Interactions Between *Trichoderma harzianum* Strain T22 and Maize Inbred Line Mo17 and Effects of These Interactions on Diseases Caused by *Pythium ultimum* and *Colletotrichum graminicola*

Gary E. Harman, Rixana Petzoldt, Alfio Comis, and Jie Chen

First, second, and fourth authors: Departments of Horticultural Sciences and Plant Pathology, Cornell University, Geneva, NY 14456; and third author: Department of Crop and Soil Sciences, Cornell University, Ithaca, NY 14853.
Current address of A. Comis: Australian Centre for Plant Functional Genomics, Adelaide University, Adelaide, SA 5005 Australia.
Current address of J. Chen: School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 20030, P. R. China.
Accepted for publication 5 September 2003.

ABSTRACT

Harman, G. E., Petzoldt, R., Comis, A., and Chen, J. 2004. Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and effects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola.* Phytopathology 94:147-153.

Seed treatment with *Trichoderma harzianum* strain T22, which results in colonization of plant roots but little or no colonization of shoots or leaves, had substantial effects on growth of and disease expression in maize inbred line Mo17. Shoots and roots of 10-day-old seedlings grown in a sandy loam field soil were larger (roots were nearly twice as long) in the presence of T22 than in its absence. Both main and secondary roots were increased in size and area and the root hair area was greater with T22. However, root hair area per unit of root length was greater in control plants. Increased growth probably was due to direct stimulation of plant growth in addition to effects from biological control of deleterious microflora. Seedlings of Mo17 grown in autoclaved or mefenoxam-treated sandy loam field soil were larger than those produced in untreated soil. However, seedlings grown in the presence of T22, either in treated or untreated soil, were larger than those produced in its absence. Infestation of soil with *Pythium ultimum* had little effect upon growth of Mo17. The presence of T22 increased protein levels and activities of β-1,3 glucanase, exochitinase, and endochitinase in both roots and shoots, even though T22 colonized roots well but colonized shoots hardly at all. With some enzymes, the combination of T22 plus *P. ultimum* gave the greatest activity. Plants grown from T22-treated seed had reduced symptoms of anthracnose following inoculation of leaves with *Colletotrichum graminicola*, which indicates that root colonization by T22 induces systemic resistance in maize.

Additional keywords: pathogenesis-related proteins, root hairs.

Trichoderma spp. long have been known for their abilities to control plant-pathogenic fungi. Mechanisms primarily have included direct effects upon target fungi via competition, mycoparasitism, and antibiosis (6). In addition, these fungi have been shown to directly increase plant growth by unknown mechanisms (5), even under axenic conditions (22,31). They also have abilities to solubilize plant nutrients (1) and to increase plant nutrient uptake (11,31).

Moreover, recent studies have indicated that these fungi also induce localized or systemic resistance systems in plants (8,10, 15,18,28–30). Thus, these fungi have a variety of effects upon plants, including direct control of fungal pathogens, enhancement of plant growth and nutrition, and induced systemic resistance. This variety of effects indicates that these beneficial fungi have multiple modes of action (15).

Among the most useful *Trichoderma* strains are those that are rhizosphere competent (i.e., able to colonize and grow with root systems of most plants). *T. harzianum* strain T22, when applied as a seed or soil treatment, colonizes the entire root system of most plants and increases the depth of rooting of maize even when plants are fully mature. This effect occurs across many soil types and pH levels (13) and results in a variety of beneficial effects, including resistance to abiotic stresses such as drought (11) and improved nitrogen fertilizer use efficiency (11,12,14). It also protects plants from a variety of pathogens when the organism is applied as a foliar, soil, or root protectant (11). However, even though the organism is becoming widely used in commercial agriculture, knowledge of its multiple modes of action is sketchy at best.

Proteomic and genomic tools permit holistic examination of protein and gene expression but require rapid and reproducible systems for optimal use. Thus, field research done in the past is poorly suited to such studies.

The purpose of this article is to describe a maize–T22 interactive system that is suitable for studying (i) growth enhancement, including increased root production, and (ii) the mechanisms of control of the seed and root pathogen *Pythium ultimum* and the foliar pathogen *Colletotrichum graminicola* in maize. The research also examined levels of activity of defense-related enzymes in maize in response to T22, T22 + *P. ultimum*, and *P. ultimum* alone and is intended to define this model system for proteomic studies that are underway.

MATERIALS AND METHODS

Maize line and microbial strains. In preliminary experiments, we identified a genetically uniform maize line that was highly responsive to T22 and that is used in commercial agriculture. Mo17, an inbred maize line, has been used extensively in commercial hybrid production and is well characterized genetically. Recombinant inbred lines between Mo17 and other inbreds are available (17,21). This line was used in all studies and is among the lines that respond most strongly to root colonization by T22.

Corresponding author: G. E. Harman; E-mail address: geh3@cornell.edu

Publication no. P-2003-1124-03R

T. harzianum strain T22 (also known as 1295-22, KRL-AG2, and ATCC 20847) was used in all studies. This strain was produced using protoplast fusion; its development and commercial uses have been described (11). In all cases, a commercial formulation (PlantShield HC; BioWorks, Inc., Geneva, NY) containing $\approx 1 \times 10^9$ CFU g^{-1} (primarily conidia) was used. The primary inert ingredient is montmorillonite clay. This material was dusted onto seeds, resulting in a thin film on the seed surface. This is the method of commercial application for the EPA-registered product.

Sporangia of *P. ultimum* strain P4 were added to an Arkport sandy loam field soil following our standard procedures (16). This pathogen causes severe seed rot of many dicotyledonous plants, such as beans; however, on maize, it usually causes only a stunting of seedlings.

Strain Cg151NY82 (26) of *C. graminicola* was grown on oatmeal agar to obtain conidia. Conidia were scraped from plates and placed in sterile deionized water containing 0.004% Triton X-100. Conidia were enumerated by counting in a Petroff-Hauser chamber at 200-fold magnification. This pathogen causes severe disease on maize and includes seedling blight, leaf blight, and stalk rot, during which it becomes an aggressive vascular pathogen. Its initial infection is as a biotrophic pathogen; however, as disease develops, the organism functions as a necrotroph (2).

Enzyme and protein assays. Following various treatments, 5-day-old plants were removed from planting medium, weighed, and immediately flash frozen in liquid nitrogen. They then were extracted in an acetate buffer that contained sodium dodecyl sulfate, sodium EDTA, and mercaptoethanol (3). After centrifugation, endochitinase (EC 3.2.1.14) and *N*-acetylhexosaminidase (exochitinase) (EC 3.2.1.52) activities were measured in reaction mixtures by release of fluorescent methylumbelliferone from 4-methylumbelliferyl β-D-*N*,*N*′-diacetylchitobiose or 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, respectively (Sigma-Aldrich, St. Louis) (9). Activity of β-1,3 glucanase (EC 3.2.1.58) was measured in reaction mixtures containing 0.1% laminarin (Sigma-Aldrich) in 12.5 mM sodium acetate, pH 5.0. Polysaccharide degradation was detected with a modification of a fluorimetric method (7); after incubation for 30 min at 37°C, samples were reacted with decolorized aniline blue and the reduction in fluorescence over time was measured using a Cytofluor II fluorescence plate reader (PerSeptive Biosystems, Framingham, MA). Data were compared with a control without plant extracts. One unit of exochitinase or endochitinase activity was defined as the amount of enzyme required to release methylumbelliferone at 1 nmol min^{-1} from the substrates in a 1-ml reaction volume at 37°C. One unit of β-1,3 glucanase (laminarinase) activity is defined as the amount of enzyme required to hydrolyze laminarin at 1 ng min^{-1}.

Experimental designs and data analysis. For assays that continued for only 5 days, 10 ml of deionized water was placed in a 3-by-11-by-11-cm plastic box and a seed germination blotter (11 by 11 cm) was placed in the liquid. Ten seeds with or without treatment with T22 were placed in the box. Moist Arkport sandy loam field soil infested or not infested with *P. ultimum* (16) was used to fill the box to a depth of 2 cm; the inoculum level used was sufficient to cause 50 to 80% mortality of cucumber seed, which was a measure of inoculum potential. Another sheet of moist blotter paper was added to the surface of the soil and the box was covered with a fitted plastic lid to prevent moisture loss. Boxes were incubated for 5 days at 25°C and seedlings were removed for further analysis. Each experiment was conducted in triplicate and data were analyzed using Fisher's protected least significant difference (LSD) test (SuperAnova, Berkeley, CA).

Other assays were conducted in larger boxes (5 by 11 by 11 cm). Four or five seeds, either treated or not treated with T22, were planted in each box. Plants were grown for various lengths of time with 12 h of diurnal fluorescent lighting and the boxes were watered as needed. In some experiments, soils were autoclaved to examine maize growth responses in the absence of natural soil microbes. In addition, some soils were drenched with mefenoxam ([R]-2-[(S2,6-dimethylphenyl)-methoxyacetylamino]-propionic acid methyl ester; Subdue MAXX; Novartis, Research Triangle, NC) according to the manufacturer's directions. This fungicide primarily controls Oomycetes.

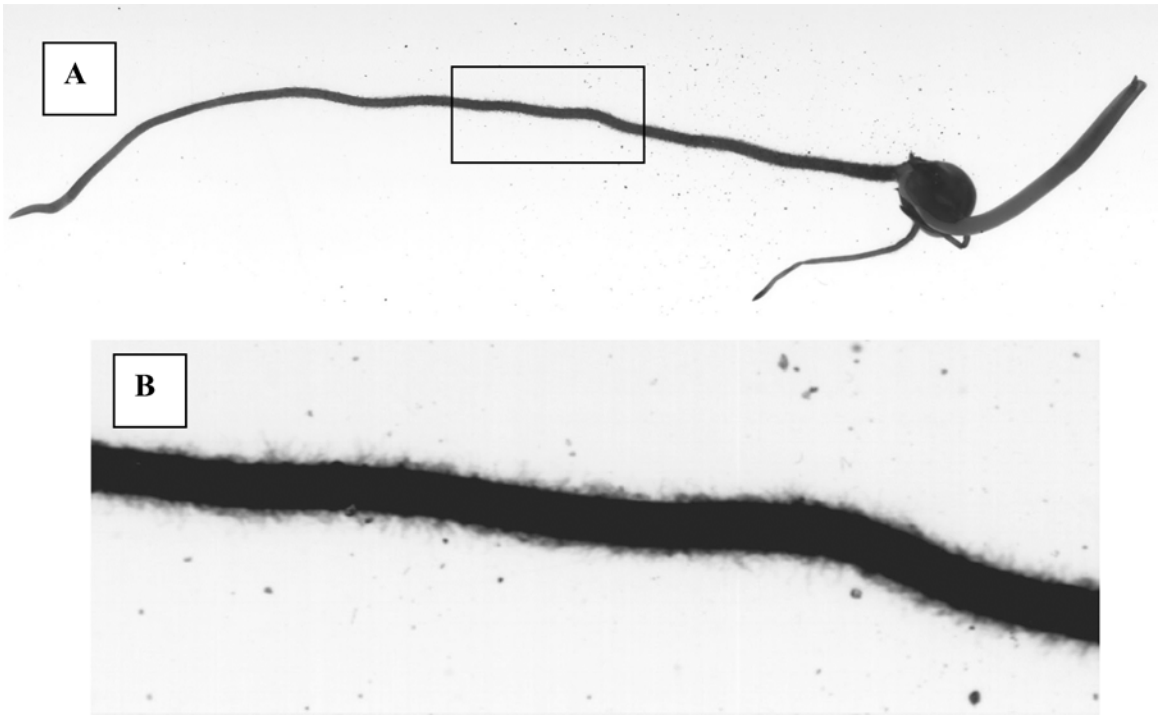

Fig. 1. A, Appearance of a scanned 5-day-old seedling of Mo17, following staining with thionin, after growth in Arkport sandy loam soil. **B,** In the insert, root hairs can be seen. With the MacRhizo program, different thresholds can be set that integrate areas of total roots, including root hairs or roots without their root hairs. The difference between the two values is the area of root hairs on each root system.

Colonization of plant surfaces by T22 was measured. Plant roots, shoots, or individual leaves were excised from plants at different times. Care was taken to avoid seeds or seed coats because this was the site of application and we wished to measure levels of T22 on other plant parts. Plant tissues were placed in tared 250-ml Erlenmeyer flasks with a known volume of sterile deionized water containing 0.004% Triton X-100 and shaken at 150 RPM for 2 h. Serial dilutions of the resulting suspension were plated onto 9-cm petri dishes containing acid potato dextrose agar (Difco Laboratories, Detroit) containing 0.1% Igepal Co630 as a colony restrictor (11). This medium allows growth of a wide range of fungi, but *Trichoderma* spp. are evident by their growth characteristics and, to a limited extent, different strains or species of *Trichoderma* are identifiable by their appearance. For example, on this medium, *T. virens* strains are white when viewed from the underside of the plate and T22 is tan to brown (11). The tissue remaining after spores were removed for dilution was dried. The levels of T22 were expressed as colony-forming unit per gram of plant tissue.

In some experiments, shoot and root growth were examined in detail. Plants were removed from soil, adhering soil was rinsed away, and the final residual soil clinging to the root hair regions was removed carefully by stroking with a small paintbrush. The plants were immersed in 0.02% (wt/vol) thionin in deionized water, which resulted in light blue staining of the root system. The stained plants then were placed in water and scanned (Hewlett Packard 4C/T ScanJet) and the images were stored in electronic memory. Images were analyzed using MacRhizo 3.8 software (Régent Instruments, Quebec City, Quebec, Canada). With the stained roots, the root hair region appeared as a light gray fringe adjacent to black root images. Changes in the threshold settings in the software allowed measurement of only the black root areas or of the black root plus the gray fringe of root hairs (Fig. 1). From this data, the area of root hairs on each root system could be calculated as the difference between the two measurements. Areas, lengths, and volumes of total and different size classes of roots also were quantitated using the MacRhizo software.

Data analysis considered each individual plant as a replicate and experiments consisted of 15 to 30 plants per treatment in each experiment. Mean values for each treatment were analyzed by LSD (SuperAnova) tests to give probability values.

Data suggested that T22 may induce systemic resistance in maize. In order to examine this possibility, T22-treated or untreated seed were planted in the sandy loam field soil in pots as described above. When plants reached the three- to four-leaf stage (15 to 20 days after planting), they were inoculated with *C. graminicola*. In initial experiments, 10- to 15-day-old plants were sprayed with conidial suspensions of the pathogen (2×10^4 conidia ml^{-1}) to runoff. Results of these experiments were difficult to quantify due to uneven distribution of lesions; therefore, in later experiments, leaves were inoculated by placing droplets on specific sites on leaves. Pots were placed on their sides and leaves were held flat by pinheads on styrofoam supports (pinheads held edges of leaves and did not pierce the leaves). Droplets (20 µl), each containing 400 conidia, were placed on two defined spots on the youngest fully expanded leaf. In some cases, leaves were wounded by piercing leaves with a needle immediately adjacent to the inoculation site. In all cases, plants were placed in a moist chamber at 25°C for 24 h immediately after inoculation. Seven days later, disease incidence was measured as the number of inoculated locations at which disease was present and disease severity was measured as the length of lesions that resulted from inoculation sites at which infection occurred. Each leaf was considered a replicate and data were analyzed using LSD tests.

RESULTS

Effects of T22 on root and shoot size and root hair area. Seedlings of Mo17 produced from T22-treated seed had larger roots and shoots than similar seedlings in the absence of T22 (Table 1; Fig. 2A). Root systems from T22-treated seed were nearly twice as long as those from control plants and growth of both fine and main roots were similarly enhanced (Table 1). Root areas also were measured and were proportional to root length; therefore, the total areas and volumes of roots in the presence of T22 also were approximately twice that of check plants (data not shown). Root hair area also increased in the presence of T22 (Table 1) but the root hair area per unit of root length was greater in control plants than in those with T22 (Table 1). These differences could be noted in 5-day-old seedlings (Tables 2 and 3) and persisted in larger plants (Fig. 2B).

Effects of T22 and *Pythium* spp. on seedling growth, protein expression, and enzyme activity. Infestation of soil with *P. ultimum* had a small and inconsistent effect upon Mo17 growth at 5 days (Table 2) or 10 to 15 days (data not shown) after planting, even though this pathogen reduced shoot and root growth of sweet corn (16) or other maize inbreds such as W23 (G. E. Harman and R. Petzoldt, *unpublished data*).

However, as noted earlier, T22 substantially increased plant growth and we investigated whether the increase measured in Arkport sandy loam field soil was a biocontrol effect or a direct effect upon the plant. Treatment of field soil with mefenoxam (Subdue MAXX), which primarily controls pythiaceous oomycetes, or autoclaving tended to increase the growth of Mo17 (Table 4). However, seed treatments with T22 increased plant growth more than either soil treatment. Further, T22 increased shoot growth in plants grown in mefenoxam-treated or untreated soil, which suggests that control of deleterious soil microflora was not the primary mechanism by which T22 increased Mo17 seedling growth.

Past research has demonstrated that T22 added to seed, soil, or roots results in colonization of roots (27) but little or no colonization of shoots (24). This was tested again in the current research. Five seedlings from each of three boxes were extracted and dilution plating was done. On 5-day-old plants in the absence of a seed treatment with T22, levels of *Trichoderma* spp. were near the limit of detection; log 0.84 ± 0.8 (standard deviation) or log 0.12 ± 0.12 CFU g^{-1} for roots or shoots, respectively. From seed treated with T22, levels of *Trichoderma* spp. were log 4.23 ± 0.2 and 0.45 ± 0.6 CFU g^{-1} on roots or shoots, respectively. Thus, the level of colonization of roots was ≈3,600-fold greater than shoots grown from T22-treated seed, and the levels of colonization of

TABLE 1. Effects of T22 on shoot and root size and root parameters of maize line Mo17 10 days after planting in a sandy loam field soil[y]

Treatment[z]	Shoot length (cm)	Total root length (cm)	Fine root length (cm)	Main root length (cm)	Root hair area (cm^2)	Ratio root hair area/ root length
None	4.5	28	11	17	0.88	0.031
T22	7.0	51	22	29	1.3	0.025
$LSD_{0.05}$	1.06	10.63	5.90	5.63	0.31	0.0073

[y] In this experiment 30 treated and 30 untreated plants were measured. Each plant was considered a separate experimental unit and values are means across the 30 plants. Shoot length was measured with a ruler but all other measurements were made using MacRhizo software following scans of the root systems.

[z] LSD = least significant difference.

shoots either in the presence or absence of T22 were similar. At 15 days, roots from plants grown from T22-treated seed supported log 4.2 ± 0.1 CFU g^{-1}; whereas on leaves of the same plants, we detected log 1.3 ± 0.4 CFU g^{-1}. On similar plants without T22 seed treatments, roots or shoots had log 1.4 ± 0.8 or 1.2 ± 0.2 CFU g^{-1}, respectively. Thus, levels of *Trichoderma* spp. on shoots of 15-day-old plants were similar with or without T22. However, there were 614-fold more *Trichoderma* propagules on roots of T22-grown plants than on shoots of the same plants.

Either seed treatments with T22 or soil infestation with *P. ultimum* affected levels of proteins in roots or shoots and activities of three enzymes with putative defensive roles: endochitinase, exochitinase, and β-1,3 glucanase. T22 treatment increased protein levels in both roots and shoots in the presence or

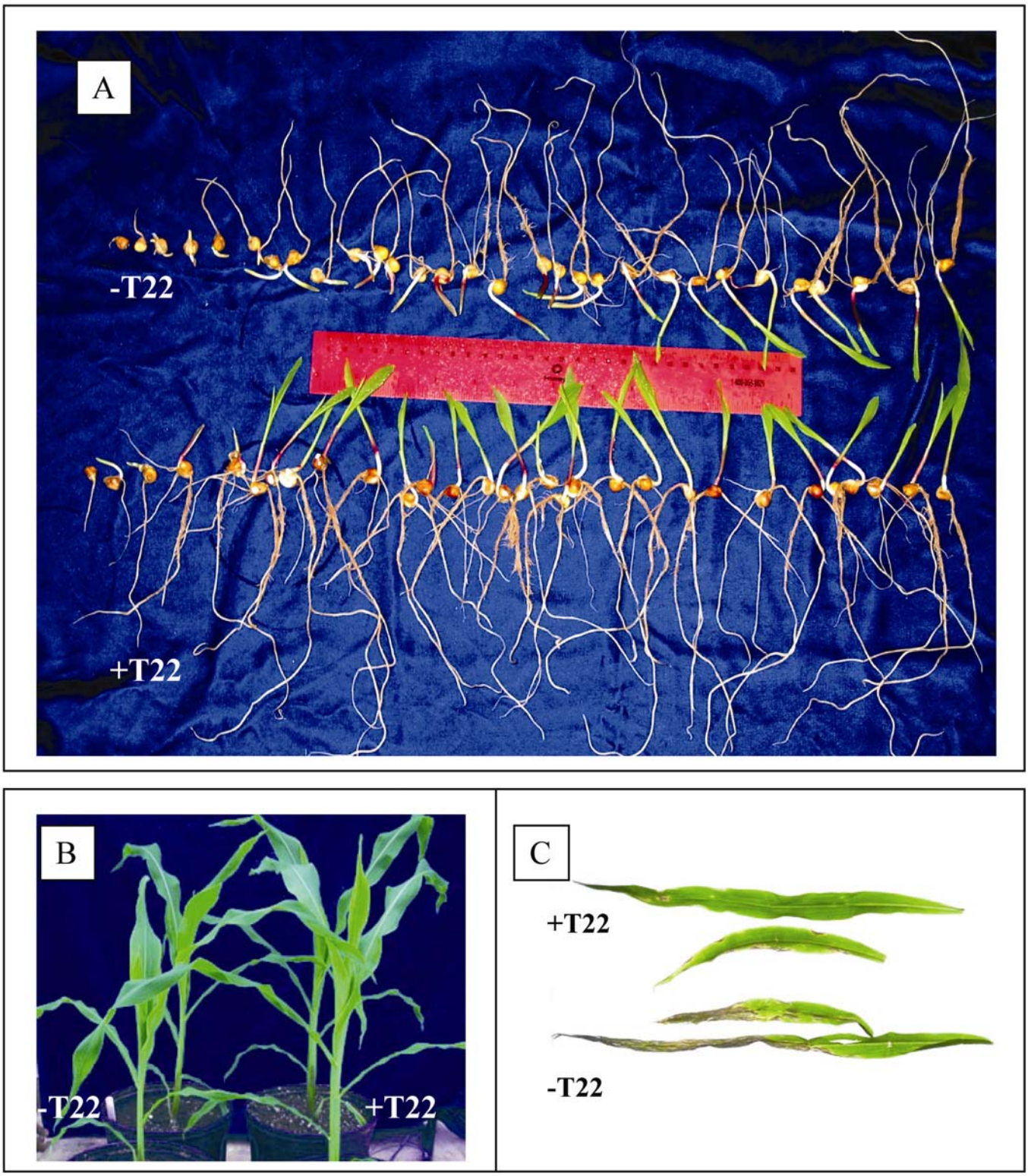

Fig. 2. A, Seedlings of maize line Mo17 (10 days old) grown in Arkport sandy loam soil with or without a seed treatment with T22, from an experiment similar to that reported in Table 1. **B,** Eight-week-old plants of maize line Mo17 in the presence or absence of T22; note that both plant height and stalk diameter are greater in the presence of T22. Differences in seedling sizes translate into larger plants throughout the life of the plant. **C,** Anthracnose lesions on maize line Mo17 leaves 7 days after spraying with conidia of *Colletotrichum graminicola.* Plants were grown from seed either treated or not treated with T22.

absence of *P. ultimum* (Table 2), even though T22 does not colonize shoots significantly. In the presence of the pathogen, protein levels decreased in one experiment but not in a second (Table 2). Treatment of seed with T22 in the absence of *P. ultimum* increased levels of all three enzymes in both shoots and roots. Moreover, β-1,3 glucanase activity was greatest in roots whereas exochitinase activity was greatest in shoots when both microbes were present. Conversely, soil infestation with *P. ultimum* resulted in little change in activity of the enzymes in either shoots or roots (Table 3). The differences between levels of enzyme expression of β-1,3 glucanase and endochitinase in seedlings in the presence of both organisms sometimes were 1.5- to 2-fold more than in the presence of *P. ultimum* alone (Table 3).

Effects of seed treatment with T22 on foliar disease caused by *C. graminicola*. Initial studies with anthracnose were conducted with spray inoculation of the pathogen on plants grown from seed with or without T22. Lesion sizes appeared to be smaller on plants from T22-treated seed (Fig. 2C) but were difficult to quantitate due to uneven distribution of lesions. Therefore, we used droplet inoculation, either with or without wounding, on specific sites on the leaves. When leaves were wounded, 70 to 80% of inoculation points became diseased regardless of the presence or absence of T22 (Table 5). However, in the absence of wounding, 20 or 0% of inoculation sites developed lesions in the absence or presence of T22, respectively. In the presence of wounding, lesion length from seedlings grown from T22-treated seed was 67% that in control plants. In the absence of wounding, lesion sizes in control plants were less than with wounding and, as already noted, no lesions developed in the presence of T22 (Table 5).

TABLE 2. Effects of a seed treatment with *Trichoderma harzianum* strain T22, soil infestation with *Pythium ultimum,* and the combination on shoot and root growth and on protein levels of 5-day-old seedlings of maize inbred Mo17 in two separate experiments[z]

	Fresh weight (g)		Protein level (mg/g fresh weight)	
Treatment	Root	Shoot	Root	Shoot
Experiment 1				
None	1.7 b	1.9 a	1.7 b	2.0 bc
T22	2.3 a	2.0 a	2.2 a	2.2 ab
P. ultimum	1.6 b	1.6 b	1.6 c	1.7 c
T22 + *P. ultimum*	2.0 a	2.1 a	2.2 a	2.5 a
Experiment 2				
None	2.2 b	1.8 b	1.5 b	2.0 b
T22	3.5 a	2.5 a	2.6 a	2.8 a
P. ultimum	2.4 b	1.7 b	1.6 b	1.9 b
T22 + *P. ultimum*	3.9 a	2.3 a	2.5 a	2.4 a

[z] Values within experiments followed by the same letter are not significantly different at $P = 0.05$ according to Fisher's protected least significant difference test.

DISCUSSION

Seed treatment with T22 had a number of effects on Mo17 maize seedlings and plants. T22 has been shown to be very efficient at colonizing roots of maize and other plants as metabolically active hyphae that exert effects, including enhanced root growth and depth, that extend at least for the life of annual crops.

As has been demonstrated in the past, T22 added as a seed treatment was effective in colonizing roots (27) but not shoots. In another study, transformants of T22 that expressed β-glucuronidase were applied to the root-soil zone and colonization of roots but not leaves was observed. However, if the organism was added as a spray to foliage, then hyphae of the organism developed both on leaves and roots (24).

Among the most dramatic effects noted in this study was an increase in shoot and root growth, which is likely a consequence

TABLE 4. Effects of soil treatments and seed treatments with T22 on shoot length of maize line Mo17 7 days after planting in Arkport sandy loam field soil[z]

Seed treatment	Soil treatment	Shoot length (mm)
None	None	58 ad
T22	None	85 bcf
None	Mefenoxam	63 abde
T22	Mefenoxam	96 cf
None	Autoclaving	79 abcde
T22	Autoclaving	92 cf

[z] Plants were grown in boxes and four seed were planted per box. Each plant was considered an experimental unit and there were 12 plants per treatment. Seed were not treated or treated with T22 and soils were either untreated, drenched with mefenoxam, or autoclaved for 90 min. Numbers followed by the same letter are not significantly different at $P = 0.05$ (a–c) or $P = 0.1$ (d–f) by Fisher's protected least significant difference test.

TABLE 5. Effect of root colonization by T22 on mean lesion size of anthracnose on maize line Mo17 7 days after inoculation with *Colletotrichum graminicola*[z]

Seed treatment	Leaf treatment	Lesions per leaf	Mean lesion length (mm)
None	None	0.4 a	7.5 a
T22	None	0.0 a	…
None	Wounded	1.4 b	33 c
T22	Wounded	1.6 b	22 b

[z] The second leaves of plants were inoculated at two separate sites. Wounding was done by penetrating each leaf with a needle just before inoculation. Lesions per leaf indicate the mean numbers of sites that developed disease per leaf (two maximum) while mean lesion length was measured on inoculation sites that developed disease. For each treatment, 9 to 14 plants were used. Numbers followed by the same letter are not significantly different at $P = 0.05$ by Fisher's protected least significant difference test.

TABLE 3. Effects of a seed treatment with *Trichoderma harzianum* strain T22, soil infestation with *Pythium ultimum,* and the combination on defense-related enzyme activities of 5-day-old seedlings of maize inbred Mo17 in the same two separate experiments reported in Table 2[z]

	β-1,3 glucanase				Exochitinase				Endochitinase			
	Root		Shoot		Root		Shoot		Root		Shoot	
Treatment	U/mg	U/μg	U/mg	U/μg	U/mg	U/μg	U/mg	U/μg	U/mg	U/μg	U/mg	U/μg
Experiment 1												
None	59.5 b	33.4 c	84.8 b	39.8 b	32.6 c	18.5 d	44.1 b	20.0 c	40.1 b	19.7 c	55.7 b	25.2 b
T22	65.9 b	36.6 bc	92.8 a	46.9 a	50.7 a	27.2 a	48.2 b	22.7 bc	51.4 a	27.8 a	75.9 a	34.9 a
P. ultimum	69.6 b	40.2 b	62.5 c	32.7 c	41.0 b	20.7 c	53.3 b	24.1 b	37.8 b	18.6 c	42.8 c	22.1 b
T22 + *P. ultimum*	93.0 a	49.3 a	a	49.6 a	49.4 a	25.2 b	66.2 a	32.4 a	44.8 a	23.2 b	76.7 a	36.4 a
Experiment 2												
None	31.8 b	18.4 bc	30.0 b	14.9 b	39.9 b	20.6 b	70.3 b	31.7 c	83.1 b	42.8 b	78.2 b	35.0 c
T22	48.4 a	25.0 ab	66.6 a	27.9 a	50.0 a	26.7 a	76.8 b	35.6 c	93.1 a	49.7 a	94.5 a	43.3 b
P. ultimum	24.5 c	13.4 c	29.4 b	15.5 b	38.5 b	20.1 b	81.1 b	43.4 b	75.0 c	35.1 c	67.7 b	37.1 c
T22 + *P. ultimum*	56.9 a	27.6 a	56.7 a	23.8 a	48.1 a	26.2 a	95.6 a	52.3 a	92.1 a	50.2 a	106.4 a	55.1 a

[z] Enzyme activity is expressed in units/mg (U/mg [fresh weight of tissue]) or specific activity (U/μg [protein in extract]). Values within experiments followed by the same letter are not significantly different at $P = 0.05$ according to Fisher's protected least significant difference test.

of both control of deleterious soil microflora and direct stimulation of plant growth. Even though addition of *P. ultimum* did not substantially affect plant growth, sterilization of soil by autoclaving or addition of mefenoxam tended to increase maize growth. These results indicate that the Arkport sandy loam field soil contained deleterious microflora even though no root disease was noted in plants grown in untreated soil. The identities of the deleterious microflora are unknown but may be pythiaceous because mefenoxam treatment increased plant growth. T22 increased growth of plants more with either soil treatment, which suggests that biological control was not the only mechanism of increased plant growth. Growth of Mo17 in the presence of T22 also is increased even on germination blotters (G. E. Harman and R. Petzoldt, *unpublished data*), which further suggests that there is a component of direct growth enhancement due to the beneficial microbe. However, it is likely that biological control of deleterious microflora also is a factor even in the absence of obvious disease, which is consistent with conclusions with other biocontrol agents (4). T22 is known to control a wide range of plant-pathogenic microbes; therefore, this result is not surprising (11).

These results are consistent with data from the commercial sector, including over 500 field trials on maize across the United States, indicating that T22 applied as a seed treatment provides a general grain yield increase averaging ≈5%. However, individual field trials have given widely different results, with yield increases ranging from less than 0 (yield decreases) to ≈50% (*personal communication,* Advanced Biological Marketing, Van Wert, OH). There are many reasons for this wide difference; for example, T22 can increase nitrogen use efficiency and, therefore, percentage yield increases are likely to be greater under conditions of nitrogen deprivation. Further, the enhancement of deep rooting in maize may partially overcome abiotic stresses such as drought (11). In some cases, large yield increases were noted in fields heavily infected with anthracnose or rust, suggesting that resistance to biotic stresses may have occurred. However, in many cases, there have been substantial yield increases even under very good growing conditions that provide high yields (11; *personal communication*, Advanced Biological Marketing).

There is a strong maize genetic component in the response to T22. Yield decreases have been noted in commercial trials and we verified these results in replicated field trials at Cornell in 2002 with the hybrid Sgi1860 × Sgi1861. A seed treatment with T22 reduced yields in plots fertilized with a range of nitrogen levels and types (ammonium nitrate or composted chicken manure). Tests with a range of inbred lines suggest that the eventual yield response can be predicted from seedling growth tests similar to those shown in Figure 2A. In these tests, several inbreds gave strongly positive growth responses similar to those of Mo17, whereas others gave weak positive responses and a few responded negatively. A rapid screening procedure for inbreds and hybrids should result in greater and more consistent yield increases in maize with T22 seed treatment than has been the case thus far.

Recent results, such as those reported here and in similar studies in other labs with other plant–*Trichoderma* spp. combinations, indicate that our understandings of the mechanisms and nature of biological control by *Trichoderma* spp. have been incomplete (15). In older literature, the primary mechanisms were considered to include direct effects on plant-pathogenic fungi, such as mycoparasitism, antibiosis, and competition (6). More recently, many more mechanisms, including competition for fungal germination elicitors (18,19) and inhibition of enzymes required for infection by *Botrytis cinerea* (32), have been described. Probably among the most important general mechanisms are direct responses of plants to *Trichoderma* spp. A number of recent papers indicate that induced resistance is a widespread and important mechanism (8,10,15,28–30); these data have been consolidated and interpreted and a new model of biological control by *Trichoderma* spp. has been formulated (15). Induction of disease resistance occurs on many dicotyledonous plants and rice in addition to maize (8,15,18,20,23,28–30). Induced resistance has been described in maize (25) but, so far as we are aware, has not heretofore been reported to be induced by beneficial root-colonizing microbes. In the present study, systemic resistance was induced in maize by a seed treatment with T22 as evidenced by both enzymatic activities and reduced anthracnose symptoms. Resistance may be either localized (20) or systemic (30) and there are at least three separate classes of proteinaceous or small molecular weight elicitors from *Trichoderma* strains that induce resistance responses in plants (15). In at least some cases, induced resistance responses are the primary determinants of biocontrol rather than the classical mechanisms of mycoparasitism or antibiosis (10,15,30). In cucumber whose roots are colonized by *T. asperellum* strain T-203, there is a transitory production of pathogenesis-related proteins. Within a few days, this reaction subsides but the plants are potentiated to respond to infection by pathogens by production of pathogenesis-related proteins and antimicrobial compounds over the entire plant, including leaves where T-203 is absent (30).

Together, these studies plus a developing body of literature indicate that *Trichoderma* spp., especially highly rhizosphere competent ones, have long-lasting and diverse effects on plants. They can increase plant root and shoot growth, probably by direct effects on plants and by biological control. Moreover, they also may increase plant growth by solubilization of nutrients in soil or by directly enhancing plant uptake of nutrients (1,31). Clearly, no single mechanism can explain the effects of these fungi in agroecosystems, but our improving understanding implies that these fungi are likely to be highly useful. Our current state of knowledge is inadequate for full exploitation of their abilities.

It is unlikely that systemic resistance systems induced by *Trichoderma* spp. will be adequate to confer levels of resistance approaching immunity. However, T22 and other strains of *Trichoderma* already are being deployed in increasingly large quantities for other purposes, such as increased yield in maize. In these systems, even a moderate increase in resistance to pathogens may be highly useful in reducing losses to biotic stresses.

Given the complexity of the *Trichoderma*–other microbe–plant interactions, holistic systems to examine changes in expression of the proteome or genome of the component organisms singly and in combination is the only approach that is likely to be successful. Rapid and reproducible biological systems to examine these interactions are essential and development of the T22-Mo17 system was conducted with this in mind. We already have examined in some detail changes in the proteome of 5-day-old Mo17 roots in the presence or absence of T22. Of the proteins envisioned on 2D gels in the presence of T22, ≈40% could not be detected in its absence (15). These data indicate that T22 induces profound changes in expression of maize proteins. Characterization of selected proteins is underway.

ACKNOWLEDGMENTS

This research was supported in part by Advanced Biological Marketing (ABM), Van Wert, OH, and by the Cornell Center for Advanced Technology. We thank T. Brutnell, Boyce Thompson Institute, T. Setter, Cornell University, and L. Bird and D. Custis, ABM, for their helpful suggestions; G. Bergstrom, Cornell University, for the strain of *C. graminicola* and for advice on handling of this pathogen; and K. Ondik for editorial assistance.

LITERATURE CITED

1. Altomare, C., Norvell, W. A., Björkman, T., and Harman, G. E. 1999. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. Appl. Environ. Microbiol. 65:2926-2933.

2. Bergstrom, G. C., and Nicholson, R. L. 1999. The biology of corn anthracnose. Knowledge to exploit for improved management. Plant Dis. 83:596-608.
3. Bolar, J. P., Norelli, J. L., Wong, K.-W., Hayes, C. K., Harman, G. E., and Aldwinckle, H. S. 2000. Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. Phytopathology 90:72-77.
4. Burr, T. J., Schroth, M. N., and Suslow, T. 1978. Increased potato yields by treatment of seedpieces with specific strains of *Pseudomonas fluorescens* and *P. putida*. Phytopathology 68:1377-1383.
5. Chang, Y.-C., Chang, Y.-C., Baker, R., Kleifeld, O., and Chet, I. 1986. Increased growth of plants in the presence of the biological control agent *Trichoderma harzianum*. Plant Dis. 70:145-148.
6. Chet, I. 1987. *Trichoderma*—Application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. Pages 137-160 in: Innovative Approaches to Plant Disease Control. I. Chet, ed. J. Wiley and Sons, New York.
7. Coté, F., Letarte, J., Grenier, J., Trudel, J., and Asselin, A. 1989. Detection of β-1,3-glucanase activity after native polyacrylamide gel electrophoresis: Application to tobacco pathogenesis-related proteins. Electrophoresis 10:527-529.
8. De Meyer, G., Bigirimana, J., Elad, Y., and Höfte, M. 1998. Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. Eur. J. Plant Pathol. 104:279-286.
9. Donzelli, B. G. G., and Harman, G. E. 2001. Interaction of ammonium, glucose, and chitin regulates the expression of cell wall-degrading enzymes in *Trichoderma atroviride* strain P1. Appl. Environ. Microbiol. 67:5643-5647.
10. Hanson, L. E., and Howell, C. R. 2004. Elicitors of plant defense responses from biocontrol strains of *Trichoderma virens*. Phytopathology 94:171-176.
11. Harman, G. E. 2000. Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T-22. Plant Dis. 84:377-393.
12. Harman, G. E. 2001. Microbial tools to improve crop performance and profitability and to control plant diseases. Pages 71-84 in: Proc. Int. Symp. Biological Control Plant Dis. New Century—Mode of Action and Application Technology. D. D.-S. Tzeng and J. W. Huang, eds. National Chung Hsing University, Taichung City, Taiwan.
13. Harman, G. E., and Björkman, T. 1998. Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. Pages 229-265 in: *Trichoderma* and *Gliocladium*, vol. 2. G. E. Harman and C. P. Kubicek, eds. Taylor and Francis, London.
14. Harman, G. E., and Donzelli, B. G. G. 2001. Enhancing crop performance and pest resistance with genes from biocontrol agents. Pages 114-125 in: Enhancing Biocontrol Agents and Handling Risks. M. Vurro, J. Gressel, T. Butt, G. E. Harman, A. Pilgeram, R. J. St. Ledger, and D. L. Nuss, eds. IOS Press, Amsterdam.
15. Harman, G. E., Howell, C. R., Chet, I., Viterbo, A., and Lorito, M. *Trichoderma* spp.—Opportunistic avirulent plant symbionts. Nature Rev. Microbiol. (In Press.)
16. Harman, G. E., Taylor, A. G., and Stasz, T. E. 1989. Combining effective strains of *Trichoderma harzianum* and solid matrix priming to improve biological seed treatments. Plant Dis. 73:631-637.
17. Ho, J. C., McCouch, S. R., and Smith, M. E. 2002. Improvement of hybrid yield by advanced backcross QTL analysis in elite maize. Theor. Appl. Genet. 105:440-448.
18. Howell, C. R. 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Dis. 87:4-10.
19. Howell, C. R. 2002. Cotton seedling preemergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with *Trichoderma* spp. Phytopathology 92:177-180.
20. Howell, C. R., Hanson, L. E., Stipanovic, R. D., and Puckhaber, L. S. 2000. Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. Phytopathology 90:248-252.
21. Lee, M., Sharopova, N., Beavis, W. D., Grant, D., Katt, M., Blair, D., and Hallauer, A. 2002. Expanding the genetic map of maize with the intermated B73 × Mo17 (IBM) population. Plant Mol. Biol. 48:453-461.
22. Lindsey, D. L., and Baker, R. 1967. Effect of certain fungi on dwarf tomatoes grown under gnotobiotic conditions. Phytopathology 57:1262-1263.
23. Lo, C.-T., Liao, F. T., and Deng, T. C. 2000. Induction of systemic resistance of cucumber to cucumber green mosaic virus by the root-colonizing *Trichoderma* spp. (Abstr.) Phytopathology 90(suppl.):S47.
24. Lo, C.-T., Nelson, E. B., Hayes, C. K., and Harman, G. E. 1998. Ecological studies of transformed *Trichoderma harzianum* strain 1295-22 in the rhizosphere and on the phylloplane of creeping bentgrass. Phytopathology 88:129-136.
25. Morris, S. W., Vernooij, B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C. C., Frederiksen, R. A., Bhandhufalck, A., Hulbert, S., and Uknes, S. 1998. Induced resistance responses in maize. Mol. Plant-Microbe Interact. 11:643-658.
26. Muimba-Kankolongo, A., and Bergstrom, G. C. 1992. Wound predisposition of maize to anthracnose stalk rot as affected by internode position and inoculum concentration of *Colletotrichum graminicola*. Plant Dis. 76:188-195.
27. Sivan, A., and Harman, G. E. 1991. Improved rhizosphere competence in a protoplast fusion progeny of *Trichoderma harzianum*. J. Gen. Microbiol. 137:23-29.
28. Yedidia, I., Benhamou, N., and Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl. Environ. Microbiol. 65:1061-1070.
29. Yedidia, I., Benhamou, N., Kapulnik, Y., and Chet, I. 2000. Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. Plant Physiol. Biochem. 38:863-873.
30. Yedidia, I., Shoresh, M., Kerem, K., Benhamou, N., Kapulnik, Y., and Chet, I. Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans* in cucumber by *Trichoderma asperellum* (T-203) and the accumulation of phytoalexins. Appl. Environ. Microbiol. (In Press.)
31. Yedidia, I., Srivastva, A. K., Kapulnik, Y., and Chet, I. 2001. Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. Plant Soil 235:235-242.
32. Zimand, G., Elad, Y., and Chet, I. 1996. Effect of *Trichoderma harzianum* on *Botrytis cinerea* pathogenicity. Phytopathology 86:1255-1260.