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Control of Postharvest Pathogens and Colonization of the Apple Surface by Antagonistic Microorganisms in the Field

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ABSTRACT

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Selected isolates of *Aureobasidium pullulans*, *Rhodotorula glutinis*, and *Bacillus subtilis* reduced the size and number of lesions on wounded apples caused by the postharvest pathogens *Penicillium expansum*, *Botrytis cinerea*, and *Pezicula malicorticis*. Combinations of the antagonistic microorganisms were applied to apple trees in the field late in the growing season of two consecutive years. The population dynamics of the introduced microorganisms and the incidence of fruit decay were determined. Population sizes of introduced antagonists on apple surfaces increased in the field following application of treatments until harvest. After transfer of the fruit from the field into cold storage, the populations of the introduced antagonists remained higher than in the control treatments. Identification of the applied isolates of *A. pullulans* and *R. glutinis* during the experiments was achieved by isolate-specific DNA probes generated from random amplified polymorphic DNA. A combination of two strains of *A. pullulans* and one strain of *R. glutinis* suppressed rotting of apple to the same extent as the commonly used fungicide Euparen. Our data demonstrate that the application of antagonistic microorganisms in the field represents a promising alternative to fungicide treatments to control postharvest diseases of apple.

Postharvest pathogens cause major losses in apple production. More than 90 fungal species have been described that cause decay of apples during storage (15). The relative importance of each pathogen depends on climatic and storage conditions. In Germany, bull's-eye rot caused by *Pezicula malicorticis* (H. Jacks.) Nannf. is the most important postharvest disease of apple (17). The pathogen infects the fruit through wounds or lenticels late in the growing season or after harvest (16). Two other important postharvest diseases are blue mold caused by *Penicillium expansum* Link and gray mold caused by *Botrytis cinerea* Pers.:Fr. (31). Both organisms are wound pathogens that cause extensive postharvest losses in nearly all regions where apples are cultivated.

Control of postharvest pathogens still relies mainly on the use of synthetic fungicides, but the development of fungicide-resistant pathogens and the public demand to reduce pesticide use have increased the search for alternative control strategies (14,36). The use of yeast or bacterial strains to control postharvest decay of several fruits by phytopathogenic fungi has been extensively studied, and several examples of successful disease control exist (11,18,25, 26). In the United States, *Candida oleophila* strain 182 (Aspire; Ecogen, Inc., Langhorne, PA) and *Pseudomonas syringae* strains ESC-10 and ESC-11 (Bio-Save 10 and Bio-Save 11; EcoScience, Worcester, MA) already have been commercialized as postharvest biofungicides (20).

Microorganisms as biological control agents have a relatively narrow spectrum of activity as compared with synthetic fungicides (13). Increased and broader activity against postharvest pathogens can be achieved by the use of combinations of microorganisms. In previous studies, mixtures of antagonistic strains were successfully employed for biocontrol of root disease (5,6) and postharvest rot of fruit (8,10,13) and potatoes (30).

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Biocontrol of decay of fruit in storage with microbial antagonists has been studied mainly under controlled environmental conditions (37). Because infection of fruit by postharvest pathogens often occurs in the field prior to harvest (2,26), it may be advantageous to apply antagonists before harvest. For this approach to be successful, putative biocontrol strains must be able to tolerate low nutrient availability, UV radiation, low temperatures, and climatic changes. *Aureobasidium pullulans* (de Bary) G. Arnaud, *Rhodotorula glutinis* (Fresen.) F.C. Harrison, and *Bacillus subtilis* are common inhabitants of leaf and fruit surfaces and have a high tolerance to desiccation and irradiation (7,21,27). Applications before harvest are also of interest, because European regulations of integrated pest management do not allow postharvest treatments of apples (28).

The current study was conducted to determine if mixtures of antagonistic microorganisms can be established on the apple surface before harvest and, subsequently, suppress apple pathogens in storage. By means of isolate-specific random amplified polymorphic DNA (RAPD) markers, we analyzed the population dynamics of introduced strains and naturally occurring populations of *A. pullulans* and *R. glutinis* on the apples in the orchard and after harvest during cold storage. A preliminary account of this work was published (24).

MATERIALS AND METHODS

Isolates. The antagonists used in this study were recovered from the surface of untreated apples in southwest Germany and were described previously (8,29). Isolates of *Penicillium expansum*, *Pezicula malicorticis*, and *Botrytis cinerea* were obtained from infected fruit tissue with symptoms of decay. The pathogens and the antagonistic isolate *A. pullulans* were identified as described by Arx (1). The identification of *R. glutinis* was confirmed by the CBS (Centraalbureau voor Schimmelcultures) Institute, Baarn, the Netherlands. The antagonistic bacterial isolates were identified by physiological characteristics as described by Claus and Berkeley (4). Isolates were suspended in 10% glycerol and frozen in liquid nitro-

gen for long-term storage or kept on potato dextrose agar (PDA) at 4°C for short-term storage.

In vivo assays with wounded apples. For each experiment, fresh aliquots of a pathogen and an antagonist were thawed and diluted to concentrations of 10^5 CFU/ml for the pathogens, 10^7 CFU/ml for the antagonistic fungal strains, and 10^8 CFU/ml for the antagonistic bacterial strains. Concentrations were determined by dilution-plating. For combination treatments, equal volumes of the suspensions were mixed. For tests with a low concentration of antagonists, suspensions were diluted 10-fold. Mixture M1 consisted of *A. pullulans* strains CF10 and CF40 and *R. glutinis* strain CF35. Mixture M2 consisted of *Bacillus subtilis* strains AG704 and HG77 and *A. pullulans* strain CF10.

Apples ('Golden Delicious') that had not been treated with late-season fungicide applications were used for laboratory tests. Apples were stored for up to 8 weeks at 2°C and 95% relative humidity (RH) before being used. They were surface-sterilized by soaking in 70% ethanol for 3 min and then wounded by removing plugs 5 mm in diameter and 3 mm in depth from the surface. Each apple was wounded four times halfway between the calyx and the stem end. Two of the wounds were inoculated with 20 µl of a single antagonistic isolate or with a mixture of strains, and the other two wounds were inoculated with 20 µl of distilled water (control). All wounds were then treated with 20 µl of a conidial suspension of the pathogen. The apples were placed in boxes that were sealed in plastic bags to maintain high humidity. Diameters of lesions were determined after 4 weeks at 4°C. Treatments were arranged in a

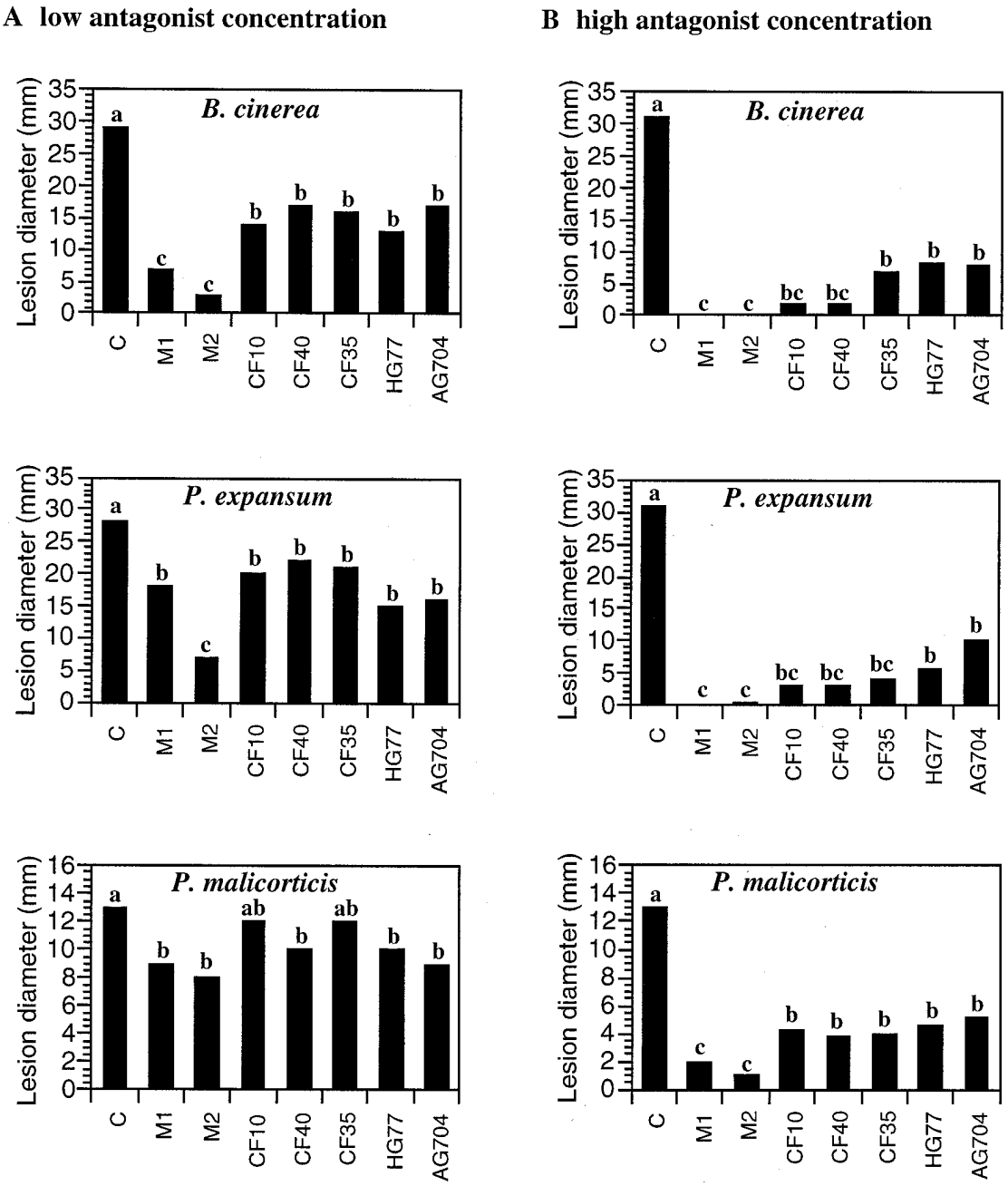

Fig. 1. Influence of single strains and strain mixtures on rot of apples caused by *Botrytis cinerea*, *Penicillium expansum*, or *Pezicula malicorticis.* Apple wounds were inoculated with 20 µl of an antagonist or a mixture. Concentrations of fungal antagonists were **A,** 10^6 and **B,** 10^7 cells/ml and concentrations of bacterial antagonists were **A,** 10^7 and **B,** 10^8 cells/ml. Control wounds were treated with water. All wounds were then treated with 20 µl of the pathogen (10^5 spores/ml). Lesion diameters were determined after 4 weeks of incubation at 4°C. C = control, M1 = strains CF35 plus CF10 plus CF40, and M2 = strains CF10 plus AG704 plus HG77. Bars with different letters are significantly different ($\alpha = 0.05$).

randomized block design. All experiments were repeated, with five replicates per treatment.

Field trials. Field trials were conducted on the same trees in 1993 and 1994 in a conventionally managed apple orchard in Meckenbeuren (southwest Germany) near Lake Constance. The plot consisted of 80 trees of the variety Golden Delicious planted in 1983. Application of fungicide treatments was stopped in June. Inoculum of the antagonistic microorganisms was prepared by transferring each isolate individually into 100-ml Erlenmeyer flasks containing 20 ml of potato dextrose medium (PDM) and incubating for 3 days at 20°C on a rotary shaker. These cultures were used to inoculate 1 liter of PDM in 5-liter Erlenmeyer flasks. Cell densities were determined by haematocytometry. Cultures were harvested at the late log phase of growth. Cells were pelleted once by centrifugation, resuspended in distilled water, and kept on ice. Before application, the suspensions were diluted with distilled water to final concentrations of 10^7 cells/ml for *A. pullulans* and *R. glutinis* and 10^8 cells/ml for *Bacillus subtilis*, and mixed as described above. Distilled water and the fungicide Euparen (dichlofluanid; Bayer AG, Leverkusen, Germany) served as controls. The fungicide was applied as a 0.15% (wt/vol) solution. Each treatment consisted of five replicates arranged in a randomized block design. Each replicate consisted of four trees.

The treatments were applied on 31 August 1993 and 23 August 1994 and were repeated 14 and 28 days later. Treatments were applied by motor-driven back sprayers (nozzle size, 0.6 mm; Mesto Spritzenfabrik, Freiberg, Germany) at a rate of 1 liter per tree. To prevent spray drift, all trees were individually covered with a thin polyethylene cover on the day of the treatment. Climatic data including temperature, leaf wetness, rainfall, and RH were recorded during the field trial with a HP 100 climatological station (Lufft Meβ- und Regeltechnik GmbH, Fellbach, Germany). Sensors were positioned at a height of 2 m in the middle of the plot. Apples

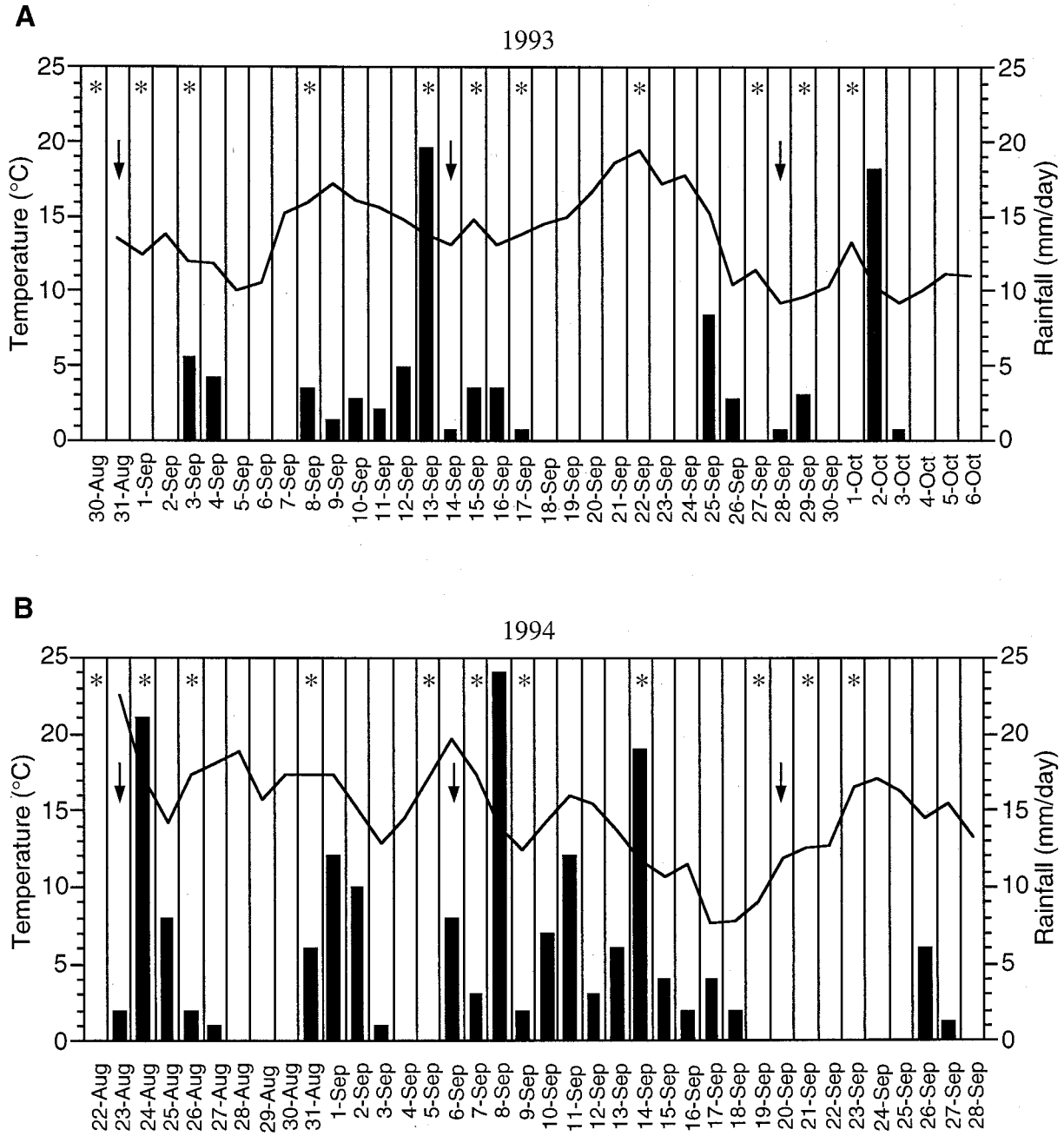

Fig. 2. Climatic conditions in the orchard during the field trials in **A,** 1993 and **B,** 1994. Rainfall is shown by columns and the mean temperatures by lines. Arrows indicate application of treatments. Asterisks indicate sampling dates.

were harvested on 6 October 1993 and 4 October 1994. Apples from different treatments were stored in separate boxes and kept at 2°C and 95 to 100% RH in a cold room for 6 months.

Colonization of the apple surface in the field and in cold storage. At each sampling, four apples from each replicate were selected for evaluation. Sampling dates in the field are given in the Results. In cold storage, apples were sampled on 28 October 1994, 23 February 1995, and 30 March 1995. In the first trial (1993/1994), the evaluation ended after harvest, because of cross-contamination by apples infected with *Penicillium expansum*. To evaluate colonization, 20 disks, each 2 cm in diameter, were cut out of the apple surface and transferred into flasks containing 200 ml of distilled water. After shaking for 1 h at 200 rpm, serial (1:10) dilutions were made of each of the washing solutions. Four aliquots were taken from each dilution and spread on the surface of malt extract agar. Petri dishes were incubated for 3 days at 20°C, and the CFU of red yeasts (*Rhodotorula* spp. and others), *A. pullulans*, and *Bacillus* spp. were determined. For identification of *A. pullulans* and *Bacillus* spp., colony morphology and microscopical characteristics were compared with the introduced strains.

Identification of antagonistic isolates by RAPD analysis and DNA hybridization. Colonies from the petri dishes were selected randomly and transferred into 2-ml reaction tubes. PDM (1.5 ml) was added, and the cultures were shaken at 200 rpm for 3 days at 20°C. Cells of individual isolates were centrifuged, and DNA was extracted following the protocol of Cenis (3). Approximately 25 ng of total DNA of each isolate was transferred to a 25-µl polymerase chain reaction (PCR) mix containing 100 µM of each of the four dNTPs, 0.2 mM of the appropriate primer, and 0.5 units of *Taq* DNA polymerase (Pharmacia Biotechnology Inc., Uppsala, Sweden). Amplification was performed in 0.5-ml reaction tubes in an Autogene II thermocycler (Grant Instruments, Cambridge). Amplification products were separated by electrophoresis in 1.2% agarose gels and detected by staining with ethidium bromide.

DNA of *A. pullulans* strains CF10 and CF40 was amplified with the minisatellite primer $(GACAC)_3$. The temperature profile for the minisatellite primer consisted of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 90 s.

DNA amplification of *R. glutinis* strain CF35 was performed with the primer GACCGCTTGT (Operon Technologies Inc., Alameda, CA). The temperature profile consisted of 94°C for 4 min; followed by 46 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min; and finally 72°C for 9 min. A strain CF35-specific fragment of 1.4 kb was eluted and labeled with digoxigenin (DIG) by random priming (9). Amplification products of red yeasts obtained under the same PCR conditions were denatured and dotted onto a nylon membrane. The hybridization of the membrane-bound amplification products with the DIG-labeled DNA fragment was performed according to standard protocols supplied by Boehringer Mannheim GmbH (Mannheim, Germany).

Evaluation of decay in cold storage. Every 4 weeks, apples were evaluated for rot incidence in each replicate. Healthy apples were transferred to new boxes to avoid cross-contamination. Infected apples were removed from cold storage, and fungal material was isolated from the lesions and used for identification. If spores or conidia were missing, parts of the lesions were transferred onto PDA, and the fungi were identified according to the specifications of Arx (1). The last evaluations were conducted on 1 March 1994 and 30 March 1995. Approximately 2,500 apples per treatment were evaluated each year.

Data analysis. All data were analyzed by using the STATISTICA-Mac program (StatSoft, Tulsa, OK). Data were checked for conformity to the main assumptions of the analysis of variance (ANOVA). Data not meeting the assumptions of ANOVA were analyzed by nonparametric procedures. Means of lesion diameter of wounds were analyzed as a factorial between-group design by using the least significance difference (LSD) test. Population sizes of *A. pullulans*, red yeasts, and *Bacillus* spp. on the apple surface were analyzed following log-transformation of data. Population sizes throughout the season for each treatment were pooled and, for *A. pullulans* and red yeasts, means were separated by the LSD test. Data of the population dynamics of *Bacillus* spp. were analyzed by the Median test (33). The LSD test was also used to compare means of disease incidence in the field trials. Unless otherwise stated, differences significant at $P < 0.1$ are reported.

RESULTS

Inoculation tests with wounded apples. Antagonists differed in ability to suppress disease caused by *Penicillium expansum*, *Botrytis cinerea*, and *Pezicula malicorticis* (Fig. 1A and B). Strain combinations had greater biocontrol activity compared with individual strains ($P = 0.0001$). Mixture M2 was more effective than mixture M1 ($P = 0.0012$). Individual strains and strain combinations were more effective against *Bacillus cinerea* and *Penicillium expansum* than against *Pezicula malicorticis* ($P = 0.0001$). The concentration of the antagonists significantly affected the biocontrol activity against *Botrytis cinerea* ($P = 0.0001$), *Penicillium expansum* ($P = 0.0001$), and *Pezicula malicorticis* ($P = 0.0001$).

Microclimatic conditions. Microclimatic conditions during the field trials are shown in Figure 2. In 1993, few showers occurred between the first and second treatments in which leaf wetness periods lasted more than 24 h (data for leaf wetness not shown). The second treatment was followed by rainfalls during the next days and several showers after September 24. In 1994, extensive rainfall periods occurred after the first and second treatments. The amount of rainfall between the first treatment and harvest was much higher in 1994 (167 mm) than in 1993 (96 mm).

Colonization of the apple surface. In both field trials after application of mixture M1, populations of *A. pullulans* increased to a population size greater than that in the water control (1993, $P = 0.0001$; 1994, $P = 0.0001$) (Fig. 3A). In the period from the first application in August until harvest in October, the number of CFU increased from 10^2 CFU/cm^2 to 8×10^3 CFU/cm^2 of apple surface. Population sizes of *A. pullulans* resulting from treatment M2 were less than those from M1 (1993, $P = 0.094$; 1994, $P = 0.0097$). In the water control, the population sizes remained between 10^2 CFU/cm^2 and 6×10^2 CFU/cm^2 each year. Natural populations of *A. pullulans* on the fungicide-treated apples were less than those on the water control (1993, $P = 0.061$; 1994, $P = 0.097$) (Fig. 3A).

Red yeast populations were higher on fruits sprayed with M1 than on fruits sprayed with water (1993, $P = 0.012$; 1994, $P = 0.0001$) (Fig. 3B). In 1993, they reached a peak population size of 2×10^4 CFU/cm^2. Natural populations of the red yeasts on the fungicide-treated apples were less as compared with populations on the water control (1993, $P = 0.09$; 1994, $P = 0.012$) (Fig. 3B). Population sizes of *Bacillus* spp. varied considerably among treatments (Fig. 3C). After treatment with mixture M2, populations of *Bacillus* spp. were significantly greater than those on water-treated apples (1993, $P = 0.0001$; 1994, $P = 0.0001$), and they peaked at about 8×10^3 CFU/cm^2 (Fig. 3C).

In cold storage, the population sizes of *A. pullulans* on water-treated apples remained below 2×10^2 CFU/cm^2 (Fig. 4A). Population sizes of *A. pullulans* on apples treated with mixture M1 decreased from 8.8×10^2 CFU/cm^2 on 28 October 1994 to 4×10^2 CFU/cm^2 on 30 March 1995 and on apples treated with mixture M2 from 4.8×10^2 CFU/cm^2 to 3×10^2 CFU/cm^2 (Fig. 4A). In cold storage, population sizes of red yeasts in the water control remained below 2.2×10^2 CFU/cm^2 (Fig. 4B). On apples treated with mixture M1, they reached 8×10^2 CFU/cm^2 on 28 October 1994 (Fig. 4B). Subsequently, the population size decreased to 2×10^2 CFU/cm^2 on 30 March 1995. The population size of *Bacillus* spp. reached 2.1×10^2 CFU/cm^2 on 28 October 1994 on apples treated with mixture M2. Subsequently, *Bacillus* spp. were not detectable in cold storage (Fig. 4C).

Identification of antagonistic isolates. For molecular identification of *R. glutinis* strain CF35, a DIG-labeled fragment from RAPD analysis was used as a hybridization probe. Specificity of the probe was verified in hybridization experiments with DNA from a *R. glutinis* isolate (CBS 322) and 25 other red yeast strains (data not shown).

Before the start of the field trial in 1993, none of the DNA from yeasts with red colonies hybridized with the CF35-specific DNA probe, indicating that *R. glutinis* strain CF35 was not naturally present in the orchard (Table 1). In 1993 after application of the mixtures, up to 57% of the red yeast detected on M1-treated fruits were strain CF35. In 1994, CF35 composed 53% of the population of red yeast after the second treatment and 74% before harvest. Strain CF35 was present on the apples even after 6 months of cold storage. On water-treated apples before harvest, strain CF35 composed 5% of the red yeast in 1993 and 16% in 1994 (Table 1).

Similar results were obtained with *A. pullulans*. On 22 August 1994 prior to the first application of M1, none of the 40 colonies that were tested had RAPD patterns characteristic of *A. pullulans* strains CF10 and CF40. On 28 October 1994 after 3 weeks in cold storage, 15 were identified as CF10 and 13 as CF40, out of 38 colonies tested (Fig. 5).

Disease suppression in storage. The antagonists applied in the field suppressed postharvest pathogens in storage (Table 2). Compared with the water-treated controls, mixture M1 (1994, P = 0.0031; 1995, P = 0.0226) and mixture M2 (1994, P = 0.0094;

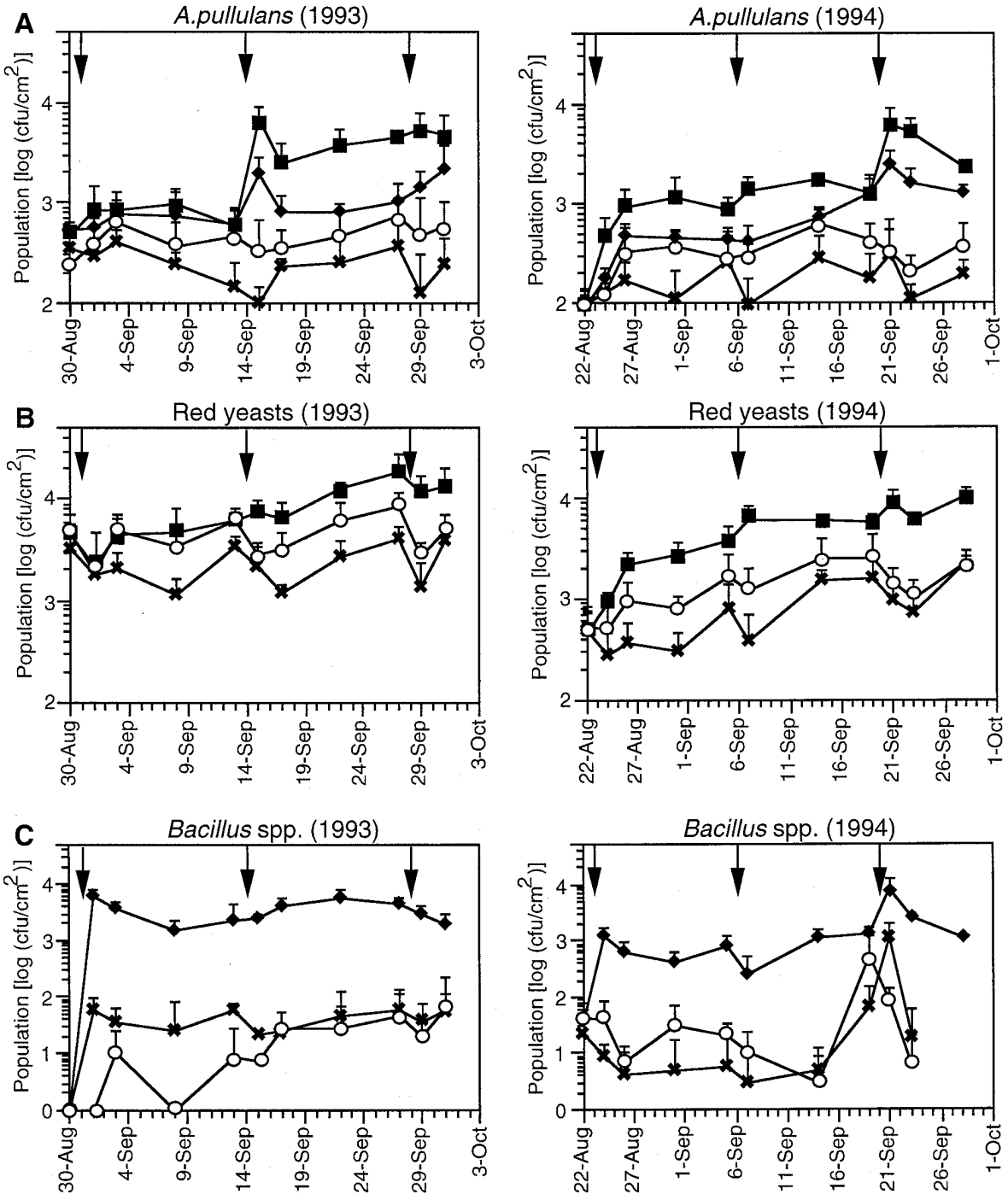

Fig. 3. Populations of **A,** *Aureobasidium pullulans*; **B,** red yeasts; and **C,** *Bacillus* spp. on apple surfaces in the field. M1 (■), M2 (◆), water (○), and fungicide (✕). Arrows indicate application of treatments. Bars represent standard deviations.

1995, $P = 0.0371$) significantly reduced infections per apple. Mixture M1 reduced the number of diseased apples in both trials (1994, $P = 0.0228$; 1995, $P = 0.0432$), whereas mixture M2 was effective only in 1994 (1994, $P = 0.0471$; 1995, $P = 0.121$). There was no significant difference between the control activities of the fungicide and of the antagonists (Table 2). In all treatments, most infections on apples were caused by *Pezicula* spp., *Penicillium* spp., and *Monilinia fructigena*. The pathogens *Botrytis cinerea* and *Alternaria alternata* were only of minor importance (data not shown).

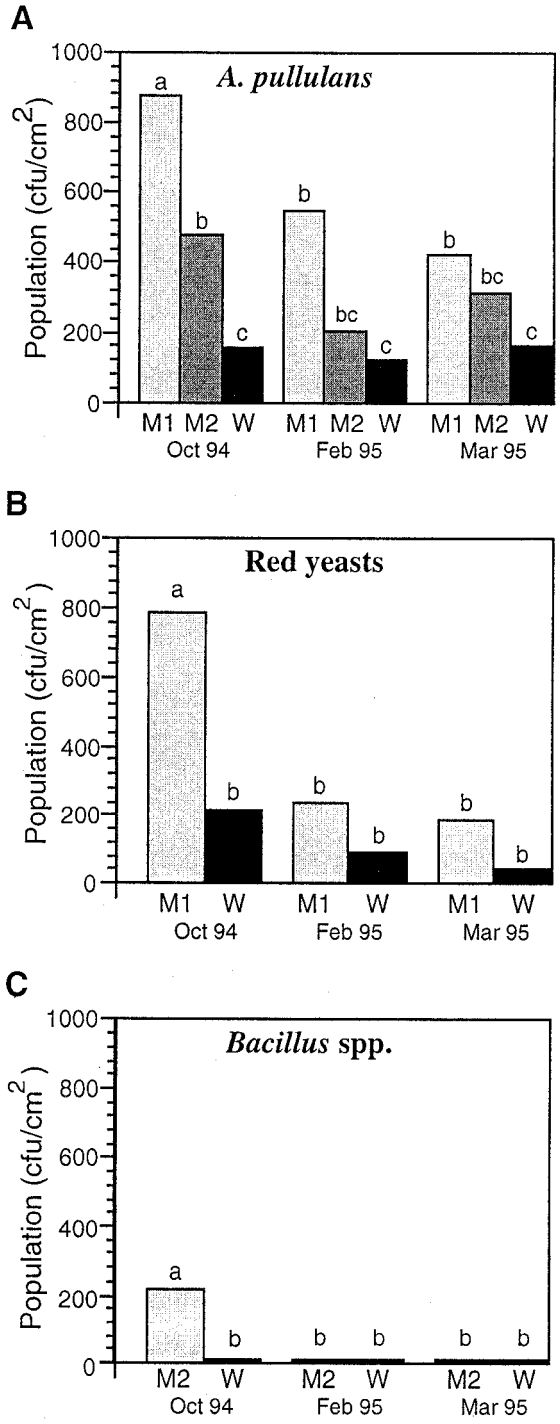

Fig. 4. Populations of *Aureobasidium pullulans*, red yeasts, and *Bacillus* spp. on apple surfaces in storage. W = water, M1 = strains CF35 plus CF10 plus CF40, and M2 = strains CF10 plus AG704 plus HG77. Statistical comparisons were made only between means within the same date of evaluation. Bars with the same letter are not significantly different ($\alpha = 0.05$).

DISCUSSION

Research on biological control of fruit rots has focused on application of biocontrol agents after harvest and studies conducted under controlled environmental conditions (37). However, a significant number of latent infections originate in the field, which cannot be satisfactorily controlled by postharvest treatments (26). Strains of *Trichoderma harzianum* have been tested under field conditions for control of dry eye rot caused by *Botrytis cinerea* (34,35). The current study demonstrates that application of antagonists to apple prior to harvest results in stable populations of antagonists in cold storage and a reduction in postharvest diseases. The antagonistic species used in this study are members of the epiphytic microflora of fruit (8,19,23,32) and, thus, appear to be adapted to the climatic conditions in the field to which they are exposed prior to harvest.

The greater biocontrol activity of strain mixtures against the postharvest pathogens *Penicillium* spp., *Botrytis cinerea*, and *Pezicula malicorticis* as compared with the same strains applied individually was demonstrated in bioassays under controlled environmental conditions. These results are in agreement with observations by Janisiewicz and Bors (13), who demonstrated that *Pseudomonas syringae* and *Sporobolomyces roseus* controlled blue mold on apple more effectively when mixed than when used individually. The diversity of phenotypes within strain combinations is likely to result in a community that is able to survive a wider range of environmental conditions and possesses a greater variety of traits responsible for disease suppression as compared with individual strains (12,37). In addition, combinations of biocontrol mechanisms may also suppress a broader range of pathogens (10,13).

Application of mixture M1, a combination of strains of *A. pullulans* and *R. glutinis*, increased the population sizes of *A. pullulans* and red yeasts more than 10-fold on apple, and they composed 95% of the total population of microorganisms on the fruit

TABLE 1. Frequency of *Rhodotorula glutinis* strain CF35 on the apple surface as detected with a specific random amplified polymorphic DNA marker after the application of mixture M1 or water

	1993[x]			1994/1995[x]		
Treatment	8/30/93	9/8/93	9/27/93	9/14/94	9/28/94	3/30/95*
Water control	0% (25)[y]	0% (20)	5% (18)	11% (18)[y]	16% (24)	16% (19)
Mixture M1[z]	0% (20)	15% (40)	57% (30)	53% (45)	74% (50)	64% (52)

[x] Sampling dates (* = postharvest sampling). Dates of application are given in Figure 2. Apples were harvested on 6 October 1993 and 4 October 1994.
[y] Values are the percentage of yeasts identified as strain CF35. Total number of red yeasts tested at each sampling date are given in parenthesis.
[z] Mixture M1 = *Aureobasidium pullulans* strains CF10 and CF40 and *Rhodotorula glutinis* strain CF35.

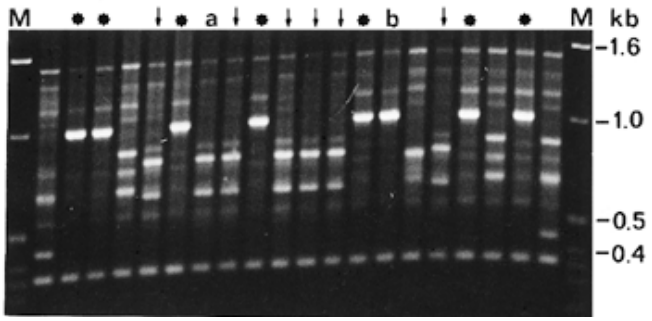

Fig. 5. Sample of random amplified polymorphic DNA profiles of *Aureobasidium pullulans* strains isolated from apple surfaces treated with mixture M1 after cold storage. a = strain CF10, b = strain CF40, arrows indicate isolates identified as CF10, asterisks indicate isolates identified as CF40, and M = molecular weight standards.

surface (data not shown). In contrast, populations of *A. pullulans* were substantially reduced when this fungus was applied in combination with *Bacillus subtilis* in mixture M2. The basis of this incompatibility is not known, but it could result from the production of antibiotic compounds by *Bacillus subtilis* (30). This incompatibility may be one reason why mixture M2 was slightly less effective than mixture M1 in the suppression of disease symptoms in both years. Similar results were obtained when *S. roseus* and *Pseudomonas syringae* were mixed; populations of the red yeast were lower when applied with the bacteria than when applied alone (13).

Bacillus spp. were found in low numbers on water-treated apples, and their population densities varied considerably on apples treated with mixture M2. Furthermore, in cold storage, their population densities remained much lower than the yeast populations. This was especially unexpected, because many *Bacillus* spp. form endospores that should facilitate their survival on the fruit surface. Possibly, conditions for growth and survival of these bacteria (32) were unfavorable during cold storage.

We used RAPD analysis and hybridization with an isolate-specific probe to track *A. pullulans* and *R. glutinis* isolates introduced into the field. To our knowledge, this is the first report of the use of these approaches for the identification of specific antagonistic yeasts during a biocontrol experiments in the field. These techniques allowed the introduced isolates to be distinguished from other strains that naturally colonize apples in the field. Thus, we were able to confirm that the introduced isolates of *A. pullulans* and *R. glutinis* had successfully colonized the apple surface and persisted during cold storage. In fact, the introduced isolates composed the majority of total recoverable isolates at the end of the cold storage periods. The unexpected recovery of the *R. glutinis* isolate CF35 from water-treated apples is probably the result of spread from inoculated trees, e.g., during heavy rainfalls. We assume that persistence of the isolate over the winter period in the field is responsible for increased recovery in 1994. Genetic approaches to characterize different isolates of a species are important, because isolates with the same phenotype can differ significantly in their biocontrol activity (B. Breuker, *unpublished data*).

The mechanisms of suppression by the introduced microorganisms are not yet understood. Previous studies established that antibiotic substances of *Bacillus subtilis* reduced development of several plant pathogens (27). Nutrient competition is a mechanism of biocontrol that is likely to be used by many yeast isolates (12). *R. glutinis* and *A. pullulans* are known to utilize the same nutrients that are required for germination and hyphal growth by *Mucor piriformis*, *Penicillium expansum*, and *Botrytis cinerea* (S. Wandmacher, W. Leibinger, and K. Mendgen, *unpublished data*). For the control of postharvest diseases, the use of microorganisms that compete with pathogens for nutrients may be preferable to the use of antibiotic-producing microorganisms, because of potential issues related to human toxicity and build-up of antibiotic resistance within the pathogen population (22,36).

We think that latent infections occurring during summer (2) are responsible for the high levels of disease caused by *Pezicula* spp. in storage. Therefore, applications of mixtures of biocontrol agents earlier in the growing season should be useful in reducing diseases of apples in the field and later in storage.

TABLE 2. Diseased apples and infections per apple after 6 months of cold storage[t]

Treatment	1993/1994		1994/1995	
	Diseased apples[u] (%)	Infections per apple[v]	Diseased apples[u] (%)	Infections per apple[v]
Control	5.6 a[w]	0.142 a	9.9 a	0.176 a
Euparen[x]	2.6 b	0.034 b	6.1 b	0.081 b
Mixture M1[y]	3.1 b	0.041 b	6.2 b	0.101 b
Mixture M2[z]	3.5 b	0.051 b	7.4 ab	0.106 b

[t] Apples were incubated in cold storage and evaluated regularly for disease symptoms for up to 6 months. Most infections were caused by *Pezicula* spp., *Penicillium* spp., and *Monilinia fructigena*.

[u] Values are expressed as percentage of diseased apples in each treatment.

[v] Values are expressed as the average number of infections per apple in each treatment.

[w] Statistical comparisons were made only within the same column. Values with the same letter are not significantly different ($\alpha = 0.05$).

[x] Dichlofluanid.

[y] Mixture M1 = *Aureobasidium pullulans* strains CF10 and CF40 and *Rhodotorula glutinis* strain CF35.

[z] Mixture M2 = *Bacillus subtilis* strains AG704 and HG77 and *Aureobasidium pullulans* strain CF10.

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