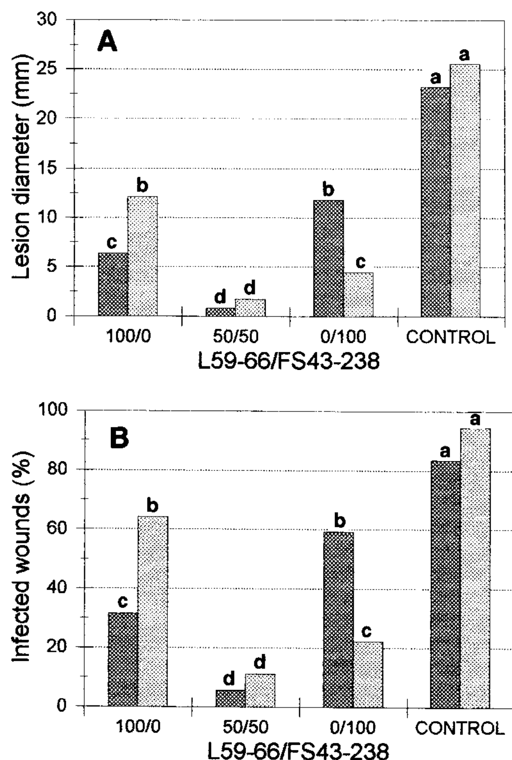

FIG. 3. Effect of L-asparagine and ratio of the antagonists (*P. syringae* L-59-66 and *S. roseus* FS-43-238) in the mixture on severity (A) and incidence (B) of blue mold on Golden Delicious apples. Fruit were wounded, inoculated with individual antagonists or a 50:50 mixture, challenged with 2.5×10^4 conidia of *Penicillium expansum* per ml, and incubated for 7 days at 22°C. Bars with the same letter are not significantly different according to the Waller-Duncan multiple-range test ($P = 0.05$). Symbols: ▩, with asparagine; ▤, without asparagine.

ments with L-asparagine, in which the *Penicillium expansum* inoculum was increased to 2.5 times that of the earlier experiments. The beneficial effect of the addition of L-asparagine with *P. syringae* in this study confirmed our earlier findings of its effect on enhancing biocontrol of blue mold on apples (13). In vitro studies on the utilization of various amino acids by *Penicillium expansum* showed that with the exception of L-cysteine and L-valine, all amino acids stimulated germination of *Penicillium expansum* conidia whereas L-asparagine induced 100% germination. This amino acid, however, had a much less stimulatory effect on radial growth of *Penicillium expansum* and was also an excellent nitrogen source for the antagonist *P. syringae* (13). The effect of L-asparagine on biocontrol by *S. roseus* was not significant in the mixture applications and significantly reduced effectiveness in individual applications. It appears that L-asparagine does not enhance the effectiveness of biocontrol with mixtures of these antagonists. This is also supported by the results of the in vitro studies, in which the addition of L-asparagine to the antagonist mixture resulted in an overall decline in *S. roseus* populations after 36 h.

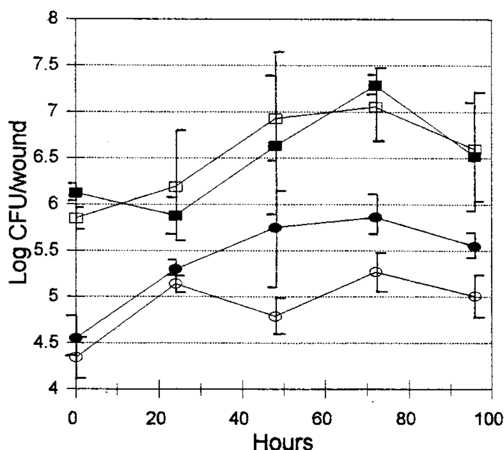

FIG. 4. Population dynamics in wounds of Golden Delicious apples inoculated with the antagonists *P. syringae* L-59-66 and *S. roseus* FS-43-238 individually or in a 50:50 mixture and incubated at 22°C. Fruit samples were removed at various times to recover antagonists from the wounds. Bars represent standard deviation. Symbols: ▭, 50% *P. syringae*; ■, 100% *P. syringae*; ⬭, 50% *S. roseus*; ⬬, 100% *S. roseus*.

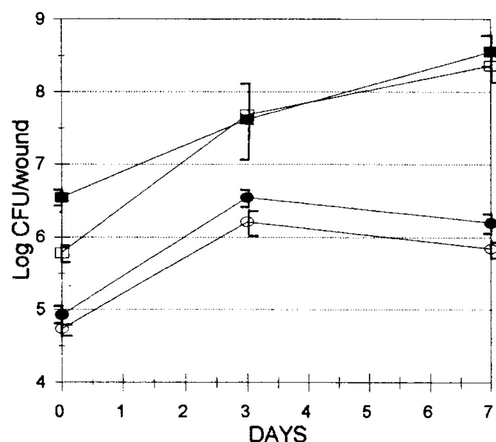

FIG. 5. Population dynamics in wounds of Golden Delicious apples inoculated with the antagonists *P. syringae* L-59-66 and *S. roseus* FS-43-238 resuspended in 80 mM L-asparagine individually or in a 50:50 mixture and incubated at 22°C. Fruit samples were removed at various times to recover antagonists from the wounds. Bars represent standard deviation. Symbols are as in Fig. 4.

P. syringae and *S. roseus* are very effective in colonizing apple wounds (9, 11). The population increase of *P. syringae* applied in the mixture to the same levels as in the individual application indicates a lack of any negative interference from *S. roseus*. It appears that populations of *P. syringae* from individual and mixed applications reached the carrying capacity of the wounds. However, the populations of *S. roseus* in mixed applications never reached the same levels as in the individual applications, and the differences at the end of the experiment appeared to be even greater than at the beginning. The more than 100-fold increase in the population of *P. syringae* after addition of L-asparagine confirms an earlier report that this compound strongly stimulates the population of *P. syringae* in apple wounds (13). Here again, populations of *P. syringae* from both applications reached similar levels, indicating that the bacterium reached the wound-carrying capacity under these conditions. Thus, scarcity of nitrogen, rather than metabolic products, limited the growth of this antagonist in apple wounds. As in earlier experiments, populations of *S. roseus* from applications in the mixture did not reach the levels from individual applications. This could result from direct interaction with *P. syringae*. Results from the in vitro studies support this, because populations of *S. roseus* in a mixture with *P. syringae* were always smaller than those of *S. roseus* applied alone. However, inhibitory substances are probably not involved, because *P. syringae* did not inhibit growth of *S. roseus* or

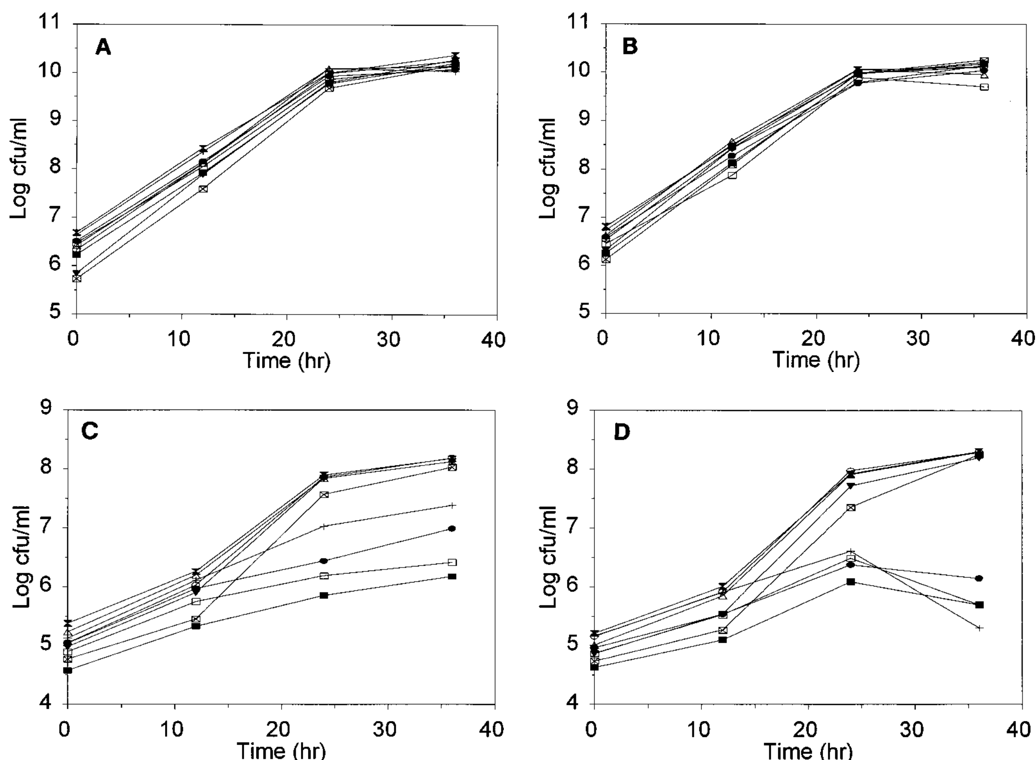

FIG. 6. Population dynamics of *P. syringae* (A and B) and *S. roseus* (C and D) alone or in mixtures at various proportions (from R1 to R.2) in NYDB (A and C) or NYDB plus L-asparagine (B and D). The flasks with NYDB were inoculated with the antagonists alone or in mixtures at various proportions (0.8:0.2, 0.6:0.4, 0.4:0.6, 0.2:0.8) and incubated on a shaker at 24°C. The samples were collected at 12-h intervals. Symbols in panels A and B: ⌶, *P. syringae* alone at 1 ml; △, *P. syringae* alone at 0.8 ml; ○, *P. syringae* alone at 0.6 ml; ▼, *P. syringae* alone at 0.4 ml; ⊠, *P. syringae* alone at 0.2 ml; +, *P. syringae* in a mixture at 0.8 ml; ●, *P. syringae* in a mixture at 0.6 ml; ▭, *P. syringae* in a mixture at 0.4 ml; ▬, *P. syringae* in a mixture at 0.2 ml. Symbols in panels C and D: as for panels A and B but for *S. roseus.*

Penicillium expansum on NYDA (unpublished results) in tests similar to these developed by Xu and Gross (26).

Recently, Wilson and Lindow found that coexistence of epiphytic bacterial strains in the phyllosphere of potato and bean plants is mediated by nutritional-resource partitioning (24, 25). The level of coexistence between epiphytes was proportional to the degree of niche differentiation, as defined by the ability to utilize limiting sources not utilized by the other (competing) strain. The authors quantitated this by using the niche overlap index, which was defined as the number of nutrient (e.g., carbon) sources mutually utilized by two strains as a proportion of the total number of nutrient sources utilized by one of these strains. A small ecological variation or niche differentiation between two bacterial strains (high niche overlap index) results

TABLE 1. Compounds tested for their ability to support growth of *S. roseus* and *P. syringae* when incorporated into a minimum salt medium

Type	Compounds
Amino acids	L-Alanine,[a,b] L-arginine,[a,b] L-asparagine,[b] L-cysteine,[b] L-glutamine,[b] glycine,[b] L-histidine,[b] hydroxy-L-proline, L-isoleucine,[a,b] L-leucine,[a,b] L-lysine,[a,b] L-methionine,[a,b] L-phenylalanine,[a] L-proline,[b] L-serine,[a,b] L-threonine,[a,b] L-valine,[a,b]
Amides and amines	D-Glucosamine, *N*-ethylmaleimide, acetamide,[b] aminoisobutyric acid, ethylenediamine, formamide,[b] methylamine
Miscellaneous (nitrogenous)	Choline, creatine, taurine,[b] L-tartaric acid diammonium salt,[a,b] casein enzymatic hydrolyzate[a,b]
Inorganic (nitrogenous)	Ammonium chloride,[a,b] ammonium phosphate dibasic, ammonium phosphate monobasic,[a,b] urea
Carbohydrates	D-Arabinose, L-arabinose,[b] D-cellobiose,[a] 2-deoxy-D-galactose,[a] 2-deoxy-D-glucose, 2-deoxy-D-ribose,[b] D-fructose,[a,b] D-fucose,[a] L-fucose, D-galactose,[b] α-D-glucose,[a,b] D-glycero-D-guloheptose, α-D-lactose, β-D-lactose, D-lyxose,[a,b] L-lyxose, D-maltose,[a] D-mannose,[a,b] L-mannose, melezitose,[a] α-D-melibiose, palatinose,[a] D-raffinose,[a] L-rhamnose, D-ribose,[a,b] L-ribose, L-sorbose,[a] stachyose, sucrose,[a,b] D-trehalose,[a] D-xylose,[a,b] L-xylose, methyl-D-glucopyranose
Sugar alcohols	D-Mannitol,[a,b] D-sorbitol,[b] *myo*-inositol[b]

[a] Compounds utilized by *S. roseus.*
[b] Compounds utilized by *P. syringae.*

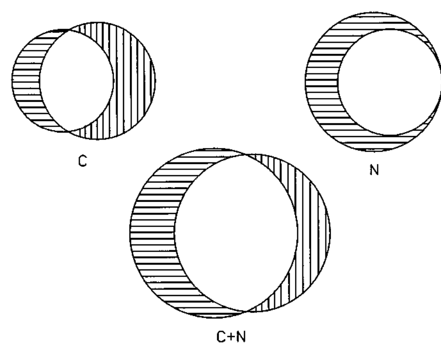

FIG. 7. Nutritional pool of the antagonists *P. syringae* (horizontal lines) and *S. roseus* (vertical lines). The antagonists were evaluated for utilization of 36 carbon sources and 35 nitrogen sources in minimum salt media. The sizes of the circles are proportional to the number of compounds utilized. Open areas within overlapping circles indicate compounds utilized by both antagonists. C indicates utilization of carbon sources, N indicates utilization of nitrogen sources, and C+N indicates data combined from utilization of carbon and nitrogen sources.

in a low level of coexistence. Carbon was the limiting source in their studies. In the case of apple wounds, the limiting resource appears to be nitrogen (13). Nutritional studies indicate that *P. syringae* has a broader nitrogen-utilizing capacity than *S. roseus*, because it utilizes all the compounds utilized by *S. roseus* plus an additional nine (39%) compounds. The niche overlap indices for nitrogen sources for *P. syringae* and *S. roseus* are 0.61 and 1.0, respectively. Thus, *P. syringae* may be a better competitor for limiting nitrogen sources in apple wounds. This may explain why *P. syringae* showed a high level of coexistence with *S. roseus* and why *S. roseus* showed a lower level of coexistence with *P. syringae* in apple wounds. *S. roseus*, on the other hand, has a broader carbon-utilizing capacity, but carbon is not the limiting factor for *P. syringae* (13), because apple wounds are rich in readily available sugars such as sucrose, D-glucose, and D-fructose (23). These sugars are utilized by both antagonists and also stimulate germination and radial growth of *Penicillium expansum* (13). Although the threshold of sugar required for conidial germination and initiation of the pathogenic process by *Penicillium expansum* was not determined, the high concentration of sugars in apple wounds (unpublished data) suggests that competition for carbon sources is unlikely to play a significant role in this biocontrol system. Most of the amino acids in apple and pear juices are readily utilized by *Penicillium expansum* (1, 13). The broad nitrogen-utilizing capacity of *P. syringae* and perhaps the effective utilization of carbon sources by *S. roseus* allowed both antagonists to flourish in the same wound. This caused even greater depletion of nutrients essential for development of *Penicillium expansum* than by either antagonist alone and resulted in better biocontrol.

The enhancing effect of the mixture of *P. syringae* and *S. roseus* appears to be additive, because decay reduction by the mixture was smaller than the sum of decay reduction by the individual antagonists. This is in contrast to the work of Limanceau et al. with fusarium wilt of carnations, in which biocontrol was synergistically enhanced by adding *Pseudomonas putida* to the antagonistic, nonpathogenic strain of *Fusarium oxysporum* (14, 15). The bacterium by itself did not provide any biocontrol, but it produced a siderophore, pseudobactin 358, which reduced uptake of Fe^{3+} by the pathogenic strain of *F. oxysporum* that was more dependent on iron availability for growth than was the nonpathogenic strain. This reduced the competitiveness of the pathogenic strain for glucose, for which Fe^{3+} is a necessary electron acceptor in the oxidation process (15). This elegantly described mechanism, however, is unlikely to operate in our system, because our strain of *P. syringae* did not produce a siderophore inhibitory to *Penicillium expansum* on NYDA (unpublished results).

This research has demonstrated that the preemptive colonization of apple wounds with two antagonists well adapted to apple wounds may result in a relatively stable assemblage of the populations as a climax community, which is more effective in controlling *Penicillium expansum* than is either population alone.

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