Combining Biocontrol Agents to Reduce the Variability of Biological Control

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ABSTRACT

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Two biocontrol agents, a yeast (*Pichia guilermondii*) and a bacterium (*Bacillus mycoides)*, were tested separately and together for suppression of *Botrytis cinerea* on strawberry leaves. The aims of the research were to determine whether the use of their combination would broaden the environmental conditions under which biological control is effective, and to test the hypothesis that it would reduce the variability of control efficacy under diverse conditions. Applied separately, the biocontrol agents significantly inhibited spore germination, lesion formation, and lesion development at most temperatures, relative humidities, and spray-timing combinations (temperatures: 10, 15, 20, 23, 25, and 30°C; relative humidities: 78, 85, 96, and 100%; and spray-timings: 0, 4, and 7 days before inoculation). However, control efficacy was highly variable, and under certain combinations it was not adequate. Control efficacy achieved by the biocontrol agents applied separately ranged between 38 and 98% (mean 74%) and the coefficient of variation ranged from 9.7 to 75%. The mixture of *Bacillus mycoides* and *Pichia guilermondii* suppressed *Botrytis cinerea* effectively (80 to 99.8% control) under all conditions, and the coefficients of variation were as low as 0.4 to 9% in all cases. Thus, application of both biocontrol agents resulted in better suppression of *Botrytis cinerea*, and also reduced the variability of disease control. Application of more than one biocontrol agent is suggested as a reliable means of reducing the variability and increasing the reliability of biological control.

Additional keyword: integrated pest management.

Saprophytic bacteria, yeast, and filamentous fungi are common inhabitants of plant surfaces (3,16,18,21,22,24,45,48). By various mechanisms, they may alter the growth of pathogens and reduce the diseases they cause (3–5,12,14,23,37,39,49,53). Significant reduction in *Botrytis cinerea* Pers.: Fr. (gray mold) severity has been achieved by filamentous fungi such as *Gliocladium roseum*, *Trichoderma viride*, *T. harzianum*, and *Penicillium* spp. The filamentous fungi were very effective, and some of the isolates were at least as effective as the fungicide used for comparison (16,19, 40,46,52). Reports on the efficacy of yeast and bacteria vary. Although in some studies the isolates resulted in low to moderate disease suppression, their efficacy in other studies was high (52). Yeast strains of the species *Rhodothorula glutinis* and *Cryptococcus albidus* reduced the sporulation of *Botrytis cinerea* and effectively controlled the disease on bean and tomato plants (17). *C. laurenti* effectively protected or prevented apple wound infections by *Botrytis cinerea*, and was comparable in effectiveness to application of benomyl at the postharvest recommended rate (41). Similarly, several species of bacteria (e.g., *Bacillus* spp. and *Pseudomonas* spp.) were effective against *Botrytis cinerea* in tomato (42), grape (20), and strawberry (34,45,46).

Most of the studies reporting high efficacy of the biocontrol agents were conducted under controlled environments. However, effective commercial use of control agents is more problematic. Occasionally, introduction of antagonists that have been highly effective in controlled environments to the phyllosphere of commercially grown plants is only moderately effective, sometimes totally ineffective. For example, a survey of 64 greenhouse experiments conducted all over the world revealed that in approximately 70% of them, *T. harzianum* T39 suppressed *Botrytis cinerea* infections in tomato and cucumber as effectively as the chemical fungicide applied for comparison. However, in 20% of the experiments, the control efficacy (CE) of the biocontrol agent was significantly inferior to that of the fungicide. In 10% of the experiments, disease intensity in plots treated with the biocontrol agent was not significantly different from that in the untreated control plots (43).

Discrepancies in the efficacy of biological control between controlled conditions and commercial situations may have several causes. It was suggested that environmental conditions that are not fully controlled (or not controlled at all) in commercial production may influence the survival, establishment in the phyllosphere, and activity of the biocontrol agents (8,27,28). Under commercial conditions, the phylloplane is subjected to fluctuating temperatures, relative humidities, surface wetness, gases, and air movement (8). These conditions affect the phyllosphere microflora (including the biocontrol agents) directly, or may have an indirect affect by modifying the characteristics of the host plant, e.g., the metabolic state and surface chemistry of the leaves (9,27). The inconsistent efficacy of biological control hinders its implementation on a large scale, especially in high-value crops (7–9,14). One of the ways to overcome the ineffectiveness of most of the biocontrol agents under diverse environmental conditions is to apply them in mixture or in alternation with chemical fungicides (43) or to apply more than one biocontrol agent at a given time (17). Introduction of two or more biocontrol agents to the phyllosphere, assuming that each has different ecological requirements, may facilitate disease control without affecting the efficacy of a single organism under diverse conditions, and may result in increased control consistency.

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The gray mold disease caused by *Botrytis cinerea* inflicts serious losses in many crops (32,45). In strawberry, the fungus attacks flowers, setting fruits, mature fruits, and leaves (43–45). The main sources of inoculum for the disease in strawberry are dead leaves, mummified fruits, straw mulch (where used), neighboring crops, and weeds (45,46). Infected flower parts shed after bloom adhere to the fruit surface. After infecting the flowers, the pathogen may cause quiescent disease until the fruit ripens (31). Diseased fruits are often covered with gray tuft consisting of mycelia, conidiophores, and conidia of the fungus; eventually, the fruits rot. Chemical control is the primary means by which gray mold is controlled. However, this may occasionally be ineffective due to the occurrence of resistant fungal populations (11,17, 30,32). Moreover, there is increasing demand to reduce the use of chemicals for environmental and safety reasons. Thus, the development of nonchemical control measures is of great importance to the strawberry industry worldwide (38,47).

Bacillus mycoides is a gram-positive rod-shaped bacterium that forms an endospore and a relatively thick cell wall (6) and belongs to the *Bacillus cereus* group (10). *Bacillus mycoides* is a rhizosphere inhabitant (Correct word?) that exhibits antagonistic activity toward fungi (35). Isolates of this bacterium controlled *Pythium mamillatum* damping-off in cucumber seedlings (36), stimulated mycorrhizal activity (50), and were effective in controlling rust (*Uromyces phaseoli*) on beans (1). *Pichia guilermondii* is a white yeast with multilateral budding containing one to four hat-shaped ascospores (2). Because the ecological requirements of bacteria and yeast are markedly different (2,6), it was hypothesized that a mixture of the two agents would broaden the range of environmental conditions under which biological control of *Botrytis cinerea* would be feasible. In the present work, we test this hypothesis by introducing the two biocontrol agents, separately or in combination, to the strawberry phyllosphere under a wide range of temperatures, relative humidities, and pre-infection spray timings. Preliminary reports have been published elsewhere (25,26).

MATERIALS AND METHODS

Organisms. *Botrytis cinerea* was cultured on potato dextrose agar (PDA, Difco Laboratories, Detroit) in petri plates. The cultures were incubated for 10 to 14 days at 20°C. Conidia were harvested from the cultures by agitating small pieces of agar bearing mycelium and spores in a glass tube containing 2 ml of tap water and 0.01% (wt/vol) Tween 80 (or Tween 20). The suspension was filtered through cheesecloth, and the spore concentration was calibrated with a hemacytometer, and adjusted to 5×10^5 to 1×10^6 cells per ml. Glucose (0.05%, wt/vol) and KH_2PO_4 (0.05%) were added to the spore suspension.

Two biocontrol agents, a yeast and a bacterium, were used in all trials. The biocontrol agents were isolated from tomato leaves. In a previous work they were selected out of several microbial candidates according to their performance. *Pichia guilermondii* (isolate Y2) was grown on PDA for 24 to 48 h at 25°C before use. *Bacillus mycoides* (isolate B16) was grown on nutrient agar medium (Difco) or on Luria-Bertani agar medium (Difco) for 24 h at 30°C. The yeast and the bacteria were washed from the agar media in 10 ml of saline solution supplemented with 0.01% Tween 80 (or Tween 20). Cell concentrations were determined and adjusted to 1×10^7 cells per ml.

Strawberry plants (*Fragaria ananassa*) cv. Oso-Granade were used in all experiments. Cultivar Oso-Granade is highly susceptible to *Botrytis cinerea*. Seedlings were planted at the beginning of September each year in 1-liter plastic pots (containing growth mixture with a peat base) and maintained in a greenhouse under temperatures of 20 to 30°C. Plants were irrigated and fertilized as needed.

Effects of temperature on generation time. Effect of temperature on generation time of *Pichia guilermondii* and *Bacillus mycoides* was determined in vitro. The biocontrol agents were plated in petri dishes and incubated at different temperatures (2, 5, 10, 15, 20, 25, 30, and 36°C). Cultures from different plates were harvested at different times from plating (0, 7, 25, 32, 48, 120, and 192 h) by scraping the culture from the surface of the agar media with a bacterial loop. Population size of the microorganisms was determined with a hemacytometer. Generation time was calculated for the linear phase of the growth curves with the formula: $g = (e - b) \times ln\ (2X_b)/ln\ (X_e)$, where g = generation time; b = time at the beginning of the linear phase of growth; e = time at the end of the linear phase of growth; X_b = the number cells at time b; and X_e = the number of cells at time e. There were three replicates (petri dishes) for each combination of biocontrol agent × temperature × timing. The experiment was repeated once.

Effects of biocontrol agents on stages in disease cycle of pathogen. Effects of a separate or combined application of *Pichia guilermondii* and *Bacillus mycoides* on *Botrytis cinerea* spore germination were determined on detached strawberry leaflets under a range of temperatures. Suspensions of the bacterium, the yeast, their mixture, or water were sprayed on strawberry leaflets by means of an atomizer sprayer (Desaga GmbH, Wiesloch, Germany). Soon after spraying, drops (20 µl each) containing spore suspension of *Botrytis cinerea* were placed on the leaflets. The leaflets were put in plastic boxes filled with 250 ml of distilled water and covered with polyethylene bags to maintain high relative humidity. The boxes were placed in incubators at temperatures of 4, 10, 15, 20, 25, 30, and 36°C. After 26 h of incubation, germination of *Botrytis cinerea* spores was determined as follows: leaflet pieces bearing a drop of the interacting microorganisms were placed on glass slides, stained with aniline blue, incubated at room temperature (20 to 22°C) for 60 min, and observed microscopically. For each treatment (four microorganism combinations × seven temperatures), germination was determined in samples of 50 conidia of *Botrytis cinerea* that were examined in each of 5 drops from three different leaflet replicates. The experiment was conducted twice (repetitions will be called experiments 1 and 2).

Detached leaflets were also used to determine the effects of the biocontrol agents on lesion development. Strawberry leaflets were sprayed with the biocontrol agents, separately or in combination, or with water as already described. The leaflets were then centrally inoculated with mycelium plugs (5 mm in diameter) cut from 4-day-old cultures of *Botrytis cinerea*. The inoculated leaflets were incubated in plastic boxes at different temperatures (20, 25, 28, and 30°C) and relative humidities (78, 85, 96, and 100%). The relative humidities of less than 100% were achieved by creating saturated salt solutions (51). The saturated solutions, at volumes of 250 ml, were poured into the plastic boxes (30 × 17 × 12 cm in size), which were put in incubators adjusted to the temperatures.

The diameters of the necrotic lesions (i.e., a measure of disease severity) were measured 7 days after inoculation and their areas were calculated. CE for each treatment was calculated by using the values of the disease severity measurements in the biocontrol-treated and water-treated leaflets (D_t and D_u, respectively) as follows: $CE(\%) = 100 - (D_t/D_u) \times 100$. There were five replicates (inoculated leaflets) for each treatment (four microorganism combinations × four temperatures × four relative humidities). The experiment was conducted twice (experiments 3 and 4).

Detached leaflets were used to determine the ability of the biocontrol agents to suppress lesion formation by *Botrytis cinerea* under diverse conditions. The leaflets were first sprayed with suspensions of the separate biocontrol agents, their mixture, or water. Then, 20-µl drops containing spore suspensions of *Botrytis cinerea* (10^6/ml) were placed on the surface of each of five strawberry leaflets. Leaflets were put in plastic boxes under various temperature and relative humidity regimes. Conditions varied slightly among the experiments as follows: in experiment 5, leaflets were incubated at temperatures of 10, 15, 20, and 25°C

and relative humidities of 78, 85, 96, and 100%. In experiment 6, leaflets were incubated for 18 h after inoculation at 20°C and 100% relative humidity and exposed to the same conditions as in experiment 5. In experiment 7, leaflets were preincubated for 26 h at 20°C and 100% relative humidity after inoculation and were transferred to temperatures of 20, 23, 28, and 30°C and relative humidities of 78, 85, 96, and 100%. In experiment 8, whole leaves were used and the petioles were placed in 20-ml glass tubes containing 5 ml of tap water. The tubes were put into plastic boxes at high (>95%) or low (<85%) relative humidities, at temperatures of 20, 25, and 30°C under a 16-h photoperiod. Disease severity in all experiments was determined by estimating the size of the necrotic area where 100% severity = a lesion of 20 mm^2.

Data analysis. Statistical analyses of the data were performed with the JMP-in software, version 3 for Windows (SAS Institute Inc., Cary, NC). Effect of temperature on generation time of the biocontrol agents was determined by means of regression analyses. One-way analysis of variance was performed to determine the effect of temperature on CE of the biocontrol agents. Two-way analysis of variance was used to determine the influence of temperature and relative humidity on lesion size. Three-way analysis of variance was used to determine the influence of temperature, relative humidity, and the time elapsed between spraying with the biocontrol agents and inoculation with *Botrytis cinerea* on lesion size. Analysis of the effects of the abiotic parameters on the ability of the biocontrol agents to suppress *Botrytis cinerea* was performed separately for each agent and for the combination of all the agents.

RESULTS

Effect of temperature on generation time. *Bacillus mycoides* and *Pichia guilermondii* grew and multiplied under a wide range of temperatures. Below 15°C, the bacteria multiplied more rapidly than the yeast. At 5°C for example, generation time of *Pichia guilermondii* was nearly twice that of *Bacillus mycoides* (Fig. 1). Between 15 and 37°C, both biocontrol agents multiplied rapidly (generation time <2.5 h), and the regression curves describing the effect of temperature on generation time were close to one another. Analysis of variance revealed that generation times for both microorganisms were not significantly different within this range of temperatures. Two-way analysis of variance showed that the interaction between the biocontrol agents and temperature was significant ($P \leq 0.05$).

Effects of biocontrol agents on spore germination of *Botrytis cinerea*. Most *Botrytis cinerea* spores germinated on untreated leaflets at temperatures between 10 and 25°C (98 to 100% germination). Although germination decreased markedly at higher and lower temperatures, more than 20% of the spores germinated at as low as 2°C and as high as 37°C (Fig. 2). The biocontrol agents, when applied separately, significantly inhibited spore germination at most temperatures (with the exception of 30°C for *Pichia guilermondii* and 2°C for *Bacillus mycoides*). *Pichia guilermondii* was significantly more effective than *Bacillus mycoides* at temperatures between 2 and 25°C, but at higher temperatures the *Bacillus mycoides* isolate was more effective. A mixture of the two biocontrol agents was effective at all temperatures tested, resulting in 75 to 98% CE (Fig. 2). Inhibition of spore germination was significantly higher in the mixture compared with the separate activities of each biocontrol agent at temperatures above 20°C. Results of a second experiment resembled those shown in Figure 2.

Effects of biocontrol agents on lesion development. Temperature and relative humidity interactively affected the size of *Botrytis cinerea* lesions in unprotected leaflets (Fig. 3). The leaflets were centrally inoculated with mycelium grown on PDA medium, which is a very rich food base. This method of inoculation is similar to natural infections of healthy plant organs touched by infected dead organs. In general, necrotic area increased as ambient relative humidity increased. The necrotic area exceeded 5 cm^2 at relative humidities lower than 100% only when temperatures were 23 or 28°C. At lower or higher temperatures, lesions were formed only at 100% relative humidity. Similarly, the effect of temperature on lesion formation was related to the ambient relative humidity. At 23 and 28°C, lesion size was larger than 10 cm^2 under relative humidity ranging from 85 to 100%. Under lower and higher temperatures, lesions were formed only at 100% relative humidity (Fig. 3). The ability of *Pichia guilermondii* and *Bacillus mycoides* to suppress *Botrytis cinerea* development was governed by the temperature and relative humidity regimes of the test. Both agents were highly effective (i.e., lesion size was <3 cm^2) at temperatures of 20 and 23°C under all relative humidities tested. However, at 28 and 30°C, they were not as effective at

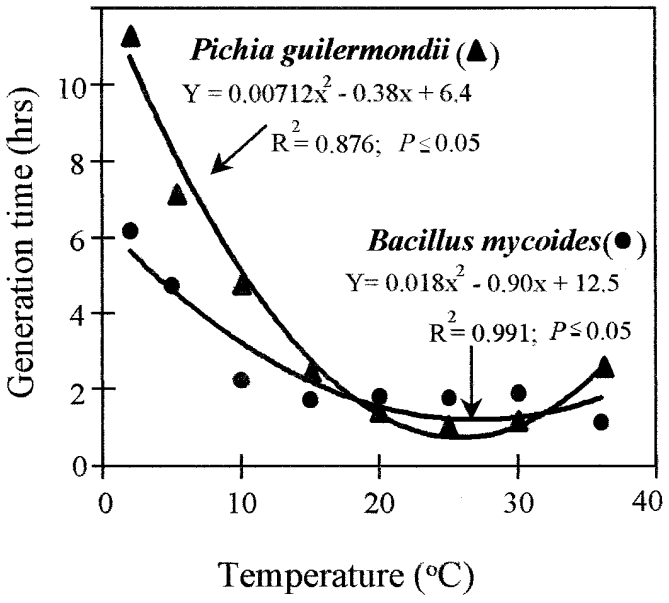

Fig. 1. Effect of temperature on generation time of *Pichia guilermondii* and *Bacillus mycoides*.

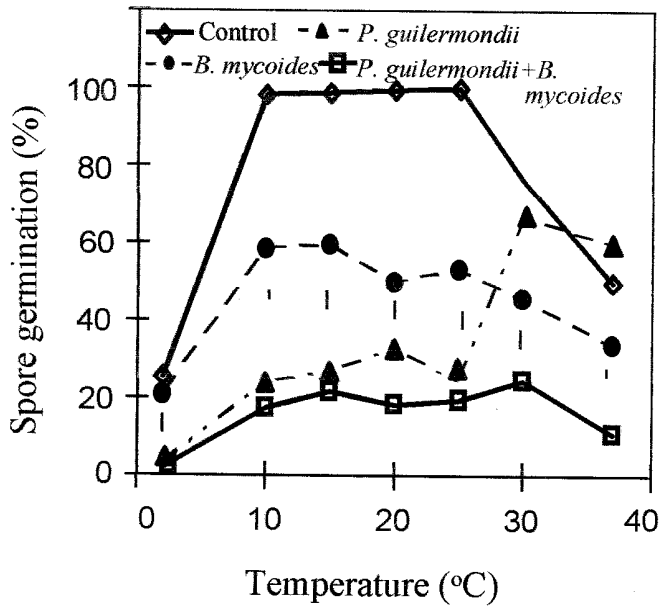

Fig. 2. Effect of temperature on inhibition of *Botrytis cinerea* spore germination by *Pichia guilermondii*, *Bacillus mycoides*, or their mixture. Bars represent the least significant differences (at $P \leq 0.05$) for each temperature.

96 and 100% relative humidity, and lesion size exceeded 10 cm^2 in some cases (Fig. 3).

The variable efficacy of *Pichia guilermondii* in suppressing lesion development of *Botrytis cinerea* under the diverse conditions resulted in significant effect of the abiotic parameters (temperature and relative humidity) and their interaction in the analysis of variance (Table 1). For *Bacillus mycoides*, none of the parameters was significant at $P \leq 0.05$. Combined application of both biocontrol agents resulted in significantly better suppression of *Botrytis cinerea* lesion development at 28°C and 96% relative humidity, and also reduced the variability of disease control. Consequently, the effects of the parameters and their interaction in the analysis of variance were insignificant in the statistical test (Table 1).

Effects of biocontrol agents on lesion formation. Both *Bacillus mycoides* and *Pichia guilermondii* suppressed lesion formation of *Botrytis cinerea* significantly under most temperature, relative humidity, and spray timing combinations. However, under certain combinations CE was not as pronounced. For example, *Bacillus mycoides* failed to suppress *Botrytis cinerea* when applied on the date of pathogen inoculation on leaves maintained at 20°C and high relative humidity (Fig. 4A). Accordingly, the

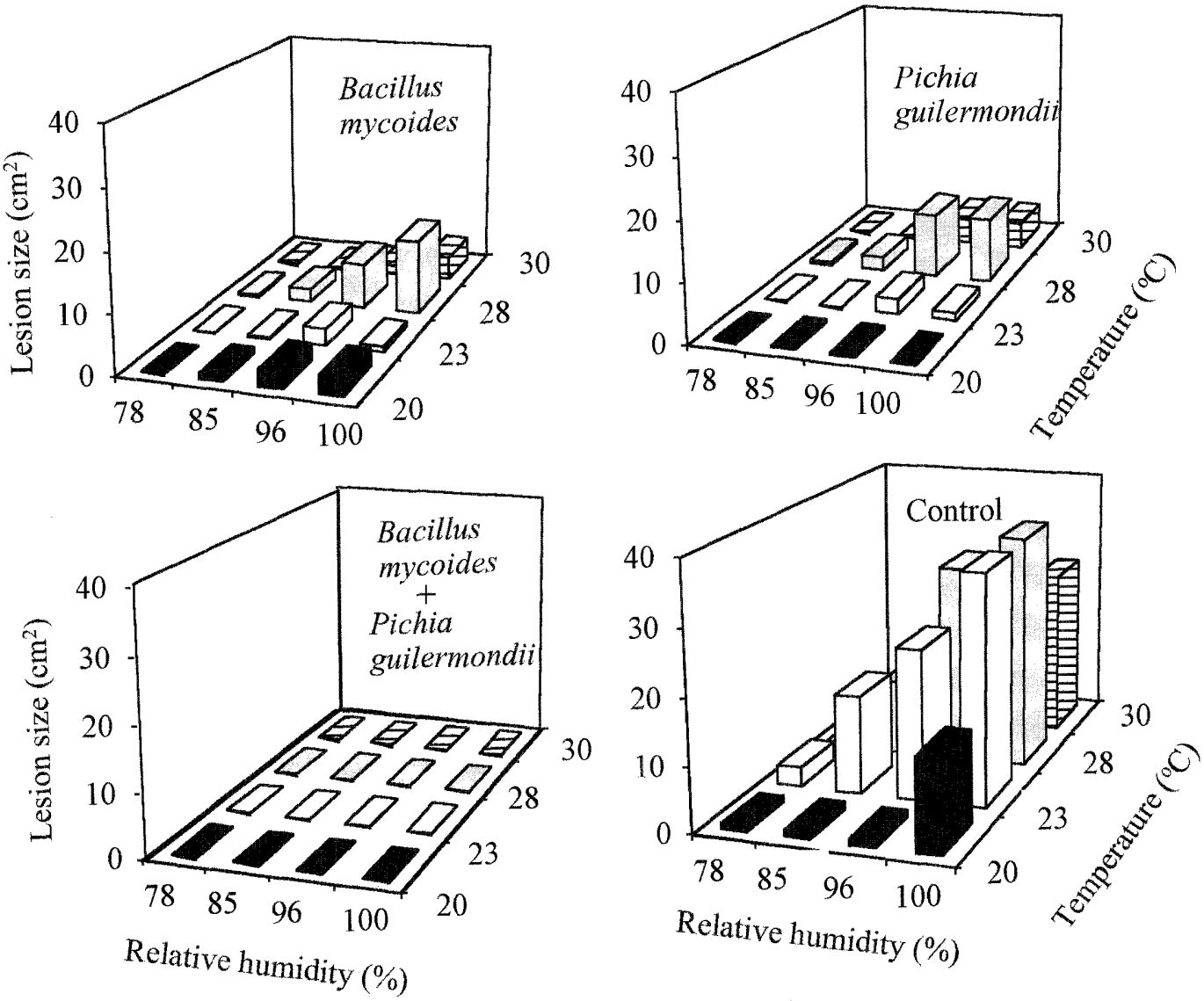

Fig. 3. Effect of temperature and relative humidity on suppress of *Botrytis cinerea* lesion size on strawberry leaflets by *Bacillus mycoides*, *Pichia guilermondii*, or their mixture. Analysis of variance for the data is presented in Table 1.

TABLE 1. Analysis of variance for the influence of temperature and relative humidity on lesion size of *Botrytis cinerea* on detached strawberry leaflets[z]

		Pichia guilermondii (*Pgl*)			*Bacillus mycoides* (*Bmy*)			*Pgl + Bmy*		
Source of variability	df	SS	*F*	*Pr > F*	SS	*F*	*Pr > F*	SS	*F*	*Pr > F*
Temperature	3	15.61	7.2	0.0005	28.85	1.4	0.2519	0.1909	1.6	0.76
Relative humidity	3	37.64	17.3	<0.0001	54.35	2.6	0.0596	0.0646	2.6	1.20
Temperature × relative humidity	9	31.04	4.7	0.0002	57.27	0.9	0.5059	0.2490	1.3	1.85
Block	3	0.98	0.4	0.7146	39.38	1.9	0.1389	0.1997	1.6	0.75
Error	45	32.47	…	…	306.73	…	…	…	…	6.98
Total	63	117.74	…	…	486.58	…	…	…	…	11.54

[z] SS = sum of squares. The leaves were treated with *Pichia guilermondii*, *Bacillus mycoides*, or their mixture.

effects of some of the main factors and their interactions in the analysis of variance were significant. This indicates that CE achieved by the biocontrol agents, when applied separately, was governed by environmental conditions (Table 2). The combined *Bacillus mycoides* and *Pichia guilermondii* treatment suppressed *Botrytis cinerea* effectively (80 to 99.8% control) under all conditions. Moreover, the effects of the main abiotic factors and their interactions (from analysis of variance of the data) were insignificant (Table 2). This indicates that CE achieved by the mixture was independent of the environmental conditions. Results observed in the other experiment resembled those shown in Figure 4.

Variability of disease suppression. Results of the eight experiments conducted in this study were analyzed consecutively. For that purpose, of each of the treatments was calculated and the mean CE (±SD) of all treatments was calculated for each experiment (Table 3). Control efficacy achieved by *Pichia guilermondii* and *Bacillus mycoides* in all experiments ranged from 38 to 96% (mean 74%). Variability was high, and the coefficient of variation ranged from 9.7 to 75%. For the combined *Pichia guilermondii* and *Bacillus mycoides* treatment, control efficacy was high in most experiments (range: 80 to 99.8%; mean: 93.2%), and the coefficients of variation were as low as 0.4 to 9% in all cases (Table 3).

DISCUSSION

Effective suppression of plant diseases by biocontrol agents is largely affected by environmental conditions. The environment affects the establishment, survival, and activity of the biocontrol agents (7,13,17–19,27,28,43). Many examples are reported in the literature. For example, the biocontrol agent *T. harzianum* T39 is less effective at suppressing cucumber fruit and stem gray mold under wet conditions and temperatures below 20°C than under dry conditions and higher temperatures (19). High temperature during the day and high vapor pressure deficit during the night were associated with reduced efficacy of *Botrytis cinerea* suppression in cucumber and tomato by *Trichoderma harzianum* T39, *Aureobasidium pullulans,* and *C. albidus* (13,15). Similarly, Hannusch and Boland (28) indicated that most of the biocontrol agents they studied were highly dependent on the environment for efficacy.

In most studies dealing with the biological control of plant pathogens, only one biocontrol agent is applied. However, attempts to apply more than one have been made for several reasons. Elad et al. (17,18) suggested that application of more than one isolate may be needed to prevent *Botrytis cinerea* infection and to reduce its sporulation. In other studies, application of several biocontrol agents simultaneously was attempted to improve control efficacy (23,24,26,33). For example, combinations with four bacterial antagonists and conidia of *Trichoderma* sp. in strawberry reduced *Botrytis cinerea* spore germination markedly (32). We suggest that application of more than one antagonist, provided the antagonists have different ecological requirements, will increase the reliability and decrease the variability of biological control.

Two microorganisms were combined in this study: a yeast (*Pichia guilermondii* isolate Y2) and a bacterium (*Bacillus mycoides* isolate B16). The ecological requirements of the two microorganisms differed when conditions were extreme: below 15°C the bacterium multiplied more rapidly than the yeast (Fig. 1). Under conditions of changing temperatures, the bacterium might compensate for the inability of the yeast to multiply rapidly. Moreover, *Bacillus* spp. may produce endospores that enable them to survive stress conditions, such as extreme temperatures or drought (6). However, it should be noted that the ability of one microorganism to multiply more rapidly than the other does not necessarily indicate better disease control when either is used in combination with other biocontrol agents.

An important stage of the disease cycle is pathogen spore germination. Spores of *Botrytis cinerea* germinated at high frequency at temperatures between 10 and 25°C; at lower or higher temperatures, germination rate decreased (Fig. 2). Whereas *Pichia guilermondii* reduced spore germination at temperatures lower than 25°C, *Bacillus mycoides* was more effective above 25°C. The mixture of both microorganisms suppressed spore germination adequately (>80% control) at all temperatures tested (Fig. 2).

Effect of the biocontrol agents on lesion formation and development were tested under a wide variety of temperature and relative humidity regimes. Both biocontrol agents, when used separately, were highly effective at suppressing *Botrytis cinerea* under conditions that were suboptimal for the pathogen, but their efficacy was lower under conditions optimal for pathogen development (Figs. 3 and 4). Combined application of *Pichia guilermondii* and *Bacillus mycoides* resulted in significant suppression of *Botrytis cinerea* under all regimes tested (Fig. 4).

The coefficient of variation is a standardized measure for variability, and permits a comparison of the degree of repeatability of treatments employed under diverse conditions. The coefficients of variation for the combined biocontrol treatment were low in all experiments. Thus, combined application of both biocontrol agents resulted in better suppression of *Botrytis cinerea*; it also reduced the variability of disease control (Tables 1 to 3, Fig. 4). In

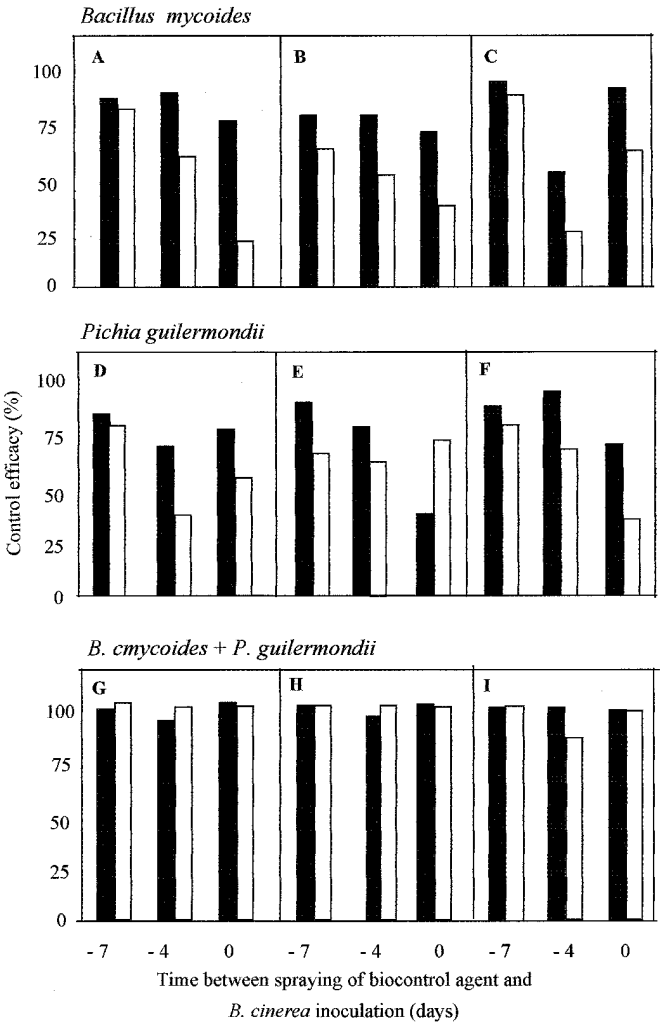

Fig. 4. Suppression of *Botrytis cinerea* on strawberry leaflets by *Bacillus mycoides*, *Pichia guilermondii*, or their mixture, under various temperatures, relative humidities and spray-timings. **A, D, and G,** temperature of 20°C; **B, E, and H,** temperature of 25ºC; and **C, F, and I,** temperature of 30°C. Open white bars: high relative humidity (>95%); solid black bars: low relative humidity (<85%). Analysis of variance of the data is presented in Table 2.

TABLE 2. Analysis of variance for the influence of temperature, relative humidity, and the time elapsed between application of the biocontrol agents and inoculation with *Botrytis cinerea*, on control efficacy of the disease by *Pichia guilermondii*, *Bacillus mycoides*, or their mixture[z]

		Pichia guilermondii (*Pgl*)			*Bacillus mycoides* (*Bmy*)			*Pgl* + *Bmy*		
Source of variability	df	SS	*F*	*Pr* >*F*	SS	*F*	*P r* >*F*	SS	*F*	*Pr* >*F*
Temperature	2	1,871	3.73	0.0303	225	0.65	0.5276	55	0.82	0.4457
Relative humidity	1	5,940	23.69	<0.0001	10,471	60.05	<0.0001	4	0.13	0.7158
Timing	2	6,537	13.04	<0.0001	6,701	0.65	<0.0001	172	2.57	0.0875
Temperature × relative humidity	2	1,655	3.73	0.0443	608	1.74	0.1844	89	1.32	0.2737
Temperature × timing	4	3,887	3.87	0.0077	4,318	6.19	0.0004	61	0.45	0.7683
Relative humidity × timing	2	923	1.84	0.1682	4,384	12.57	<0.0001	56	0.83	0.4382
Timing × temperature × relative humidity	4	1,816	1.81	0.1400	1,025	1.47	0.2239	163	1.21	0.3152
Error	54	13,536	…	…	9,416	…	…	…	…	6.98
Total	71	36,165	…	…	37,148	…	…	2,413	…	…

[z] SS = sum of squares.

TABLE 3. Control efficacy of *Botrytis cinerea* achieved by *Pichia guilermondii*, *Bacillus mycoides* or their mixture in eight experiments

	Pichia guilermondii (*Pgl*)			*Bacillus mycoides* (*Bmy*)			*Pgl* + *Bmy*		
Experiment number	% Control efficacy[x]	SD[y]	% CV[z]	% Control efficacy	SD	% CV	% Control efficacy	SD	% CV
1	53.3	40.2	75.4	38.2	10.2	26.8	80.0	7.2	9.0
2	53.3	40.1	73.3	37.8	11.1	29.4	79.9	7.1	8.9
3	87.3	19.1	21.9	83.5	20.3	24.4	96.6	6.8	7.0
4	68.8	35.7	51.9	71.9	36.9	51.4	96.3	4.5	4.7
5	72.6	18.4	25.3	72.5	23.6	32.6	96.7	2.3	2.4
6	95.8	9.3	9.7	95.1	11.1	11.7	99.8	0.4	0.4
7	79.0	22.5	28.5	78.9	22.8	22.9	97.0	5.8	5.9
8	75.7	24.0	36.6	72.0	17.2	25.1	93.2	4.5	5.0

[x] Various temperatures, relative humidities, and spraying timing treatments were applied in each experiment. Values are the mean control efficacy records calculated for all the treatments in each experiment.
[y] SD = standard deviation.
[z] CV = coefficient of variation (%).

some cases, the suppression of *Botrytis cinerea* in the combined treatment was significantly higher than that achieved by each of the biocontrol agents alone. This was not expected, and suggests that interactions (possibly synergism) between the microorganisms may affect their activity. The improved efficacy of the mixture may be the result of different modes of action of the biocontrol agents. The role of mechanisms of antagonism in the improved control by the mixture of microorganisms is currently being investigated.

As already indicated, inconsistency and low repeatability in CE are the main reasons why biological control is not utilized on a large scale (7,13,14,19,27,42). One of the ways to overcome this problem is to integrate biological and chemical control measures, either by alternating applications or by taking into account the ecological requirements of the biocontrol agents. Integrated use of biological and chemical control measures has resulted in consistent, highly effective disease suppression in trials conducted in a commercial greenhouse (43). Results of our study suggest that application of more than one biocontrol agent at a time is another means of reducing the variability and increasing the reliability of biological control.

In most countries, biocontrol agents must be registered before use. The cost of registration is high. If a combination of biocontrol agents is attempted, each one needs to be registered independently. This requirement will certainly increase the cost of developing a multi-agent product as well as the cost of its use. The higher costs may put the use of such an approach in question (29). However, these problems must be solved if we are serious about the development of commercial products for biological control of plant disease (34).

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