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Trichoderma biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry

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Abstract

Trichoderma isolates are known for their ability to control plant pathogens. It has been shown that various isolates of *Trichoderma*, including *T. harzianum* isolate T-39 from the commercial biological control product TRICHODEX, were effective in controlling anthracnose (*Colletotrichum acutatum*) and grey mould (*Botrytis cinerea*) in strawberry, under controlled and greenhouse conditions. Three selected *Trichoderma* strains, namely T-39, T-161 and T-166, were evaluated in large-scale experiments using different timing application and dosage rates for reduction of strawberry anthracnose and grey mould. All possible combinations of single, double or triple mixtures of *Trichoderma* strains, applied at 0.4% and 0.8% concentrations, and at 7 or 10 day intervals, resulted in reduction of anthracnose severity; the higher concentration (0.8%) was superior in control whether used with single isolates or as a result of combined application of two isolates, each at 0.4%. Only a few treatments resulted in significant control of grey mould. Isolates T-39 applied at 0.4% at 2 day intervals, T-166 at 0.4%, or T-161 combined with T-39 at 0.4% were as effective as the chemical fungicide fenhexamide. The survival dynamics of populations of the *Trichoderma* isolates (T-39, T-105, T-161 and T-166) applied separately was determined by dilution plating and isolates in the mixtures calculated according to the polymerase chain reaction (PCR) using repeat motif primers. The biocontrol isolates were identified to the respective species *T. harzianum* (T-39), *T. hamatum* (T-105), *T. atroviride* (T-161) and *T. longibrachiatum* (T-166), according to internal transcribed spacer sequence analysis.

Abbreviations: ITS – internal transcribed sequence; ap-PCR – arbitrarily primed polymerase chain reaction; rDNA – ribosomal DNA.

Introduction

Anthracnose caused by *Colletotrichum acutatum* and grey mould caused by *Botrytis cinerea* are two of the major fungal diseases of strawberry occurring worldwide (Elad, 2000a,b; Freeman and Katan, 1997). Anthracnose infections of mother plants develop bud and crown rot, which eventually result in the collapse and death of the plant. In the nursery, lesions, which are formed on stolons, eventually girdle the runners, resulting in the wilting and death of unrooted daughter plants. Infected transplants are capable of spreading the disease from the nursery to the field, where typical anthracnose symptoms are manifested as irregular leaf spot, crown rot, flower blight and fruit rot (Howard et al., 1992). Considerable yield loss can be inflicted by the pathogen under the appropriate environmental and cultural conditions (Maas, 1998). Following transplantation to production fields, entire beds of strawberry plants may collapse due to heavy

infections. Furthermore, symptoms of stunting and chlorosis, accompanied by decayed roots are also typical of *C. acutatum* infection (Freeman and Katan, 1997). In Israel, the current commercial control measures for anthracnose disease rely mainly on chemical pesticides (Freeman et al., 1997).

Grey mould disease of strawberry, caused by *B. cinerea*, attacks flowers, setting fruits, mature fruits and leaves (Sutton, 1990). The main sources of inoculum for the disease in strawberry are dead leaves, mummified fruits, straw mulch (where used), neighbouring crops and weeds (Sutton, 1995). Infected flower parts, shed after bloom, adhere to the fruit surface. After infecting the flowers, the pathogen may remain quiescent until the fruit ripens (Jarvis, 1980). Diseased leaves and fruits are often covered with grey tuft consisting of mycelia, conidiophores and conidia of the fungus; eventually, the fruits rot (Maas, 1998). Chemical control is the primary means by which grey mould is controlled. However, this may occasionally be ineffective due to the occurrence of resistant fungal populations (Hunter et al., 1987; Katan et al., 1989).

Trichoderma isolates are known for their ability to control plant pathogens (Elad and Freeman, 2002). Intensive research into biocontrol with *T. harzianum* has been carried out under commercial conditions, and there have been some significant achievements in greenhouse crops and in vineyards (Elad and Shtienberg, 1995). The first biocontrol agent (BCA) to be commercialized, registered and used in greenhouse crops and vineyards was isolate T-39 of *T. harzianum* (TRICHODEX), which effectively controlled *B. cinerea*, *Sclerotinia sclerotiorum* and *Cladosporium fulvum* diseases in greenhouse-grown tomato and cucumber, and in vineyards (Elad, 1994; 2000a,b). *T. harzianum* isolates were also reported to control strawberry grey mould (Tronsmo and Dennis, 1977). In a previous study, we showed that various isolates of *Trichoderma* that originated from a *Trichoderma* collection of 76 BCA isolates, including isolate T-39, were effective in controlling anthracnose and grey mould in strawberry, under laboratory and greenhouse conditions (Freeman et al., 2001; Elad et al., 2001). It has been reported on various occasions that the combination of a number of BCAs can further improve grey mould control (Elad and Freeman, 2002). For example, it was reported that co-application of T-39 of *T. harzianum* and AQ10 of *Ampelomyces quisqualis* resulted in improved control of grey mould in cucumber than with each organism alone (Elad et al., 1998). Likewise, combined application of two BCAs, a yeast (*Pichia guilermondii*) and a bacterium (*Bacillus mycoides*), resulted in better suppression of *B. cinerea* infection in strawberry and also reduced the variability of disease control (Guetsky et al., 2001).

Survival dynamics of populations of *Trichoderma* BCA isolates is of importance to determine their viability since several commercial products represented by strains of this genus exist and they may be applied for disease control in a mixture. Furthermore, differentiation between one or more strains of the mixture is required since reapplication of certain isolates may be necessary as well as the differentiation between BCAs and indigenous populations in the environment. Survival of *Trichoderma* strains may be quantified by the serial dilution method on a semi-selective medium (Elad and Kirshner, 1993), however, morphologically it is not possible to distinguish between applied isolates in a mixture or for that matter naturally occurring populations. A molecular approach using universally primed polymerase chain reaction (UP-PCR) was successfully applied to identify and monitor different *Trichoderma* isolates utilized for biocontrol in a glasshouse (Lübeck et al., 2000; Lübeck and Jensen, 2002). UP-PCR was compared to the dilution plating method and verified spread and establishment of a variety of commercial *Trichoderma* strains in ornamental pot plants.

The objectives of this study were to further assess selected *Trichoderma* strains for biocontrol of anthracnose and grey mould in large-scale strawberry experiments using different isolate combinations, timing application and dosage rates for improved disease control, and to assess a PCR-based method for evaluating survival of the selected biocontrol strains applied in a mixture.

Materials and methods

Fungal cultures and growth conditions

Four representative monoconidial *Trichoderma* culture isolates: T-39 from the commercial biological control product TRICHODEX (Makhteshim Chemical Works Ltd., Beer Sheva, Israel; Elad, 2000a,b), T-105, T-161 and T-166 were used in this study (Table 1). These isolates are representatives of a collection of 74 *Trichoderma* isolates, collected by the authors from strawberry plants growing in cultivated plots in the Kadima area of the central Sharon region, Israel and partially characterized to genotype by arbitrarily

Table 1. Trichoderma isolates

Isolate	Species	Origin[1]	EMBL accession
[2]T-39 (TRICHODEX)	*T. harzianum*	(Zimand et al., 1994)	AY222351
[3]T-105	*T. hamatum*	Crown	AY222340
[4]T-161	*T. atroviride*	Fruit	AY222344
T-166	*T. longibrachiatum*	Fruit	AY222347

[1]All Israeli *Trichoderma* isolates were collected by the authors from strawberry plants growing in cultivated fruiting fields in the Kadima area of the central Sharon region, Israel.
[2]Additional isolates belonging to this genotype based on ap-PCR: T-118, T-119, T-121, T-123, T-129, T-131, T-132, T-133, T-134, T-147.
[3]Additional isolates belonging to this genotype based on ap-PCR: T-101, T-102, T-103, T-104, T-106, T-107, T-108, T-109, T-110, T-111, T-112, T-113, T-114, T-120, T-122, T-126, T-128, T-130, T-136, T-138, T-140, T-141, T-142, T-143, T-144, T-145.
[4]Additional isolates belonging to this genotype based on ap-PCR: T-164, T-165, T-167.

primed PCR (ap-PCR) (Elad et al., 2001; Freeman et al., 2001). All fungi were cultured in the dark at 25 °C on potato dextrose agar (PDA) (Difco, Becton Dickinson, Le Pont de Claix, France) supplemented with 250 mg chloramphenicol in 1L sterile distilled water.

Large-scale biocontrol experiments

Strawberry plants (cultivar Mal'akh, susceptible to both *C. acutatum* and *B. cinerea*) were purchased at the beginning of September from Romano Strawberry Nursery (Even Yehuda, Israel) and transplanted into pots (12 cm) containing a styrofoam/coconut mix (15 : 85/v : v) used as a commercial rooting medium for strawberry cultivation. In consecutive years, 2001 and 2002, two large-scale experiments for the control of each pathogen separately, were conducted (i.e. four different experiments, two for each pathogen). The anthracnose control experiments were set up in mid-September corresponding to the commercial cultivation period in Israel. Plants were overhead irrigated every second day till run-off for the anthracnose experiments, and maintained outdoors (20–30 °C) under a shade net for the duration of the trial (6 weeks). Plants for the grey mould control experiments were maintained on elevated tables ($1 \times 2\,m^2$) in a greenhouse at 25 °C from mid-September before commencing the experiment which started beginning of January and lasted for 8 weeks. Upon commencement of the grey mould control experiments the controlled temperature conditions were terminated. The experimental conditions recorded fluctuating diurnal temperatures (10–25 °C) and RH > 92%, maintained with standing water on polyethylene sheets spread under tables. Plants were watered manually every 3–4 days. Both anthracnose and grey mould control experiments contained 16 or 24 plants per treatment, each replicated 5 or 6 times, set up in a randomized design. Plots were separated 50–70 cm from each other and barriers were erected during sprays in order to prevent drift from one treatment to another.

Three selected *Trichoderma* strains, T-39, T-161 and T-166, shown previously to control *B. cinerea* and *C. acutatum* (Elad et al., 2001; Freeman et al., 2001), were assessed in a large-scale experiment using different timing application, dosage rates and mixtures for reduction of strawberry grey mould and anthracnose. Different combinations of single, double or triple mixtures of the *Trichoderma* strains, applied at 0.4% and 0.8% concentrations, and at 2-, 7- or 10-day intervals, were applied till run-off, at a volume of 10 ml per plant. The concentrations of each isolate (T-39, T-161 and T-166) at 0.4% were approximately 4×10^7, 3×10^7 and 2.5×10^7 conidia per plant, respectively. Three days following the first *Trichoderma* treatment, the plants were infested with aggressive isolates of the two pathogens by spraying conidial suspensions on the foliage. Pathogen isolates included *B. cinerea* (isolate BcI16, Guetsky et al., 2002) and *C. acutatum* (isolate TUT-149, Freeman and Katan, 1997) at concentrations of 10^5 and 10^6 conidia per ml, respectively, which were produced from 14- and 10-day-old cultures of *B. cinerea* and *C. acutatum*, respectively, and applied at volumes of 500 ml and 5 l per experiment of each pathogen respectively, till run-off. A chemical treatment using Teldor (0.15% of fenhexamid 50% WP, Bayer AG, Germany) for grey mould control was

also included, and at a concentration of 0.15% once a week to the foliage till run-off, applied initially 3 days prior to pathogen application.

Anthracnose was assessed according to number of diseased apexes resulting in plant mortality, whereas grey mould was assessed according to number of blighted flowers. Disease incidence was evaluated twice a week. The results are presented as the area under the disease progress curve (AUDPC), which is a multiplication of severity by days (Dik and Elad, 1999). Statistical analyses of the data were performed using the JMP-in software, version 3 for Windows (SAS Institute Inc.). Differences in AUDPC of disease incidence between treatments were determined using Fisher's protected least significant difference (LSD) test at $P \leq 0.05$. Since the results, based on different trials of the repeated experiments were similar, findings of one such experiment are presented.

Survival of Trichoderma *isolates on the leaf surface based on dilution plating and PCR*

Survival of *Trichoderma* isolates (T-39, T-105, T-161 and T-166) alone or in combination was assessed on leaves of strawberry (cv. Mal'akh) plants, used also for the *Botrytis* control experiment, which were maintained in a glass-covered greenhouse at $25 \pm 1.0\,^{\circ}C$ under controlled conditions. For individual isolate survival studies, each strain was applied separately in a 10 µl volume drop containing 5×10^4 conidia, 10 drops per leaf, five plants per isolate. In combined inoculation studies, the four *Trichoderma* isolates were mixed, each at concentrations of 5×10^6 conidia per ml, and 10 µl volume drops of the mixture were inoculated on leaves in the same manner as the individual isolates. After leaf inoculation the plants were covered with plastic bags to maintain RH > 92% (temperatures remained constant at $25 \pm 1.0\,^{\circ}C$ within the bags) and leaves were picked periodically to determine population survival. Each sampled leaf was washed in 5 ml sterile water with 0.01% Tween80 and vigorously shaken by vortexing for 2–3 min to remove conidia. The conidia were serially diluted on PDA chloramphenicol (250 mg/l) plates at zero time (3–4 h after application), 1, 3, 7, and 14 days after inoculation to determine population survival, in time, of the individually inoculated and mixed treatments. In both individual and mixed inoculation experiments, each treatment consisted of five plants, and four inoculated leaves per treatment were sampled at each picking period. Uninoculated water drop controls were also used for comparison to determine whether naturally occurring populations of *Trichoderma* could be detected on the phylloplane. Ten single spore isolates of each of the individual treatments and 100 single spore isolates (25 from each of four sampled leaves) from the mixed inoculations per sampling period were re-isolated at random and re-cultured in 24-plate wells for DNA extraction after dilution plating. In the individual inoculated experiments, ap-PCR was conducted (see section on ap-PCR amplification) on the extracted DNA and amplified products were compared to those of the original genotype to confirm that the population remained uncontaminated. In the mixed culture experiments, single spore colonies were re-isolated, and ap-PCR was conducted to determine the percentage of each population in the mixture by comparing to amplified products of each representative genotype. Colony forming units (CFUs) of each isolate in the mixture were calculated by multiplying the percentage of each isolate (determined by PCR) by the total population recorded, based on dilution plating. These experiments were conducted twice and since the results of the repeated trials were similar, data from one such experiment are presented.

Isolation and purification of fungal DNA

For DNA extraction, liquid cultures comprising 100 ml of potato dextrose broth (PDB) (Difco, Becton Dickinson, Le Pont de Claix, France) in 250 ml Erlenmeyer flasks were inoculated with five mycelial disks of each of the four *Trichoderma* cultures (Table 1) derived from colony margins. The cultures were grown for 5–6 days without shaking and maintained at $25\,^{\circ}C$ before DNA extraction. Thereafter, the mycelium was collected by vacuum filtration and lyophilysed until dry. DNA was extracted and purified as previously described (Freeman and Katan, 1997). The DNA was dissolved in 0.5 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0) to an approximate concentration of 200–500 µg/ml and diluted to a final concentration of 10–100 ng/µl for PCR reactions.

DNA of the representative isolates (T-39, T-105, T-161 and T-166) from survival studies was extracted using a different protocol. For this purpose, conidia from selected fungal colonies were transferred to 2 ml Eppendorf tubes containing 200 µl PDB with ~150 mg glass beads (G-9393; 710–1810 µm, Sigma, St. Louis, MO, USA) per tube. The cultures were incubated at $25\,^{\circ}C$ for 5 days without shaking before

DNA extraction. The mycelium was then centrifuged at 14,000 rpm for 5 min and the supernatant discarded. Thereafter, 600 µl breaking buffer (2% Triton-X, 1% Sodium dedocyl sulfate, 100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA adjusted to pH 8.0) and 430 µl of phenol : chloroform : isoamyl alchohol (25 : 24 : 1) was added to the tubes. The tubes were sealed and thoroughly mixed on a vortex for 5 min and centrifuged at 8000 rpm for 5 min. The supernatant of 430 µl containing DNA was collected into a new tube and 43 µl of 3 M Na acetate (pH 5.2) and 860 µl of cold 96% ethanol was added. The DNA was precipitated by centrifugation for 5 min at 10,000 rpm, washed with 70% ethanol, re-centrifuged as described and dried. The DNA was dissolved in 100 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0) to an approximate concentration of 200–500 µg/ml and diluted to a final concentration of 10–100 ng/µl for PCR reactions.

Ap-PCR amplification

For ap-PCR, primers were derived from the following microsatellite or repeat sequences: CAGCAGCAGCAGCAG [$(CAG)_5$] and GACACGACACGACAC [$(GACAC)_3$] (Freeman et al., 2000). Universal PCR primers were used (internal transcribed spacer (ITS) 1, TCCGTAGGTGAACCTGCGG and ITS 4, TCCTCCGCTTATTGATATGC) for amplification of the ITS 1 and ITS 2 regions between the small and large nuclear ribosomal DNA (rDNA), including the 5.8S rDNA, as described (White et al., 1990). PCR reactions were performed in a total volume of 20 µl, containing 10–100 ng genomic DNA, 50 mM KCl, 10 mM Tris–HCl, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase (Promega, Corp., Madison, WI) and 1 µM primer. All the reactions were incubated in a PTC-100 thermocycler (MJ Research, INC., MA, USA) starting with 5 min of denaturation at 95 °C. For ap-PCR, this was followed by 30 cycles consisting of 30 s at 95 °C, 30 s at either 60 °C [for $(CAG)_5$] or 48 °C [for $(GACAC)_3$] and 1.5 min at 72 °C. For rDNA amplification, denaturation was followed by 40 cycles consisting of 30 s at 95 °C, 30 s at 50 °C and 1.5 min at 72 °C. Amplification products were separated in agarose gels (1.5% w/v; 15 × 10 cm^2, W × L) in TAE buffer (Sambrook et al., 1989) electrophoresed at 80 V for 2 h. All PCR experiments were conducted at least four times with identical results being observed. ITS sequence data of the representative isolates (T-39, T-105, T-161 and T-166) was analyzed for species designation (see below).

Sequencing procedure and species designation

PCR amplified rDNA products from representative isolates (T-39, T-105, T-161 and T-166) of *Trichoderma* spp., using the primer pair ITS 1 and ITS 4 (White et al., 1990), resulted in fragments of between 566 and 616 base pairs in size, which were extracted from agarose gels using the Jetsorb kit (Genomed GmbH, Germany). The Big Dye Terminator DNA sequencing kit (Perkin-Elmer Inc., Branchburg, NJ) was used for determining sequence of the ITS 1–2 regions (White et al., 1990). The sequence was determined using an ABI prism 377 DNA sequencer (Applied Biosystems Inc., Foster City, CA) and was performed at the sequencing unit of Tel Aviv University, Tel Aviv, Israel. Analysis of ITS sequences of the representative isolates was carried out using the ARB program package (Strunk et al., 1998). Alignment of sequences was performed with the implemented ARB automated alignment tool and alignments were refined manually. Complete ITS 1–2 sequences of the isolates were submitted to the GenBank, with EMBL accession numbers appearing in Table 1.

Results

Control of anthracnose and grey mould disease incidence in plants

All combinations of single, double or triple mixtures of *Trichoderma* strains, applied at 0.4% and 0.8% concentrations, and at 7 or 10 day intervals, were effective in reduction of anthracnose disease in large-scale experiments (Figure 1). All treatments significantly reduced disease incidence; however, the higher concentration of 0.8% for most strains was more effective than the 0.4% treatments. Combining strains T-39 with T-161 or with T-166 resulted in a higher reduction of disease incidence as compared with the 0.4% concentration for each of the strains alone but was similar to the 0.8% treatment (Figure 1). Anthracnose disease incidence in the control reached 21.4% at the end of the experiment.

Floral infection was the major symptom that occurred in the grey mould experiments and in the control disease incidence reached 89.1% at the end of the experiment. Reduction of grey mould in flowers was

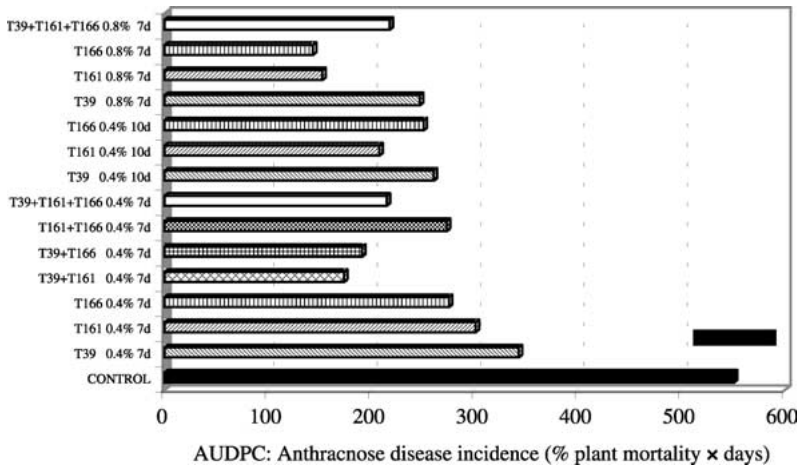

Figure 1. Effect of *Trichoderma* treatments for biocontrol of strawberry anthracnose disease caused by *Colletotrichum acutatum*. The experiments included single and combined treatments of isolates T-39, T-161 and T-166, at 0.4% and 0.8% concentrations, applied at 7 and 10 day intervals, compared with an untreated control. Disease incidence was expressed as AUDPC, percent plant mortality × days, and reached 21.4% in the control at the end of the experiment. The horizontal bar indicates the LSD at $P \leq 0.05$ for comparison between treatments.

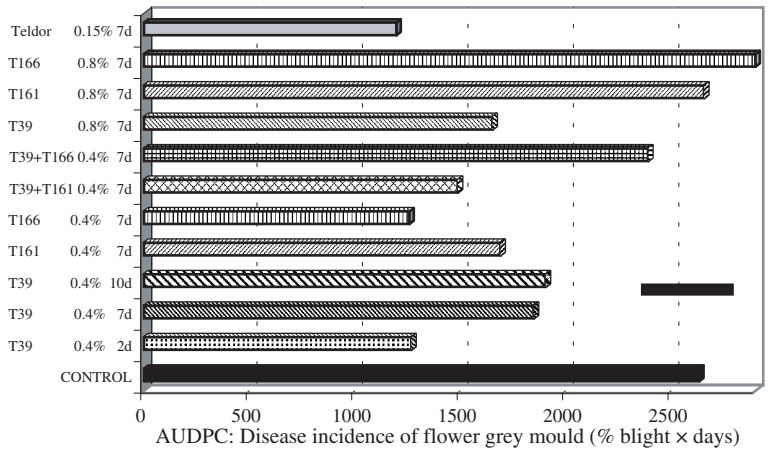

Figure 2. Effect of *Trichoderma* treatments for biocontrol of grey mould disease caused by *Botrytis cinerea* in strawberry. The experiments included single and combined treatments of isolates T-39, T-161 and T-166, at 0.4% and 0.8% concentrations, applied at 2, 7 and 10 day intervals, compared with an untreated control. Disease incidence was expressed as AUDPC, percent blight × days, and reached 89.1% in the control at the end of the experiment. The horizontal bar indicates the LSD at $P \leq 0.05$ for comparison between treatments.

highest when *Trichoderma* strain T-39 was applied at a 2-day interval at 0.4% concentration (Figure 2). Certain combinations of treatments with T-39 at 7- to 10-day application intervals at 0.4 and 0.7% concentrations were significantly better than other *Trichoderma* strains and the untreated control. Strain T-166, at a 7-day application rate, was equally effective for reducing floral grey mould infection compared to isolate T-39 at the 2-day interval. However, the combination of T-39 with T-166 did not significantly reduce disease incidence although the combined treatment of strain T-39 with T-161 produced significant results (Figure 2).

Survival of Trichoderma *isolates*

Viability of the *Trichoderma* strains T-39, T-105, T-161 and T-166, applied individually on strawberry leaf

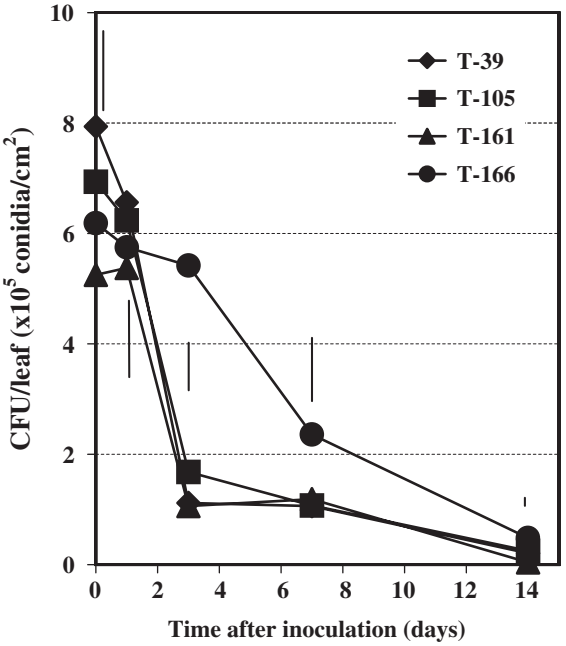

Figure 3. Survival of separate applications of *Trichoderma* isolates (T-39, T-105, T-161 and T-166) on individual strawberry leaves as assessed by the serial dilution plating method. The vertical lines indicate the LSD at $P \leq 0.05$ for each isolate at the different inoculation times.

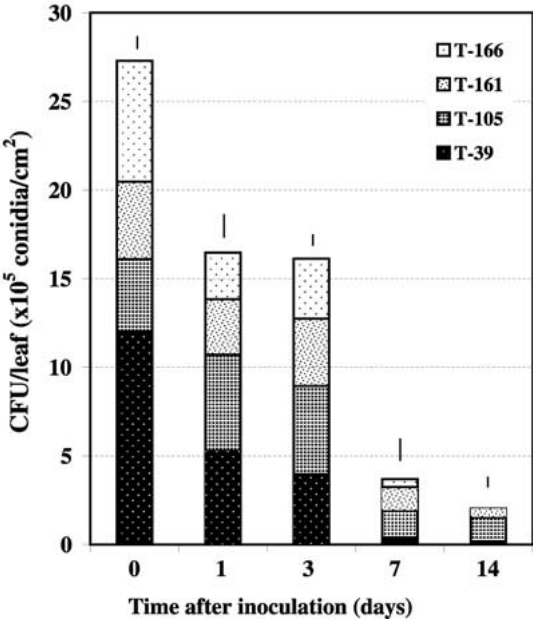

Figure 4. Survival of individual *Trichoderma* isolates (T-39, T-105, T-161 and T-166) applied as a mixture on individual strawberry leaves and assessed by the ap-PCR reaction. The isolates were removed after application at the different times and DNA was extracted from the developing colonies and compared to reference strains. Calculation of the number of CFU per isolate was determined by counting the number of individual isolates according to ap-PCR band patterns. The vertical lines indicate the LSD at $P \leq 0.05$ for each isolate in the mixture at each time period.

surfaces, declined rapidly to a lower level after 3 days, but less rapidly, 7–14 days after application (Figure 3). Although strain T-166 declined at a slower rate, differences in survival of CFU of the different strains were insignificant, 7 days after application. Furthermore, survival of the strains declined to undetectable levels after 2 weeks incubation (Figure 3).

Isolates T-39, T-105, T-161 and T-166 in the mix were serially diluted on PDA plates, DNA extracted, and ap-PCR performed to identify the percentage of each population in the mixture. Survival of isolates in the mix, calculated according to ap-PCR identification, also declined to low or undetectable levels after 14 days (Figure 4). Typical band patterns easily differentiated between these isolates when using primers $(GACAC)_3$ (Figure 5) and $(CAG)_5$ (data not shown) and allowed accurate identification of each isolate in the mix when compared to band patterns of the representatives. Strain T-105 in the mix appeared to survive better than the other isolates throughout the 14-day period of monitoring. No naturally occurring *Trichoderma* isolates could be detected on plates from uninoculated water control treatments.

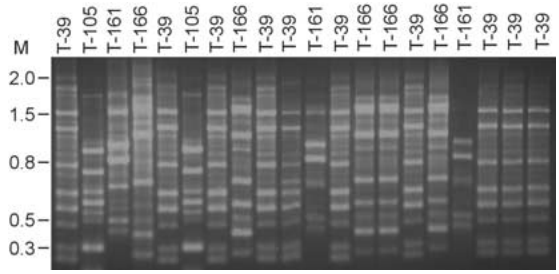

Figure 5. Band patterns of ap-PCR amplified genomic DNA using primer $(GACAC)_3$ of the representative *Trichoderma* spp. isolates (T-39, T-105, T-161 and T-166) used in the survival experiments at 0 time. Representative isolates appear in lanes 1–4, while the unknown isolates in the mix, from lanes 5–20, can easily be identified when compared to band patterns of the representatives. M: DNA sizes in kb.

Identification of Trichoderma *isolates*

In order to verify species identification of the four representative *Trichoderma* isolates (T-39, T-105, T-161

and T-166), ITS 1, ITS 2 and ITS 1–2 sequences of these isolates were analyzed with previously reported sequences of other *Trichoderma* species in the GenBank. Analysis of the ITS 1 sequence performed by applying ARB parsimony, distance matrix, and maximum-likelihood methods, grouped isolates T-39 within the *T. harzianum* clade, T-105 as *T. hamatum*, T-161 as *T. atroviride* and T-166 as *T. longibrachiatum* (Table 1).

Discussion

Variability among *Trichoderma* isolates makes it difficult to establish criteria according to which an antagonist may be regarded as a competent BCA. Moreover, biotic parameters such as the pathogen and its pathotypes, the host plant species, its cultivars and the plant structures affected by the pathogen, may also modify the efficacy of microorganisms that are selected for biocontrol.

In this work, we chose a number of potential *Trichoderma* BCAs and have shown that under commercial conditions anthracnose and grey mould in strawberry can be significantly reduced by their application. All combinations of concentrations and application time intervals were effective in anthracnose disease reduction. The higher concentration (0.8%) was superior in control whether used with single isolates or as a result of combined application of two isolates, each at 0.4%. Only few treatments resulted in significant control of grey mould. Isolates T-39 applied at 0.4% at 2-day intervals, T-166 at 0.4%, or T-161 combined with T-39 at 0.4% were as effective as the chemical fungicide fenhexamide (Figure 2). It appears that the time period between applications and concentrations are important parameters for grey mould control in strawberry. In this case, we found that 2–7 days and 0.4% concentration was preferential for *Botrytis* control (Figure 2). The effect of the rate of T-39 on control efficacy varies with the host and disease system. In earlier studies the lower rate was superior for control of tomato grey mould, similar to the findings in this study, but was inferior in controlling cucumber white and grey mould in commercial greenhouses (Elad, 2000a).

In a previous study, the *Trichoderma* isolates, as well as T-39 and TRICHODEX were used effectively in two strawberry plant bioassays, on petioles and leaves, for the control of *Colletotrichum* and *Botrytis in planta* (Freeman et al., 2001). The isolates selected represented mycoparasites, antibiotic producers and those not interacting in dual cultures that were effective in biocontrol (Elad et al., 2001). Isolates T-115, T-161, T-166, T-39 and TRICHODEX were effective in reducing *Botrytis* leaf grey mould incidence whereas T-118, T-165, T-166, T-39 and TRICHODEX were effective in reducing anthracnose in petioles (Freeman et al., 2001).

A selected number of isolates that showed promise for control of both anthracnose and grey mould were further characterized for viability and survival when applied individually or in a mixture. Since morphological criteria are inadequate for distinguishing between isolates when mixed together, ap-PCR was used for assessing the dynamics of such a population in time, similar to the UP-PCR method (Lübeck and Jensen, 2002). The isolates of *Trichoderma* that we chose possessed a variety of potential modes of action as *in vitro* tested (Elad et al., 2001). Isolate T-39 does not cause antibiosis nor acts as a mycoparasitise (Elad, 2000b); isolate T-161 parasitises *C. acutatum* but not *B. cinerea* and does not secrete antibiotics; isolate T-166 possesses antibiotic activity against *C. acutatum* but not *B. cinerea* and does not mycoparasitise; isolate T-105 is a parasite of both *C. acutatum* and *B. cinerea*. Ap-PCR indicated that isolate T-105 survived better than the other isolates in the mix (Figure 4), suggesting that the population of this isolate proliferates on the leaf surface. The mode of action of each individual isolate may partially play a role in survival of each of these populations when applied together, however, when applied separately they declined as rapidly (Figure 3). It has been reported that biocontrol can be improved when combining certain BCAs with different suppression mechanisms. For example, competition of *P. guilermondii* combined with secretor substances of *B. mycoides* resulted in improved control of *B. cinerea* (Guetsky et al., 2001; 2002). Therefore, it is possible that isolate T-105 is antagonistic to the other *Trichoderma* isolates when applied in the mix which may result in initial population increase due to decrease in the other competing populations.

Ap-PCR using repeat motif primers produced reliable, reproducible amplification results, similar to UP-PCR, indicating that these methods may be useful for determining the fate of introduced biocontrol or other agents, applied separately or in a mixture, in the environment (Cumagun et al., 2000; Lubeck et al., 2000). In this study, representative isolates were accurately grouped into the recognized clades of *T. harzianum*, *T. hamatum*, *T. longibrachiatum* and the species clade of *T. atroviride* (Hermosa et al. 2000; Kindermann et al., 1999; Lieckfeldt et al., 1999). The isolates used

in biocontrol studies in this work belong to each of the different species or sections listed above, indicating the taxonomic diversity of potential *Trichoderma* agents for biocontrol.

It can be concluded that it is possible to determine survival of isolates of *Trichoderma* applied in a mixture using PCR technology. Certain isolates appeared to survive better in a mix than others on leaf surfaces indicating diversity in survival and viability. The mode of action of the potential BCA must also be considered when applying a mixture of isolates as potential antagonism between the beneficial organisms may occur.

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