Control of Infection and Sporulation of *Botrytis cinerea* on Bean and Tomato by Saprophytic Yeasts

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ABSTRACT

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Saprophytic yeasts were screened for their ability to reduce the sporulation of *Botrytis cinerea* and the severity of gray mold. One isolate of *Rhodotorula glutinis* and two isolates of *Cryptococcus albidus* effectively controlled disease on bean and tomato plants. Their ability to reduce the germination of conidia and the severity of rot symptoms on detached leaves and to control the disease on whole plants at calculated concentrations of 750–7,500 cells per square centimeter under growth-room conditions was consistently as effective as the known biological control agent *Trichoderma harzianum* T39 (unformulated). Glucose and KH_2PO_4 (0.02 M each) were added to conidial suspensions of *B. cinerea* to enhance infection. The yeast isolates were effective at this low nutrient level. All isolates competed for nutrients with the germinating conidia of *B. cinerea*, but resistance induced in the host by live or dead yeast cells may also have been involved. Small amounts of compounds inhibitory to *B. cinerea* germination were detected on treated leaves. Establishment of yeast populations on healthy and *Botrytis*-infected leaves and flowers was investigated. Five days after application, population densities of yeasts reached 7–8 $\times$ 10^3 cfu/cm^2 of healthy bean and tomato leaves and increased 7.5- and 8.5-fold on infected leaves of the respective crops. Lesion expansion and sporulation of *B. cinerea* were reduced after its establishment on leaves.

The surfaces of the aerial parts of plants are subjected to fluctuations in temperature, radiation, relative humidity (RH), surface wetness, gases, and air movement (6). These conditions may affect phyllosphere microflora directly, or they may have an indirect effect by modifying leaf characteristics, e.g., metabolic state, morphology (7), and surface chemistry (21). On the plant surface, nutrients are necessary for the growth of saprophytes and pathogens that have an epiphytic growth phase before penetration (2,34). The quality and quantity of leachates from plants are influenced by plant age (2) and environmental parameters such as temperature, light, fertilization, pollen, and surface moisture (34,36,37). Yeast, bacteria, and filamentous fungi are common inhabitants of plant surfaces (3,8,15). The importance of nutrient competition in antagonism of necrotrophic fungi has been demonstrated (5,16,19). Yeast and bacteria are known for their ability to reduce conidial germination by competition for nutrients (4,9); however, the introduction of antagonists to the phyllosphere for the control of leaf infections by necrotrophic pathogens has been in many cases only moderately effective. It was suggested (17) that in order to provide effective control, the introduced microorganism has to be established in the phyllosphere before the arrival of the pathogen. Once infection has occurred, it might be too late for the microorganism to be effective.

Many necrotrophs sporulate abundantly on lesions as well as on dead plant material, and their conidia contribute to epidemics in the same season or in the one that follows. *Botrytis* spp. sporulate abundantly on necrotic tissue and crop remains, and the conidia from successive cycles of infection contribute to the development of an epidemic within the crop (25,35,41). Recently, Peng and Sutton (29), working with *B. cinerea* on strawberries, and Fokkema et al (18,25), working with *B. aclada* on onion, proposed to reduce pathogen sporulation by means of biocontrol agents in order to minimize the conidial load on the crop. They found several isolates of filamentous fungi effective in reducing sporulation of the established pathogens. Similarly, *Trichoderma harzianum* Rifai suppressed sporulation of *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur on excised leaves of wheat seedlings (1).

Interaction between saprophytes and germinating propagules of necrotrophic pathogens differs from their interaction with the sporulating phase of the pathogens. The time for interaction with germinating propagules is shorter than the extent to which the sporulating phase is exposed. The interaction with germination takes place on the plant surface, whereas the interaction with sporulation is in necrotic lesions.

The purpose of the present work was to study the biocontrol of gray mold by saprophytic yeasts with the goal of preventing infection. Furthermore, the effect of yeasts applied to established lesions on sporulation of the pathogen was tested.

MATERIALS AND METHODS

Yeast cultures. Twenty isolates of *Rhodotorula, Cryptococcus,* and some unidentified white and pink isolates (all from the collection of the Agricultural Research Department, Research Institute for Plant Protection [IPO-DLO] Wageningen, The Netherlands) were screened on leaves with the pathogen as detailed below. Identification of the potential biocontrol agents was carried out by Centraalbureau Voor Schimmelcultures at Delft, The Netherlands. One isolate used throughout this work was *C. albidus* (K. Saito) C. E. Skinner WCS36 isolated from rye leaves (15) and referred to here as strain 053. The remaining isolates were from potato leaves (G. J. Kessel and P. Jongebloed, Ecology Section, IPO-DLO). The yeasts were grown at 20 C on basal yeast agar (BYA) (10). Cells scraped from the surface of 3-day-

old BYA cultures were suspended in sterilized tap water, and concentrations were adjusted to 10^6–10^7 cells per milliliter. Cells were counted with a hemacytometer. If not otherwise specified, 6×10^6 cells per milliliter were used. In some experiments, the performance of the yeast cells was compared with the performance of the biocontrol agent *T. harzianum* T39 from Y. Elad's lab in Israel (12,14). It was maintained and grown on potato-dextrose agar. Conidia of *T. harzianum* from 2-wk-old cultures were suspended in sterilized tap water, and 10^6 conidia per milliliter were used.

Pathogen. *B. cinerea* Pers.:Fr. (isolate 700) was grown on tomato leaf agar (32) at 20 C. Conidia from 10-day-old cultures were suspended in water containing 0.01% Tween 80 to give 10^5–10^6 cells per milliliter. If not otherwise specified, 6×10^5 cells per milliliter were used.

Host plants. Plants of bean (*Phaseolus vulgaris* L. 'Groffy') and tomato (*Lycopersicon esculentum* Mill. 'Money Maker') were planted in 1-L plastic pots (containing a potting mixture based on peat) and grown in a greenhouse at 20–25 C, a vapor pressure deficit (VPD) of 806–352 Pa, and RH of 75–85%. Leaves were collected from 3- to 4-wk-old bean plants and 4- to 5-wk-old tomato plants. Similar plants were used for whole-plant experiments. Detached leaves were incubated in plastic trays ($30 \times 45 \times 5$ cm) on a plastic grid that was laid over water-soaked filter paper. The cut end of each petiole was inserted into wet floral foam as described by Leone and Tonneijck (27) in order to maintain freshness throughout the course of the experiments. The trays were kept in transparent polyethylene bags to allow for conditions of VPD <105 Pa (high RH) and condensation. Detached flowers from 1-mo-old bean plants and fruits from 7-wk-old bean plants were similarly incubated. Whole plants were kept in polyethylene bags on a tray containing water to ensure the same VPD, unless otherwise specified. The plant material was kept in an illuminated (10.3 $J/cm^2/h$, at leaf level) walk-in growth chamber at 18 C, unless otherwise specified. When other temperatures were required, plant material was incubated in growth chambers having a similar light intensity.

Treatment of plant material. Plant material was inoculated with a conidial suspension of *B. cinerea* in a solution 0.02 M glucose and 0.02 M KH_2PO_4 to promote infection (27). The suspension was applied to whole plants by means of an atomizer at a volume of 1.5 ml per plant (approximately 50 μl per leaf), resulting in approximately 50–100 conidia per square centimeter. Yeast suspensions were similarly applied on the wet, infested plants within 10 min, unless another spraying sequence was indicated. In one experiment, the biocontrol isolates were applied 5 days before *B. cinerea*. Densities of introduced yeasts or of T39 conidia were 7×10^2–1×10^3 cells per square centimeter of leaf, unless otherwise specified. Detached leaves were infected with 30-μl drops containing similar (unless otherwise indicated) suspensions of conidia and yeast cells. Up to four treatments of 20 drops each were applied to each leaf at a spacing of at least 1 cm.

In most of the experiments, the concentration of nutrients mentioned above (referred to as "low nutrient level") was used; however, in certain experiments, no nutrients or a 10-fold, "high nutrient level" concentration was used.

In order to trace possible inhibitory compounds produced on leaves, drops of water containing the yeasts and cells of *B. cinerea* were collected in Eppendorf tubes from the treated leaves after 2 days of incubation. The suspensions were centrifuged twice, filtered to render a cell-free liquid, and placed on fresh leaves along with a new suspension of pathogen conidia, as described above.

In certain experiments, drops containing conidia of the pathogen were placed on leaves at sites remote (2 cm) from the drops containing the antagonist. The effect of dead yeast cells was also tested. Cells from 3-day-old cultures were killed by one of the following methods: 1) exposure of a paste of cells to gamma irradiation (4.5 Mrad from a cobalt source), 2) heat treatment (100 C for 1 h), or 3) immersion in a solution of 60% ethanol for 10 min. All treated cells were washed aseptically five times by centrifugation and resuspended in sterile distilled water at the same concentration as the live cells.

Germination of conidia. The germination of *B. cinerea* conidia in water drops on detached leaves was observed with a light microscope after 20 h of incubation. Pieces of leaves bearing drops of the interacting microorganisms were excised and placed on glass slides, dyed with aniline blue, incubated at room temperature for 5 min, and observed. Two hundred conidia from five drops of each treatment were observed. The percentage of germination and the lengths of germ tubes were recorded. Estimation of germ tube biomass produced by the pathogen on leaves was calculated by multiplying the percentage of germination by the average germ tube length. Because germ tube lengths did not vary between replicates of each treatment, this was regarded as a good estimation of the germ tube biomass.

Severity of symptoms induced by the pathogen on leaves. Symptoms of the drop-infected leaves were evaluated on a 0–5 arbitrary scale of intensity of rot underneath the inoculation droplet, where 0 = symptomless leaf tissue; 1 = 1–12% rot under the droplet; 2 = 13–25%; 3 = 26–50%; 4 = 51–100%; and 5 = rot extending about 2 mm around the droplet. Symptoms on whole plants were evaluated according to a severity index of 0–5, where 0 = healthy plant and 5 = plant completely destroyed (13).

Established lesions. Leaves on whole plants or detached leaves were infected by mycelial plugs (3 mm in diameter) from the edges of 3- to 4-day-old nonsporulating cultures of *B. cinerea* on potato-dextrose agar. Lesions appeared within 2–3 days. Leaves bearing nonsporulating lesions 10–15 cm^2 in size were sprayed with the suspensions of yeasts. The treated plant material was incubated under continuous conditions of high humidity (VPD <10^5 Pa) until assessment of sporulation. In certain cases, the yeast-treated plants were exposed to a lower RH, 75–85% (VPD 524–314 Pa), for 2–4 days after treatment. Sporulation was evaluated by counting the conidiophores of all lesions under a stereo binocular microscope.

Dead plant material. Leaves of bean or tomato and stem segments (3 cm from the third internode) were killed by one of

TABLE 1. Effects of saprophytic yeast isolates *Rhodotorula glutinis* F147 and *Cryptococcus albidus* F131 and 053 and *Trichoderma harzianum* T39 on *Botrytis cinerea* infection of bean

Isolate	Germination of *B. cinerea* conidia (%)	Total germ tube length (μm/100 conidia)	Symptom severity on detached leaves[x]	Flower infection (%)	Pod infection[y]	Gray mold severity[y]
Control	86 a[z]	26,000 a	3.3 a	100 a	4.1 a	3.3 a
F147	0 c	0 c	0.5 c	66 b	0.8 b	0.6 b
F131	25 b	1,050 b	1.7 b	51 b	0.7 b	1.0 b
053	5 c	100 c	0.7 c	50 b	0.9 b	1.4 b
T39	8 c	900 b	0.4 c	26 c	1.1 b	0 b

[x]Symptoms occurring under drops of inoculum of *B. cinerea* and the antagonists. Severity was indexed on a scale of 0–5, where 0 = no symptoms and 5 = rot extending about 2 mm around the drop.

[y]Severity of gray mold on pods and whole plants was indexed on a scale of 0–5, where 0 = noninfected plant and 5 = plant completely covered with gray mold.

[z]Numbers in a column followed by a common letter do not differ significantly at $P \leq 0.05$.

two methods. 1) Plant material was placed on moistened filter paper in a glass petri dish and exposed to microwave radiation (50 leaves at 100 W energy input for 5 min). The plates with killed leaves were rinsed with sterile water five times to wash away the excess nutrients released from the plant tissues. 2) Plant material was exposed to gamma radiation (4.5 Mrad from a cobalt source) and then washed with a 20-fold volume of water for 1–3 h and placed in sterile petri dishes on moistened filter paper. Dead plant material was inoculated aseptically with a suspension of *B. cinerea* conidia (10^4/ml, resulting in approximately 20 conidia per square centimeter), allowed to colonize for 2 days, and then sprayed aseptically with a suspension of yeast cells (10^6/ml, resulting in approximately 2,000 cells per square centimeter immediately after spraying).

TABLE 2. Effects of saprophytic yeast isolates *Rhodotorula glutinis* F147 and *Cryptococcus albidus* F131 and 053 and *Trichoderma harzianum* T39 inoculated simultaneously in nutrient solution on infection of tomato by *Botrytis cinerea*

Isolate	Germination of *B. cinerea* conidia (%)	Total germ tube length (μm/100 conidia)	Symptom severity on detached leaves[x]	Gray mold severity[y]
Control	80 a[z]	12,000 a	3.4 a	2.3 a
F147	0 b	0 b	0.6 b	0.4 b
F131	9.0 b	600 b	1.2 b	1.65 b
053	0 b	0 b	0.23 b	1.35 b
T39	5.2 b	410 b	1.1 b	0.15 b

[x]Symptoms occurred under drops of inoculum of *B. cinerea* and the antagonist. Severity of symptoms was indexed on a scale of 0–5, where 0 = no symptoms and 5 = rot extending about 2 mm around the drop.
[y]Severity of gray mold was indexed on a scale of 0–5, where 0 = healthy plant and 5 = plant completely destroyed.
[z]Numbers in a column followed by a common letter do not differ significantly at $P \leq 0.05$.

Population dynamics of yeast and bacteria on plant material. Five leaves from each treatment were collected and placed immediately into 100 ml of sterile Ringer solution in a 250-ml conical flask. The leaves were washed thoroughly by agitating the flasks at 150 spm (Agitator, Stuart Scientific, Great Britain) for 30 min at room temperature. Aliquots of the proper dilution were plated on BYA, and the colonies were counted 2 days later. Results were expressed as colony-forming units per square centimeter of leaf.

In certain experiments, populations of naturally occurring bacteria and yeasts were counted. The original drops of suspensions placed on bean leaves were collected with a micropipette. The suspensions were plated on BYA supplemented with 0.25 mg of chloramphenicol per liter for counting yeasts and on nutrient agar supplemented with 100 mg of cycloheximide per liter for counting bacteria. Results were expressed as colony-forming units per milliliter of water.

Statistical analysis. Experiments were arranged in completely randomized or randomized block designs and repeated at least

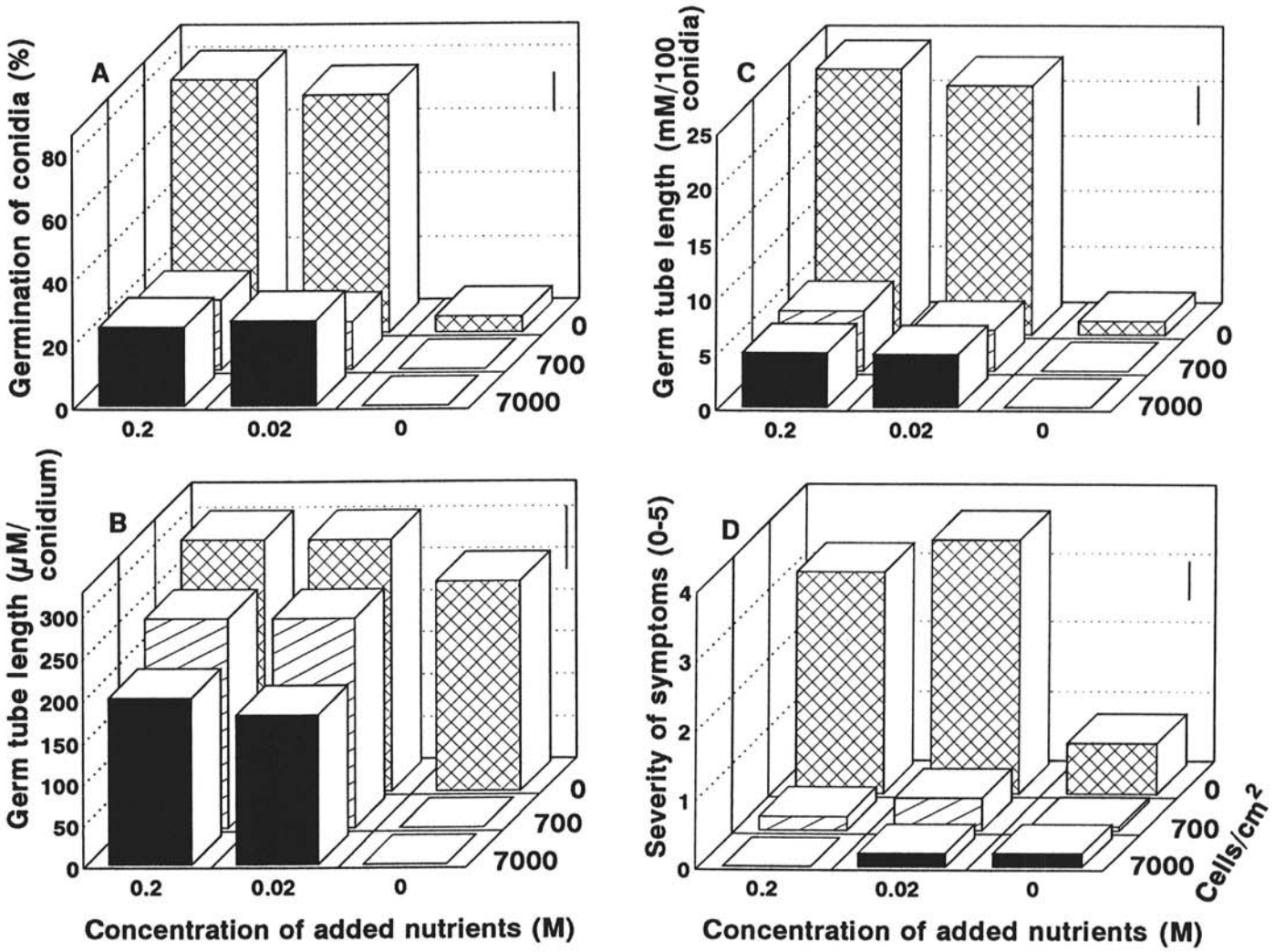

Fig. 1. Effects of nutrient concentration and density of cells of *Cryptococcus albidus* isolate 053 applied to bean leaves on **A,** the percentage of germination of conidia of *Botrytis cinerea;* **B,** average conidial germ tube length of germinated conidia; **C,** total germ tube length per 100 conidia; and **D,** severity of symptoms. The yeast cells were applied to achieve three densities on leaves, and 0.02 M glucose and 0.02 M KH_2PO_4 were applied as nutrients. Bars = LSD ($P \leq 0.05$) at 11 df.

twice with similar results. An analysis of variance was run on data from all three trials. Variance of trials of the same experiment was similar. The main effect of trial of experiments presented in Results was not significant ($P = 0.08$–0.27), and interactions between treatments and trial factors were not significant. Treatments were replicated six to 12 times. Data were arcsine transformed, analyzed by analysis of variance, and tested for significance with Student-Newman-Keuls's multiple range test. Findings of one experiment are presented in Results.

RESULTS

Control of conidial germination and gray mold. Screening of antagonists was carried out with isolates of saprophytic pink and white yeasts. Screening was based on the effect of the candidates on germination and germ tube elongation of *B. cinerea* on leaves, on severity of symptoms of infected leaves, and on sporulation of the pathogen on established lesions. The yeast isolates *R. glutinis* (Fresen.) F. C. Harrison F147 and *C. albidus* F131 and 053 were found to be most effective and were used in further experiments (Tables 1 and 2). Conidial germination on detached bean leaves and symptoms induced on leaves by drops of conidial suspensions were best reduced by isolates F147 and 053. The control of flower, pod, and whole bean plant infection was reduced significantly by the three isolates; there was no significant difference in their activity (Table 1). All three isolates significantly reduced the germination of *B. cinerea* conidia and symptoms incited by it on detached leaves and on whole plants of tomato (Table 2). Similar results were obtained with T39 (Tables 1 and 2).

Effect of temperature on yeast activity. Leaves of bean were treated simultaneously with each of the three yeast isolates and conidia of *B. cinerea* and incubated at 10 and 15 C. Germination of conidia of *B. cinerea* after 24 h on yeast-free leaves at these temperatures were 45 and 95%, respectively; germ tube lengths of the germinated conidia were 150 and 200 μm per conidium, respectively; and symptom severity ratings were 2.6 and 3.2, respectively. All yeast isolates reduced germination of conidia and germ tube lengths by more than 95%. Isolates F147, F131,

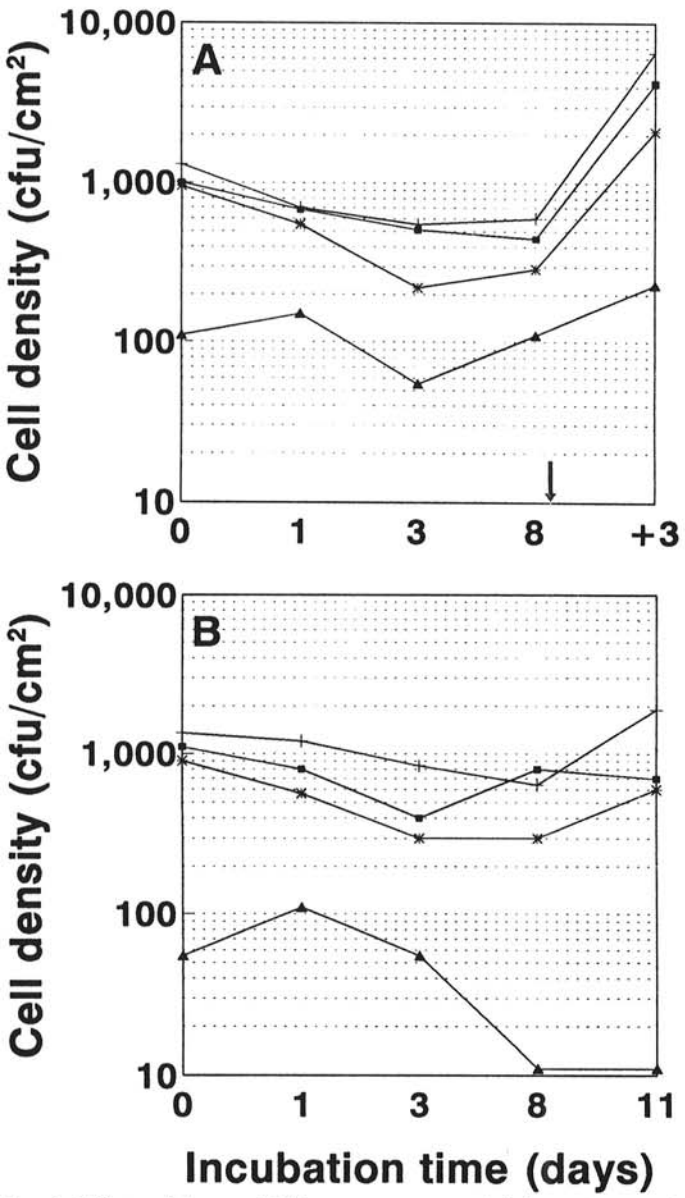

Fig. 2. Effects of low and high vapor pressure deficits on levels of introduced yeast populations on bean leaves. The plants were sprayed with *Rhodotorula glutinis* isolate F147 (+) and *Cryptococcus albidus* isolates F131 (*) and 053 (■). ▲ = Indigenous yeast population. The plants were incubated at 18 ± 1 C **A,** at 314–524 Pa (75–85% RH) for 8 days followed by 3 days (+3) at 105–209 Pa or **B,** at 105–209 Pa (90–95% RH) for 11 days. During the course of the experiment, the cell density of introduced yeasts was significantly higher than the cell density of the indigenous yeasts at $P \leq 0.05$.

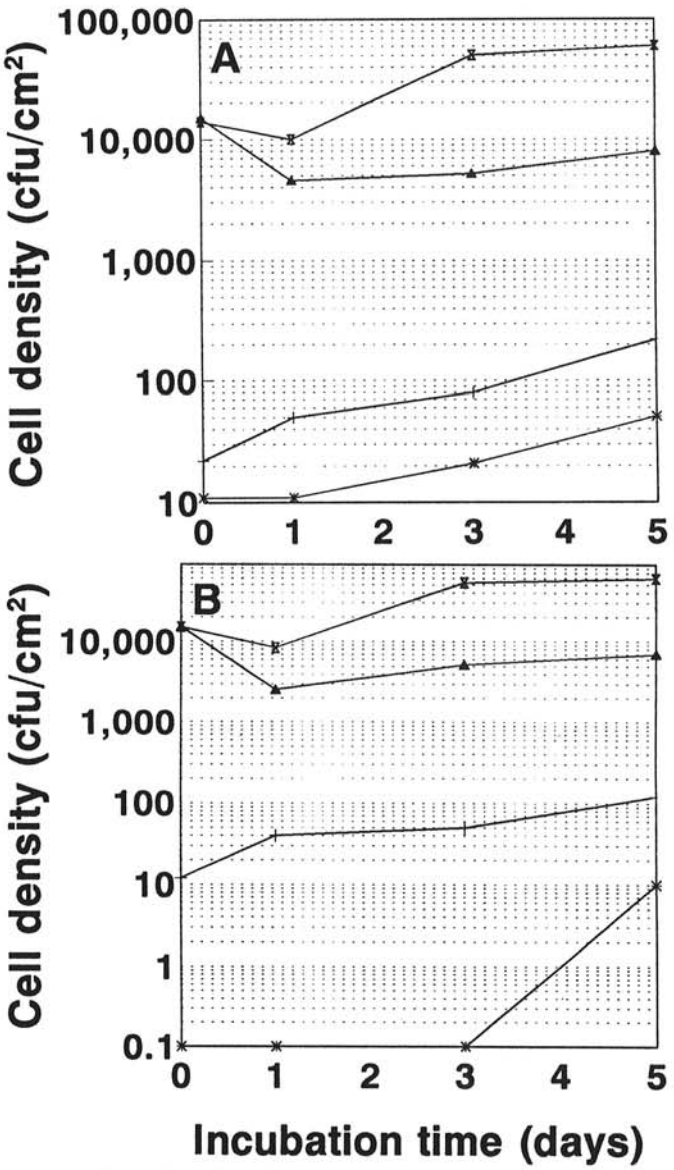

Fig. 3. Population densities of indigenous yeasts on healthy (*) and necrotic (+) leaves and of *Cryptococcus albidus* isolate 053 on healthy (▲) and necrotic (⧖) leaf tissues of **A,** tomato and **B,** bean plants. Populations of the introduced *C. albidus* and of indigenous yeasts on necrotic and healthy leaves differed significantly throughout the entire incubation period ($P \leq 0.05$).

and 053 significantly ($P \leq 0.05$) reduced disease severity ratings on leaves incubated at 10 C by 92, 80, and 81%, respectively, and on leaves incubated at 15 C by 95, 96, and 100%, respectively. The effects of isolates F131 and 053 on disease development were significantly ($P \leq 0.05$) better at 15 C than at 10 C.

Effect of nutrients on biocontrol activity. Suspensions of the three yeast isolates were applied to leaves at rates of 10^6 and 10^7 cells per milliliter along with three levels of nutrients. The effects of the added nutrients and the concentration of the saprophyte cells are exemplified by isolate 053 (Fig. 1). Reduction in the percentage of germination of conidia was similar at both concentrations of added nutrients and levels of yeast cells (Fig. 1A). Germ tube length of the germinating conidia was significantly reduced only by the high level of yeast cells when nutrients were added (Fig. 1B). Total germ tube length was significantly reduced by both concentrations of cells at both concentrations of added nutrients (Fig. 1C). Symptoms on bean leaves were reduced considerably by the yeasts; there was no significant effect of their inoculation concentration or of the level of nutrients (Fig. 1D). Similar results were obtained by the two other isolates of yeasts.

Population dynamics on leaves. Yeast suspensions were sprayed on bean plants, and the plants were incubated at a VPD of 314–524 mbar (low RH, 75–85%) and a VPD of 105–209 Pa (high RH, 90–95%) for 8 days (Fig. 2). The populations of yeasts on leaves showed a similar rate of decline under both humidities during this period, except for F147, which had a lower rate of decline during the first 3 days. At a low VPD, an increase in population was observed on the eighth day for isolate 053 and did not increase from day 8 to day 11 (Fig. 2). This was not the case for plants incubated at a high VPD. When the plants from the high VPD were transferred to low VPD conditions for another 3 days of incubation, an increase in the populations of all three yeast isolates was observed (Fig. 2).

Yeast cells were also sprayed on lesions established on bean and tomato leaves. Population densities on necrotic and noninfected leaves are shown for isolate 053 (Fig. 3). The natural yeast population on leaves infected by *B. cinerea* reached a level of 100–110 cfu/cm^2 after 5 days and was significantly higher than the population on noninfected leaves (Fig. 3A and B). Infected tomato and bean leaves showed 7.5- and 8.5-fold increases, respectively, in the population densities of the introduced yeast compared with densities on healthy leaves of the same crops (Fig. 3).

Each of the three yeast isolates was applied to tomato and bean plants along with conidia of the pathogen. Disease severity of the pathogen was reduced significantly by these treatments (Table 3). Populations of the introduced yeasts on symptomless leaves, flowers of bean, and fruits of tomato after 11 days of incubation were significantly lower in the presence of propagules of the pathogen compared with populations on plant material having no *B. cinerea* infection, except for those on tomato fruits treated with isolate F131 (Table 3). Sporulation was also reduced significantly by the yeasts, except that on tomato plants treated with isolate 053 (Table 3).

Possible mechanisms. We hypothesized that competition for nutrients is a major mechanism at work in the interaction between these antagonists and the pathogen on the plant surface. However, in order to test other possible modes of action, several tests were carried out on bean leaves. The data describing the effect of dead yeast cells on pathogenesis by *B. cinerea* are presented in Table 4. Gamma-irradiated cells reduced disease severity significantly on detached bean leaves and on whole plants (except isolate F131 on whole plants). The live cultures of isolates F147 and F131 were significantly better at reducing disease severity than were the gamma-irradiated cultures applied to detached leaves (Table 4). On detached bean and tomato leaves, heat-killed cells significantly controlled the disease at levels similar to those of the live cultures, except heat-killed isolate F147 when applied to tomato leaves (Table 4). Ethanol-killed suspensions of the yeasts were also as effective as the live suspensions (Table 4).

The presence of dead yeast cells may have affected the level of the indigenous microbial population, so the quantities of bacteria and yeasts in the drops of water placed on bean leaves were monitored. The initial bacterial and yeast population densities in water drops removed from the leaves were 90–150 and 30–60 cfu/ml, respectively. The effect of introduced dead yeast cells, live conidia of *B. cinerea,* and glucose and phosphate (0.02 M

TABLE 3. Population densities of yeast isolates *Rhodotorula glutinis* F147 and *Cryptococcus albidus* F131 and 053 on symptomless leaves[v] with and without inoculum of *Botrytis cinerea* and their effect on infection and sporulation of the pathogen

	Yeast population densities on symptomless tissue											
	Bean leaf (cfu/cm^2)		Tomato leaf (cfu/cm^2)		Bean flower (cfu)		Tomato fruit (cfu)		Disease severity[x]		Sporulation intensity[y]	
Isolate	−B[w]	+B	−B	+B	−B	+B	−B	+B	Bean	Tomato	Bean	Tomato
Control	650 b[z]	950 a	150 b	450 a	500 a	350 a	900 a	140 b	4.5 a	3.5 a	135 a	88 a
F147	12,000 a	5,500 b	45,000 a	28,000 b	35,000 a	2,400 b	42,000 a	5,400 b	0.8 c	1.8 b	58 b	45 b
F131	18,000 a	6,500 b	7,500 a	10,700 b	28,000 a	2,500 b	85,000 a	68,000 a	2.5 b	0 c	55 b	0 c
053	22,000 a	5,400 b	33,000 a	7,500 b	13,000 a	2,400 b	29,000 a	4,600 b	2.5 b	1.4 b	64 b	68 ab

[v] Leaves were incubated at high humidity (vapor pressure deficit 209–105 Pa, 90–95% RH) for 5 days with no visible water condensation in order to allow yeast development with no *B. cinerea* infection.
[w] −B = Plant material not infected with *B. cinerea*, and +B = plant material infected with a suspension of *B. cinerea*.
[x] Disease severity was indexed 8 days after infection on a scale of 0–5, where 0 = healthy plant and 5 = plant completely destroyed.
[y] Conidiophores of *B. cinerea* per square centimeter of lesion 10 days after infection.
[z] Numbers in a column followed by a common letter do not differ significantly at $P \leq 0.05$.

TABLE 4. Effect of dead yeast cells of isolates *Rhodotorula glutinis* F147 and *Cryptococcus albidus* F131 and 053 on disease severity of gray mold

	Detached bean leaves[x]						Detached tomato leaves with heat-killed cells[x]		Whole bean plants with gamma-irradiated cells[y]	
	Gamma-irradiated cells		Heat-killed cells		Ethanol-killed cells					
Isolate	−[w]	+	−	+	−	+	−	+	−	+
Control	...	3.82 a[z]	...	2.90 a	...	2.60 a	...	3.70 a	...	4.10 a
F147	0.65 c	2.35 b	1.00 b	1.30 b	1.00 b	0.30 b	1.22 b	2.40 ab	2.30 b	2.10 b
F131	0.35 c	2.55 b	1.71 b	1.50 b	0.40 b	0.80 b	0.85 b	1.00 b	2.40 b	3.05 ab
053	0.97 c	1.35 bc	1.25 b	1.30 b	0.75 b	0.80 b	1.30 b	1.50 b	2.00 b	2.45 b

[w] − = Plant material treated with live cells of the antagonist, and + = treatments with dead cells.
[x] Severity of symptoms underneath the drop of inoculum of *Botrytis cinerea* with antagonist cells was rated on a scale of 0–5, where 0 = symptomless leaves and 5 = rot extending about 2 mm around the drop.
[y] Severity of disease was rated on a scale of 0–5, where 0 = healthy plant and 5 = plant completely destroyed.
[z] Numbers in a column followed by a common letter do not differ significantly at $P \leq 0.05$.

each), alone or combined, on these populations is shown in Figure 4. Large populations of bacteria and yeasts were counted in drops containing conidia of the pathogen. The populations on *B. cinerea*-treated leaves were enhanced by the addition of the nutrients but not by the dead yeast cells. The dead yeast cells did not increase the levels of microbial populations unless conidia of the pathogen were also present in the drop of water. The maximum bacterial and yeast population densities were 10^6 and 4×10^5/ml, respectively, after 24 h of incubation with the pathogen, regardless of the presence of the dead yeast cells (Fig. 4).

Live cells of yeasts were applied in drops separate from the drops of the pathogen conidia. This remote application of the biocontrol agents did not result in a significant reduction in disease severity on detached bean leaves.

The water drops from leaves treated with the different yeast isolates were collected, filter sterilized, and applied to fresh leaves of beans along with conidia of the pathogen. No significant inhibition of the disease was observed.

Early application of biocontrol agents. Suspensions of the tested yeasts and *T. harzianum* T39 were applied either at the time of inoculation with *B. cinerea* or 5 days earlier. Delayed inoculation was accompanied by incubation of the treated plants under dry conditions (VPD of 524 Pa) before inoculation with *B. cinerea*. Disease symptoms of control treatments at these application times were rated 3.4 and 4.1, respectively.

When the biocontrol agents were applied along with the pathogen, severity was significantly reduced by 75–80%. Application of the biocontrol agents 5 days before infection resulted in a significant disease reduction of 48–62%.

Sporulation of *B. cinerea* on dead plant material. Tomato or bean leaves and stem segments were killed by microwave or gamma radiation. Yeast suspensions were applied after establishment of the pathogen (2 days after inoculation) on the dead plant material (Table 5). Sporulation was evident 2 days later. The yeasts reduced sporulation of the pathogen only on microwaved tomato and bean leaves at this sampling date. Two days later, sporulation had been reduced by the yeasts compared with the untreated control in most cases (Table 5). The yeast cultures were able to prevent the increase in conidiophore initiation on most of the treated plant organs. Sporulation on bean flowers treated with the yeasts was significantly lower than that on control flowers on the fourth day of incubation but was significantly higher than the sporulation on the yeast-treated flowers 2 days earlier (Table 5).

All isolates of yeast cells applied to detached bean leaves were effective in reducing the lesion area 6–9 days after application (Fig. 5). Sporulation was reduced significantly during the experiment (Fig. 5).

The ability of the yeasts to reduce sporulation under three temperature regimes was tested on gamma-irradiated tomato segments (Table 6). Sporulation was reduced significantly by all isolates at 10 and 18 C. At 5 C, sporulation was reduced significantly by isolates F131 and 053 (Table 6).

The effect of dryness on the ability of the yeasts to reduce sporulation was tested on leaves of beans attached to plants (Fig. 6). Twenty-four hours after yeast treatment, plants with leaf lesions were exposed to a VPD of 105 Pa for 24 h, 314 Pa for 24 h,

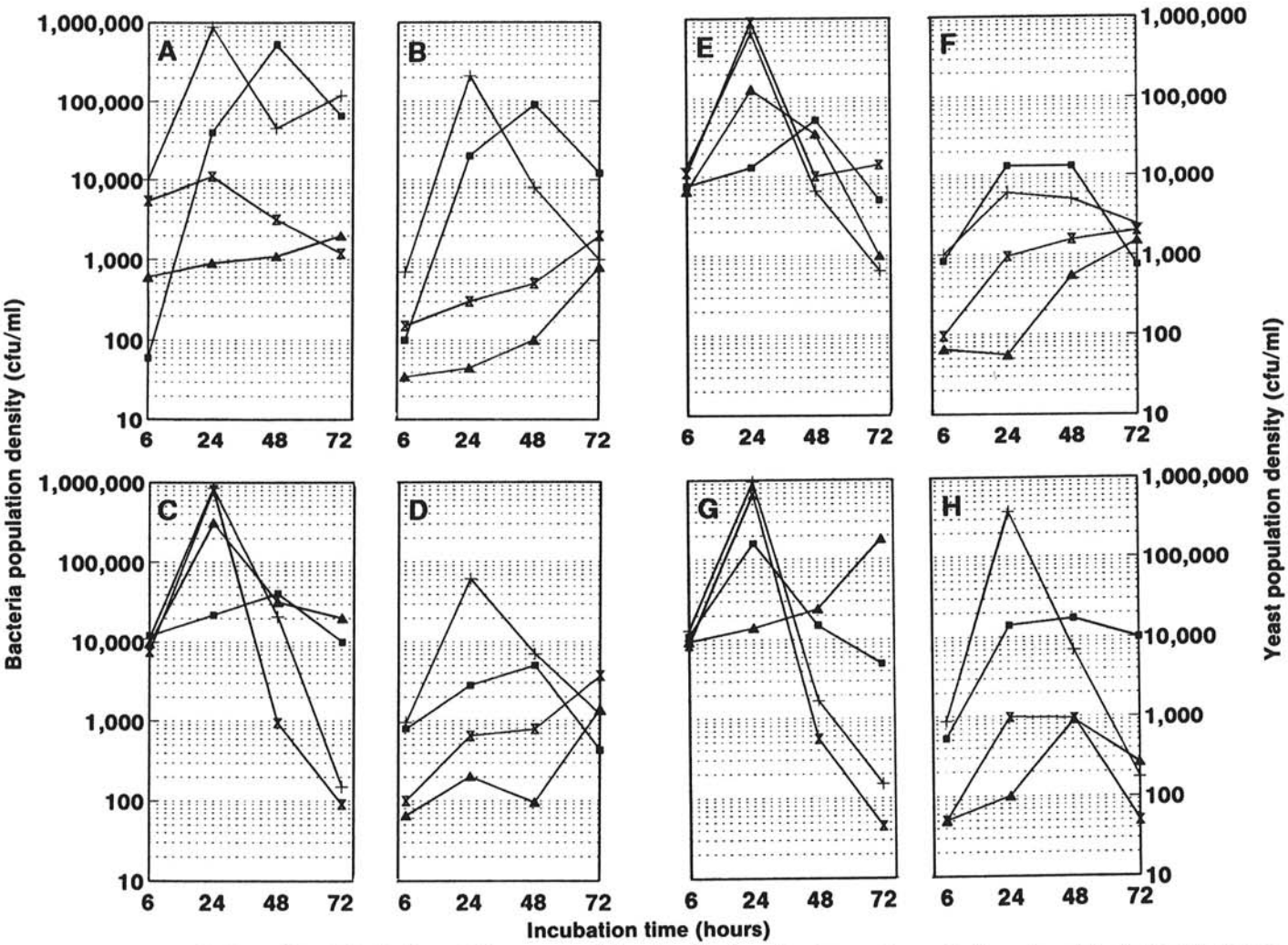

Fig. 4. A, C, E, and G, Bacterial and **B, D, F, and H,** yeast population densities in water drops after application of conidia of *Botrytis cinerea* (10^5/ml) (■), glucose and phosphate (0.02 M each, ⧖), conidia and nutrients (+), or no conidia and nutrients (▲). These treatments were supplemented with heat-killed cells (10^6/ml) of the yeast isolates *Rhodotorula glutinis* F147 **(C and D)**, *Cryptococcus albidus* F131 **(E and F)**, or *Cryptococcus albidus* 053 **(G and H)** or did not contain the introduced yeast cells **(A and B)**.

and then reexposed to a VPD of 105 Pa. A significant reduction in sporulation was obtained by isolate F131 on plants exposed to low RH. The three isolates reduced sporulation significantly after 4 days under humid conditions (Fig. 6).

DISCUSSION

The yeast isolates were selected for the present work on the basis of their effectiveness in reducing the germination of conidia of *B. cinerea* and controlling the disease on leaves, flowers, and pods of bean and on leaves of tomato. Results of the most promising isolates are presented in Tables 1 and 2. They were more effective than were other tested isolates and were at least as effective as the known biocontrol agent *T. harzianum* T39 (12–14). The yeasts survived a moderate VPD (314–524 Pa) and were active in reducing the disease after this dry period. In the presence of propagules of *B. cinerea* on symptomless leaves, the yeast population was reduced compared with that on noninfected leaves (Table 3), probably because of deprivation of nutrients by the pathogen. However, on established lesions, the yeast population proliferated (Fig. 3). When introduced to lesions incited by the pathogen, the antagonistic population was able to reduce pathogen expansion and the formation of conidiophores (Fig. 5). Similarly, when postharvest control of apple gray mold was achieved by *C. laurentii,* its population densities in wounds increased rapidly (31). The introduced yeasts probably consumed the leaking nutrients from the damaged host tissue (10), thus protecting the leaves (24) and slowing down the development of gray mold.

In order to germinate and infect plant tissues, *B. cinerea* requires exogenous nutrients. Therefore, germinating conidia of *B. cinerea* are susceptible to competition for nutrients (5). Control of gray mold by yeasts was achieved in recent years on rose flowers and various fruits after harvest (28,30,31). However, the effect of the yeasts on sporulation of a pathogen has been reported only by Williamson (38) for *Colletotrichum graminicola.*

Sporulation of *B. cinerea* was affected by our yeast isolates on various dead plant tissues precolonized by *B. cinerea,* even at a temperature as low as 5 C (Table 6) and on plants exposed to a low VPD interrupted by a higher VPD (Fig. 6).

Antagonism of microorganisms to pathogens is expressed in a variety of ways (11). Mechanisms suggested for biocontrol of foliar pathogens are production of antibiotics, competition for

TABLE 5. Sporulation of *Botrytis cinerea* on bean flowers and dead plant material treated with suspensions of yeast isolates *Rhodotorula glutinis* F147 and *Cryptococcus albidus* F131 and 053

Plant material[x]	Days of incubation[y]	Control	Yeast isolate F147	F131	053
Live (at application time)					
Bean flowers	2	30 bc[z]	20 c	12 c	12 c
	4	75 a	42 b	50 b	50 b
Microwave-irradiated					
Bean stem	2	35 c	15 c	22 c	5 d
	4	110 a	102 a	24 c	75 b
Tomato stem	2	21 bc	0 c	4 c	2 c
	4	105 a	38 b	5 c	55 b
Bean leaf	2	52 b	0 c	5 c	0 c
	4	107 a	5 c	20 c	10 c
Tomato leaf	2	85 a	20 c	2 c	20 c
	4	110 a	98 a	22 c	55 b
Gamma-irradiated					
Bean stem	2	18 b	10 b	8 b	6 b
	4	108 a	21 b	22 b	30 b
Tomato stem	2	24 b	12 b	8 b	14 b
	4	110 a	22 b	31 b	42 b
Bean leaf	2	12 bc	0 c	6 c	0 c
	4	52 a	0 c	12 bc	4 c

[x] Microwave- and gamma-irradiated plant mterial or detached flowers were inoculated with condial suspensions of *B. cinerea* (10^4/ml), incubated for 2 days at 20 C, and treated with yeast suspensions (10^6/ml). The inoculated plant material was further incubated for 2–4 days.
[y] Time after treatment with yeast suspensions.
[z] Values are numbers of conidiophores per flower for live material and numbers of conidiophores per square centimeter of irradiated material. Numbers in a column followed by a common letter do not differ significantly at $P \leq 0.05$.

TABLE 6. Effect of temperature and cells of yeast isolates *Rhodotorula glutinis* F147 and *Cryptococcus albidus* F131 and 053 on sporulation of *Botrytis cinerea* on gamma-irradiated segments of tomato stems

Temperature (C)	Incubation time (days)	Control	Yeast isolate F147	F131	053
5	4	48 a[z]	38 ab	18 b	15 b
10	5	75 a	45 b	32 b	42 b
18	8	112 a	65 b	75 b	52 b

[z] Conidiophores per square centimeter of tissue. Numbers in a column followed by a common letter do not differ significantly at $P \leq 0.05$.

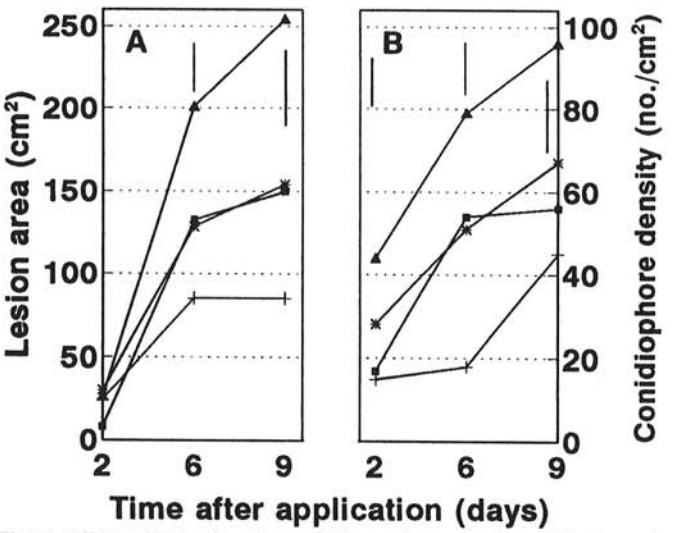

Fig. 5. Effects of introduced populations of yeast isolates *Rhodotorula glutinis* F147 (+), *Cryptococcus albidus* F131 (*), and 053 (■) **A,** on expansion of lesions incited by *Botrytis cinerea* on leaves of bean plants and **B,** on formation of conidiophores on the lesions. The yeasts (6–7 $\times 10^3$ cfu/cm^2) were sprayed on established lesions. ▲ = Untreated plants. Bar = LSD ($P \leq 0.05$) at 8 df.

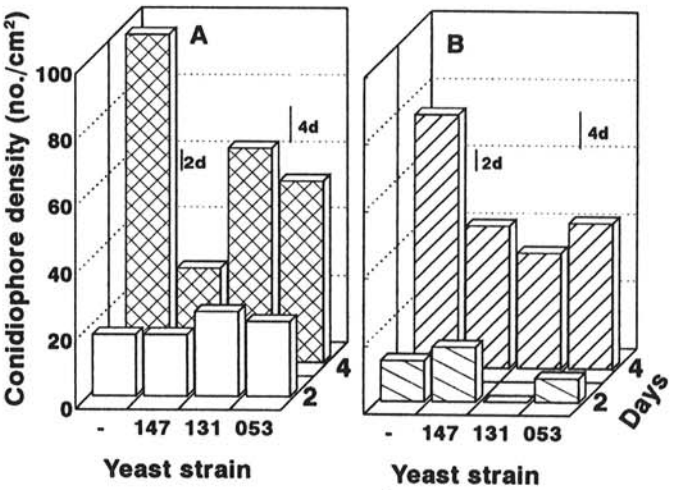

Fig. 6. Effects of populations (6–7 $\times 10^3$ cells per square centimeter) of yeast isolates *Rhodotorula glutinis* F147 and *Cryptococcus albidus* F131 and 053 introduced onto established lesions of *Botrytis cinerea* on the formation of conidiophores by the pathogen 2 days and 4 days after application. After yeast application, plants were exposed **A,** to a continuous vapor pressure deficit (VPD) of <105 Pa or **B,** to an atmosphere of 105 Pa VPD for 1 day, 314 Pa VPD for 1 day, and then back to 105 Pa VPD. Bars = LSD ($P \leq 0.05$) of data obtained after 2 days and 4 days of incubation at 8 df.

nutrients, parasitism, and induced resistance (20,22,26,33,39). The mechanism underlying the activity of our yeast isolates may be complex. Application of the yeasts at varied nutrient levels suggests competition for nutrients (Fig. 1). However, dead cells of the antagonistic yeasts were also capable of reducing gray mold. Their application was associated with an increase in the indigenous populations of bacteria and yeasts, but these populations were not likely to be high enough to reduce pathogenesis by *B. cinerea* (Fig. 4). The yeasts did not induce resistance when applied at a short distance from the pathogen and did not produce detectable inhibitory compounds (Table 4). It was suggested recently (40) that the mode of action of the postharvest biocontrol yeast *Pichia guilliermondii* lies in the secretion of cell wall-degrading enzymes, but our observations did not reveal such degradation of *B. cinerea* cell walls (results not presented). It may be concluded that the activity of the tested yeasts is associated with their cells or cell walls and at least partial activity is not associated with live cells. Thus, competition for nutrients and locally induced resistance result in the inhibition of gray mold on bean and tomato.

The epidemiological application of preinfection and postinfection biological control in the field may result in decreased disease spread. Prevention of inoculum increase followed by a suppression of its ability to infect would create a cumulative effect over several disease cycles (23,25), especially in greenhouses where a population of *Botrytis* is developing independently (23,41) from inoculum that originates from outside.

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