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Survival in the Phylloplane of an Introduced Biocontrol Agent (*Trichoderma harzianum*) and Populations of the Plant Pathogen *Botrytis cinerea* as Modified by Abiotic Conditions

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Leaf populations of *Trichoderma* were studied on tomato, pepper and geranium plants incubated under various conditions. Treatments involved high (>90%) or lower (75-85%) relative humidity (r.h.), temperatures of 15±3°C or 25±3°C, and soil fertilization with formulations of 2,2,5%, 3,3,8% or 5,3,8% NPK. The size of populations on leaves treated with the fungus *Trichoderma* harzianum differed according to plant species, leaf age, length of incubation, atmospheric conditions, and plant nutrition. *T. harzianum* populations were promoted in many cases by high r.h. and by 3,3,8% NPK. Interactions of introduced populations of *Botrytis cinerea* with populations of *T. harzianum* on tomato leaves under combinations of the above conditions showed that the population of *B. cinerea* was *ca* tenfold lower in the presence of *T. harzianum* than in the absence of this fungus.

KEY WORDS: *Trichoderma harzianum*; *Botrytis cinerea*; biological control; gray mold; phylloplane.

INTRODUCTION

The phyllosphere is the three-dimensional space of the leaf surface. The environment of the phyllosphere includes physical, chemical and biological components occupying the surrounding space (15). There are many factors that may influence microorganisms in the phyllosphere. The atmosphere of plant surfaces is exposed to fluctuating temperatures, vapor water pressure deficit and water persistence, gases, wind, radiation, light and polluting compounds. Soil-associated factors – such as chemicals exudates, which contain macro- and micro-elements, sugars, sugar alcohols, pectic substances, amino acids, and organic acids – influence physical properties of the plant surface (19). These released chemicals serve as nutrient sources for the microflora that occupy the plant surface (18). Secondary metabolites produced at the epidermis, such as sucrose esters, diterpenes, alkanes and fatty alcohols, affect leaf surface organisms (2). The resident microorganisms of leaf surfaces use leaf resources actively to sustain their growth

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(5,6,17). As nutrients fluctuate, there is an ecological succession in colonization by bacteria, yeasts, and filamentous fungi (1).

Trichoderma spp. are known antagonists to various plant pathogenic fungi (3,4). A system of biological control by *Trichoderma harzianum* Rifai (T39) of gray mold, caused by *Botrytis cinerea* Pers. ex Fr., was established recently. The biocontrol preparation was found to be effective on grapes, cucumber, tomato and strawberries (8,12-14,21). The preparation based on the effective T39 isolate (Trichodex) was sprayed over plants in many experiments (13) and proved to control gray mold when applied alone or in combination with fungicides. Both tank mix and alternation with fungicides are recommended for use although the latter is preferred (10,12). This is one of the first such preparations commercialized for use against foliar pathogens (7). The effect of *T. harzianum* on indigenous populations of plant surface bacteria and fungi was recently summarized (11). Application of this preparation resulted in an increase or decrease in comparison with untreated leaves. However, relative humidity (r.h.), temperature, plant nutrition, plant species and leaf age influenced the changes in the indigenous microflora. The ability of this biocontrol agent to survive and to establish an active population in the phylloplane may be affected by phyllosphere inhabitants, nutrients, and microclimatic conditions. The aim of the present study was to study the effects of plant nutrition, temperature and r.h. on the survival of the introduced biocontrol agent, *T. harzianum*, and on the phyllosphere's interaction with the plant pathogen *B. cinerea*.

MATERIALS AND METHODS

Plants, microorganisms and growth conditions

Tomato (*Lycopersicon esculentum* Mill.) cv. F144, pepper (*Capsicum annuum* L.) cv. Maor, and geranium (*Pelargonium domesticum* Bailey) were planted in 10-*l* plastic containers with sandy loam soil and grown for 10 weeks in a glass greenhouse at temperatures ranging from 14 to 30°C. *T. harzianum* Rifai (T39) sensitive to benzimidazole fungicides was grown and produced by Makhteshim Chemical Works Ltd., Be'er Sheva, Israel (Trichodex, 25% powder). Control plots were treated with the nonactive formulation powder. The biocontrol agent was sprayed at a concentration of 10^5 conidia/ml and a volume of 2 ml/plant. *B. cinerea*, which was resistant to

TABLE 1. Fertilizers used in the experiments

	% NPK		
	2,2,5	3,3,8	5,3,8
pH	3.5	3.5	3.5
Conductivity (mMoh)	1.12	1.17	1.28
N-NH_4 (%)	0.6	0.6	0.6
N-NO_3 (%)	1.4	2.4	4.4
Total N (%)	2	3	5
NH_4/NO_3 ratio	1:2.33	1:4	1:7.3
P_2O_5 (%)	2.1	3.0	3.0
K_2O (%)	4.8	8.0	8.0
Fe, Mn, Zn, Ca, Mo, B (ppm) (respectively, in all treatments)	475, 50, 75, 11, 8, 72		

benzimidazole fungicides, was isolated from infected cucumber fruit (13). Conidia of the pathogen were collected from 14-day-old potato dextrose agar cultures and sprayed at a rate of 10^5 conidia/ml and a volume of 2 ml/plant. Liquid fertilizers (obtained from Fertilizers and Chemical Compounds Ltd., Haifa, Israel) were applied to the soil at a rate of 1 ml/l water at each irrigation. The fertilizers were 2,2,5%, 3,3,8% and 5,3,8% NPK, containing macro- and micro-elements, as detailed in Table 1. Fertilization was started one week before treatment with *T. harzianum* and was continued for the duration of the experiment, which involved incubation at high temperature and low r.h. Plants were incubated in glasshouse compartments where the temperature was controlled by air conditioners. High r.h. was obtained by evaporating water in the chamber. Air temperature and r.h. were monitored constantly. The ambient conditions were recorded by a C21X data logger (Campbell Scientific Inc., Logan, UT, USA) at the height of the plant canopies, and r.h. was measured with a 4A-100-Hygrometer sensor (Rotronic AG, Zurich, Switzerland). Relative humidities were chosen to create vapor pressure deficits (VPD) of 0-2.09 and 2.14-5.24 mbar at 15±3°C, and 0-3.83 and 4.02-9.58 mbar at 25±3°C. VPDs of 3.46 and 0.87 mbar correspond to 80% and 95% r.h., respectively, at 15°C, whereas VPDs of 6.45 and 1.61 mbar correspond to 80% and 95% r.h., respectively, at 25°C.

Populations of T. harzianum *and* B. cinerea

Plants were either subjected to a *T. harzianum* spray, or not sprayed at all, and then incubated under conditions of low temperature (15±3°C) or high temperature (25±3°C) and high r.h. (>90%) or lower r.h. (<85%, *viz.* 75-85%). In the absence of added nutrients, symptoms of gray mold did not develop. Symptomless mature (lower) and young (upper) leaves were detached, 20 leaves from each treatment, and brought to the laboratory. Leaf area was measured visually by comparison with standard measured leaf area in shapes of leaves or leaflets of each of the crops. Each leaf was immersed in 100 ml of water containing 0.01% Tween 20 and shaken at 100 rpm in a linear shaker for 1 h at 18±2°C. The suspensions were then diluted serially and plated on separate media to allow the growth of the test microorganisms. *B. cinerea* was counted on *Botrytis*-selective medium (16) supplemented with 5 µg benomyl/ml to prevent growth of *Trichoderma* spp., and *T. harzianum* was counted on *Trichoderma*-selective medium (9).

Microscopic observations

Leaf segments (1 cm^2) were sampled 0, 7 and 15 days after application of the fungal suspensions on the plants. Drops of cotton blue solutions were placed on the leaf segments. Observations were carried out under a light microscope (Nikon Optiphot, Japan) after 10 minutes of incubation at room temperature, at high light intensity and magnifications of x200 (*B. cinerea*) or x400 (*T. harzianum*).

Experimental design

Experiments were carried out three times and each treatment was replicated five times. Dilutions from leaf samples of each treatment were replicated three to five times. Since trends were similar in the various experiments, the results of representative experiments are presented throughout. Analysis of variance was conducted for values of

Trichoderma populations (Tables 2 and 3). Population levels were log-transformed prior to analysis. Three-way analysis was performed for population counts on leaves of tomato and pepper. Two-way analysis was conducted for geranium leaves. In experiments involving populations of *Botrytis* and *Trichoderma*, the LSD was calculated for $P \leq 0.05$ at each sampling date (Fig. 1).

RESULTS

Effect of temperature and relative humidity on T. harzianum *survival*

The initial population of *T. harzianum* was *ca* 5000 colony-forming units (cfu)/cm^2. Populations of *T. harzianum* were sampled and evaluated after 7-10 days of incubation (Table 2). Generally, the level of the fungus on the leaves was low as compared with the initial level but the survival in the different treatments varied. The main effects of microclimate conditions (temperature and r.h.) and of leaf age were significant for

TABLE 2. Effect of temperature and relative humidity,[z] on *Trichoderma harzianum* population levels (cfu/mm^2 leaf)[y] on the leaf surface

Temp. (±3°C)	Relative humidity (%)	Tomato		Pepper		Geranium
		young[x]	mature	young	mature	
Sampled after 7-10 days of incubation of leaf						
25	<85(75-85)	19.0	70.0	54.0	160.0	160.0
	>90(90-100)	1150.0	1670.0	740.0	890.0	1620.0
15	<85	19.0	54.0	110.0	250.0	470.0
	>90	690.0	540.0	740.0	390.0	4050.0
Statistical analysis[w]						
Temperature (T)		<0.001		0.385		<0.001
Relative humidity (r.h.)		<0.001		<0.001		<0.001
T x r.h.		<0.001		<0.001		0.415
Leaf age (LA)		<0.001		0.001		–
T x LA		0.008		0.006		–
r.h. x LA		0.001		<0.001		–
T x r.h. x LA		0.359		0.087		–

[z]Plants were fertilized to the root zone with 1 ml/*l* water of 3,3,8% NPK solution at every irrigation.

[y]A preparation of the biocontrol agent was sprayed on the leaves 7-10 days before populations were evaluated. The initial population of *Trichoderma* on the leaves was *ca* 5.10^3 cfu/mm^2 as evaluated by serial dilutions from treated leaves. No *Trichoderma* spp. colonies were observed in dilutions from leaves not treated with the biocontrol preparation; therefore, populations of the fungus were less than 0.1/mm^2 leaf.

[x]Samples were taken from the fully expanded (mature) or expanding (young) leaves of tomato and pepper plants and from mature geranium leaves.

[w]*P* values for the analysis of variance. Three-way analysis of variance was performed for tomato and pepper, and two-way analysis for geranium.

populations of tomato and pepper leaves. The main effects of the microclimate parameters on geranium populations also were significant (Table 2). Analysis of variance conducted on the data revealed that the three-way interaction terms both for tomato and for pepper (for temperature x r.h. x leaf age) were insignificant ($P \leq 0.05$). On the other hand, the temperature x r.h., temperature x leaf age, and r.h. x leaf age interaction terms were significant for pepper and tomato leaves. The interaction term of temperature x r.h. was insignificant for geranium populations (Table 2).

As reflected in the analysis of variance, there were complexed interactions between the factors tested in the experiment. However, the leading trends were as follows: The population of *T. harzianum* on leaves evaluated from plants incubated at high r.h. was *ca* $4–17 \cdot 10^2$ cfu/cm^2 on tomato and pepper leaves (young and mature), and 100 times higher on geranium leaves. Under the lower r.h. conditions, the population of *T. harzianum* on tomato and geranium leaves was 8-16 times less than the populations under high r.h. and the difference on pepper leaves was smaller.

Analysis of variance was also done in order to examine the effect of the crop on *Trichoderma* survival (analysis of the results shown in Table 2). Populations of the fungus on mature leaves of the three crops were considered. The main effect of the crop, the interaction terms of crop x temperature, crop x r.h, and crop x temperature x r.h. were significant (analysis not shown).

Results of a sampling conducted after 15-18 days of incubation were evaluated as well (results not shown). The trends in the *T. harzianum* population at that time were similar to the first sampling date. The absolute numbers of the cfu counted from leaves at the second sampling were lower than at the first sampling, but a significant change was observed in only some of them.

Effect of fertilization on T. harzianum *survival*

Plants were fertilized with three formulations of fertilizers in which a low input of NPK (fertilizer 2,2,5%) or a high input of NPK was introduced into the soil; the two fertilizers of high phosphate and potassium input were either poor in nitrogen (3,3,8% NPK) or richer in nitrogen (5,3,8% NPK) (Table 1). Microbial populations of the leaves were sampled 7-10 days after the *T. harzianum* spray (Table 3) and 10 days thereafter (Table 3). The initial level of the biocontrol agent on the leaves was 400 cfu/mm^2. In this experiment its surviving population also was reduced drastically during the first week of incubation but the actual population was affected by the treatment (Table 3).

Analysis of variance was done in order to evaluate the interactions between fertilizer, leaf age and incubation time and their effect on *Trichoderma* survival (Table 3). The main efffects of fertilizer and incubation period were significant for the three crops and the main effect of leaf age on tomato and pepper leaves was significant. The three-way interaction term was significant for populations on pepper leaves but not for tomato leaves. The interaction term for fertilizer x incubation period was significant for all three crops, the interaction term for fertilizer x leaf age was significant for tomato leaves, and the interaction term for leaf age x incubation time was significant for pepper leaves.

Analysis of variance was also done in order to examine the effect on *Trichoderma* survival (analysis of the results shown in Table 3). Populations of the fungus on mature leaves of the three crops were considered. The main effect of the crop, the interaction

TABLE 3. Effect of fertilization[z] of plants with *Trichoderma harzianum*[y] on population[x] levels on the leaf surface (cfu/mm^2 leaf)

Fertilization (% NPK)	Tomato		Pepper		Geranium
	young[w]	mature	young	mature	
Sampled after 7-10 days of incubation[v]					
2,2,5	1.4	43.0	195.0	107.0	10.0
3,3,8	12.0	93.0	30.0	22.0	33.0
5,3,8	2.7	30.0	20.0	29.0	15.0
Sampled after 15-18 days of incubation					
2,2,5	4.0	120.0	54.0	165.0	9.0
3,3,8	9.0	64.0	12.0	21.0	30.0
5,3,8	5.0	40.0	39.0	58.0	45.0
Statistical analysis[u]					
Fertilizer (F)	<0.001		<0.001		<0.001
Leaf age (LA)	<0.001		<0.001		–
F x LA	<0.001		0.105		–
Incubation period (P)	<0.001		0.094		<0.001
F x IP	<0.001		<0.001		<0.001
LA x IP	0.432		<0.001		–
F x LA x IP	0.471		<0.001		–

[z]Fertilizers were applied in the water to the root zone at every irrigation, at a rate of 1 ml/*l* water.
[y]A preparation of the biocontrol agent was sprayed on the leaves 7-10 or 17-20 days before populations were evaluated. The initial population of *Trichoderma* on the leaves was *ca* 4.10^2 cfu/mm^2 as evaluated by serial dilutions from treated leaves.
[x]Samples were taken from the fully expanded (mature) or expanding (young) leaves of tomato and pepper plants and from mature geranium leaves.
[w]No *Trichoderma* spp. colonies were obtained in dilutions from leaves not treated with the biocontrol preparation; therefore, populations of the fungus were less than 0.1/mm^2 leaf.
[v]Plants were incubated at 25±3°C and 75-85% r.h.
[u]*P* values for the analysis of variance. Three-way analysis of variance was performed for tomato and pepper, and two-way analysis for geranium.

terms of crop x fertilizer, crop x incubation period, and crop x fertilizer x incubation period were significant (analysis not shown).

The analysis of variance revealed a complexed interaction also in the case of this experiment. Generally, survival of populations of *T. harzianum* after 7-10 days was better on young leaves of tomato plants fertilized with 3,3,8% NPK, and on young and mature leaves of pepper plants fertilized with 2,2,5% NPK. There was no effect to the fertilizer composition on the population of *T. harzianum* on geranium leaves (Table 3). At the second sampling date, survival was lower on mature tomato leaves fertilized with 5,3,8% NPK and on young pepper leaves fertilized with 3,3,8% NPK. Survival on leaves from geranium plants fertilized with 2,2,5% NPK was significantly lower than on leaves from 5,3,8% NPK plants. In this experiment *T. harzianum* survived better on mature than on young tomato leaves at both sampling dates (Table 3).

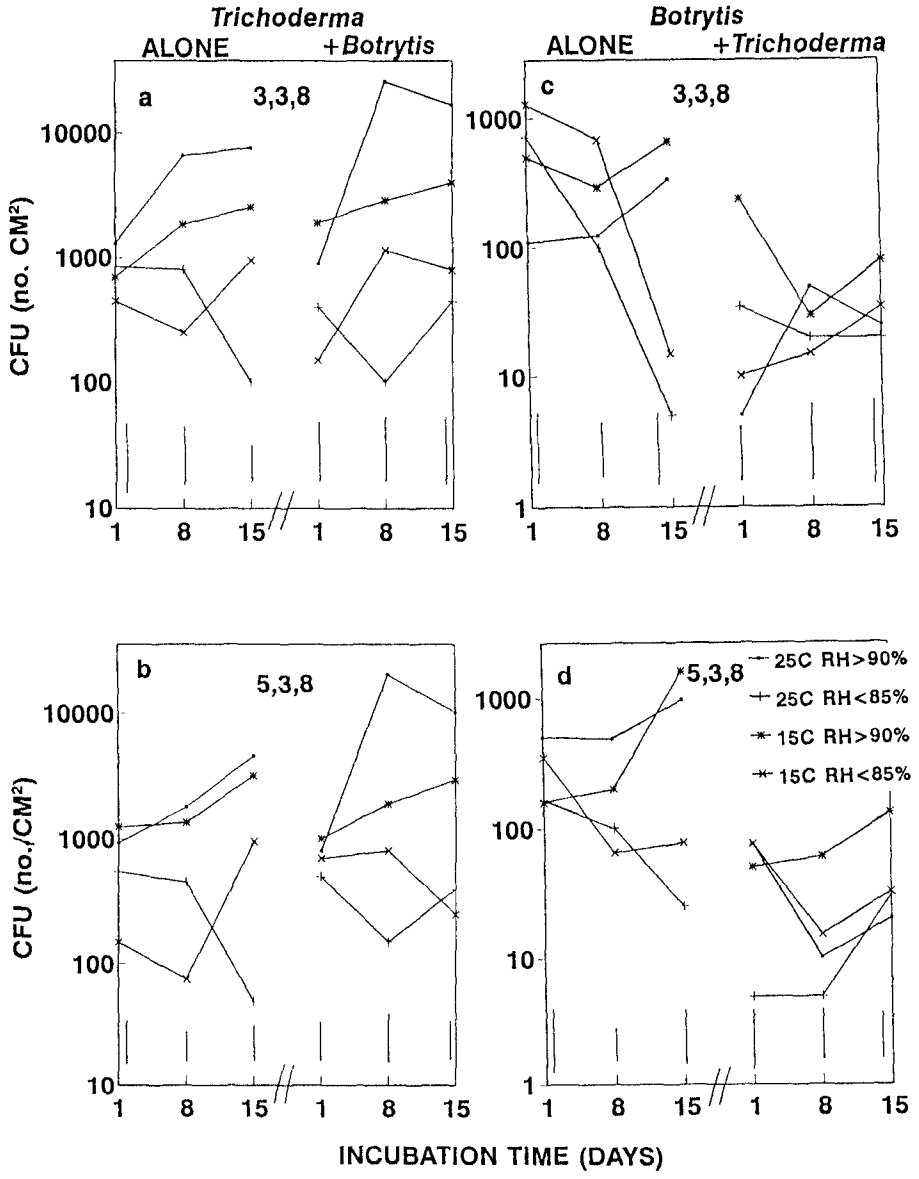

FIG. 1. Population levels of *Trichoderma harzianum* (a,b) and *Botrytis cinerea* (c,d) on mature leaves of tomato. Propagules of *Trichoderma* were counted from leaves treated with the biocontrol agent alone (left side of figs. a,b), or from leaves treated with *B. cinerea* and *T. harzianum* (right side of figs. a,b). Propagules of *B. cinerea* were counted from leaves treated with the pathogen alone (left side of figs. c,d) or from leaves treated with both fungi (right side of figs. c,d). Plants were fertilized with 3,3,8 % NPK (a,c) or 5,3,8 % NPK (b,d) as mentioned in the respective figures. Treated plants were incubated at 25°C and >90% r.h., 25°C and <85% r.h., 15°C and >90% r.h., and 15°C and <85% r.h. Bars indicate the LSD values in log scale ($P \leq 0.05$) for each sampling date.

Interaction between T. harzianum *and* B. cinerea

Due to lack of additive nutrients, production of germ tubes by conidia of both fungi was not visible on leaves, except for poor germination in the high humidity treatments. However, 4-9% of the conidia observed after 7-15 days swelled and did not produce germ tubes. Suspensions of *B. cinerea* and *T. harzianum* were applied on leaves and their conidia were observed 0, 7 and 15 days thereafter, in order to evaluate the rate of conidial spread on the leaves. Conidia of *T. harzianum* aggregated on the leaves. The clusters of conidia contained up to 30 conidia but most contained 7-11 conidia/cluster. In the first day of incubation 84% of the conidia were aggregated in clusters of seven or more conidia. One week later only 15% of the conidia were arranged in big clusters, 52% were arranged in clusters of 3-6 conidia, and the rest were isolated or in couples. After 2 weeks of incubation most of the conidia were not arranged in big clusters. The conidia of the plant pathogen were also arranged in clusters on the inoculated leaves, which consisted of up to 15 conidia. Only 3% of the conidia remained separate or in groups of up to three. Dispersal of the conidia of *B. cinerea* on the leaves after 1-2 weeks of incubation followed the same pattern as that observed for *T. harzianum.* Most of the conidia of the pathogen were observed to be separate. No sporulation of either *T. harzianum* or *B. cinerea* on the leaves, under the conditions of the experiments, was observed microscopically.

The *T. harzianum* and *B. cinerea* populations were evaluated on mature tomato leaves treated with each of the fungi alone or with the two together. The tomato plants were kept under combinations of high and low r.h. or temperature and fertilized with 3,3,8% and 5,3,8% NPK at triple dose (Fig. 1). In the absence of *B. cinerea*, the *T. harzianum* population was larger at the higher than at the lower r.h.; at the lower r.h., the *Trichoderma* population at the end of the incubation period was smaller on plants incubated at 25°C as compared with the lower temperature (Fig. 1a,b). In the presence of *B. cinerea*, a similar trend for populations of *Trichoderma* was observed.

Botrytis populations in the absence of *T. harzianum* were larger at the high r.h., and at the last sampling resulted in higher counts at 15° than at 25°C. In the presence of *T. harzianum* conidia, the *B. cinerea* population decreased dramatically, especially at 25°C and high r.h. (Fig. 1c,d).

DISCUSSION

Populations of fungi in the phylloplane react differently to atmospheric conditions and to plant nutrition. The reaction of each species of microorganism may even differ among the different plant species and leaf ages. The microclimatic conditions affect the biochemical activity of plant tissue, the rate of transpiration, and movement of solutes from the roots to the canopy. The concentration or ratio of nutrients supplied to the roots of plants affects the biochemical activity of plant tissue and the nature of leachates over the leaf surface. The leachates are composed of inorganic or organic compounds, which are produced by the plant or transferred by it to the leaf surface (19). The changes in the phylloplane populations can be attributed to the direct effect of the conditions under which plants were incubated (humidity and temperature), and to an indirect effect due to changes in the physical or chemical nature of the phyllosphere (18). Complete germination of the conidia of the tested fungi was rare under the conditions of the present

experiments. Sporulation was not observed either. Although physiological changes in the conidia in the presence of leaf leachates probably occurred, they were not investigated in this study. On the other hand, the effect of external conditions on actual survival of the fungi due to loss of viability was studied.

The effect of microclimatic conditions (Table 2) or plant nutrition (Table 3) on survival of the populations of the biocontrol agent, *T. harzianum*, was variable. This reflects the complexity of the behavior of introduced populations in the phylloplane. The effect of r.h. on the *T. harzianum* population was marked. Larger numbers survived under higher r.h. On the other hand, the effect of temperature cannot be generalized, although there was a tendency towards larger populations under lower temperatures. This may be attributed to relatively larger VPD (within the limit of up to 75-85% r.h.) that may occur at lower temperatures. Field examination of *T. harzianum* activity in a separate study (14) revealed better performance of the biocontrol agent at a high r.h. (but not wetness), but also at a high temperature (25°C).

The varied effect on survival of the *T. harzianum* population on plants fertilized with the various NPK formulations emphasizes another effect of cropping practice that is important for the establishment of the biocontrol agent in the phylloplane. The reason for variable survival of the population of the biocontrol agent on plants fertilized with the various fertilizers, or on the different leaves, is probably the changes in chemical properties which occur in the phyllosphere. The mechanism responsible for the effect of the chemical composition of the leaf surfaces on the *Trichoderma* population is not clear. Studies such as this one are important in order to obtain a better understanding of the biocontrol agent's introduction into its target space. Lack of attention to this point may explain cases of failure of biocontrol experiments when using such a biocontrol agent.

The effect of leaf age on the *T. harzianum* population was pronounced in only some of the observations on tomato leaves. The larger populations found on geranium leaves in comparison with tomato or pepper leaves may point to the possible effect of the crop itself on the *T. harzianum* population. Generally, the effects of temperature, r.h. and time of incubation on the *Trichoderma* population on the leaves of the three crops were similar. On the other hand, the effects of plant nutrition and leaf age varied with the crop. It is therefore not possible to generalize about the effect of the crop on the population of the biocontrol agent.

Biological control is based on the antagonistic properties of the agent(s) used. Apart from the agent's antagonistic activity, effective biocontrol involves also the ability of the agent to survive in the habitat where it is applied. Insufficient research efforts have been directed towards selection for characteristics of biocontrol agents which survive well, or towards environmental conditions capable of enhancing the survival and activity of the biocontrol agent (7,20). It is obvious that a biocontrol organism will not persist and be active unless it is adapted to the plant environment; moreover, to be successful in controlling the pathogen, the introduced biocontrol agent must compete with other microorganisms and establish an active population on the phylloplane. This is one of the least understood aspects of biocontrol.

The effect of the introduced *T. harzianum* on phylloplane microorganisms was reported recently (11). *Trichoderma* was either promotive or inhibitory to bacteria, yeasts or filamentous fungi, depending on plant nutrition and the conditions under which the

plants were incubated (11). The influence of *Trichoderma* may be attributed to an increased population of this introduced organism, that was affected by the experimental conditions, or to the organism's effect on the interactive balance among the microbial populations.

The changes in time of the quantities of the fungal propagules on the leaves may have been affected by two major factors, *i.e.*, a decrease in population due to loss of vitality which was affected by the abiotic conditions, and an increase in the dispersal of the conidia due to the loss of their aggregation on the leaves. In the presence of *B. cinerea* on tomato leaves, as compared with no Botrytis, the *Trichoderma* population increased under conditions of high r.h. The reason for the larger population of the biocontrol agent in the presence of the conidia of the pathogen is not clear. However, it was already shown that conidia of *B. cinerea* on petals of rose flowers induce increased leakage of nutrients from plant tissue (Shaul, Elad and Zieslin, unpublished).

The change in *B. cinerea* leaf populations in the presence of *Trichoderma*, as compared with populations of *Trichoderma*-free leaves, was more significant than the changes in *Trichoderma* in the presence of *B. cinerea*. This may reflect the fact that conidia of the pathogen are more sensitive to changes in the phyllosphere than are *Trichoderma* conidia; however, other mechanisms in the phylloplane should also be explored as a possible explanation for this phenomenon.

The results of the present study do not provide a simple picture of phyllosphere *Trichoderma* and *Botrytis* populations because of complicating feedbacks within the phyllosphere as a dynamic system involving many interdependent variables. Moreover, our results reflect the position of viable propagules at the leaf surface, which may themselves have complex relationships with the activity of mycelial phases (where these exist) inside the plant tissues. Further detailed study is needed to clarify the phenomena which were highlighted in this work.

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