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Biological control of *Rhizoctonia solani* AG1-1A, the causal agent of rice sheath blight with *Trichoderma* strains

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Summary. Sheath blight caused by *Rhizoctonia solani* is one of the most serious rice diseases worldwide. The disease is currently managed only by the excessive application of chemical fungicides which are toxic and not environmentally friendly. Therefore, greater emphasis should be given to biological control as being both safe and effective. *Trichoderma* species are ubiquitous fungi in the soil and have an antagonistic activity against several soil-borne plant pathogens including *R. solani*. The present study was undertaken to evaluate the potential of indigenous *Trichoderma* strains from Mazandaran province, Northern Iran (a Mediterranean region on the southern coast of the Caspian Sea) against *R. solani* AG1-IA *in vitro*, and against sheath blight disease in the glasshouse, in order to find biocontrol isolates for application in the field. More than 200 *Trichoderma* strains were isolated from the soil, plant debris and the phyllosphere in rice fields. Strains were first screened for their antagonism to *R. solani* by *in vitro* antagonism tests including dual culture, antibiosis, the effect of *Trichoderma* strains on the production and viability of *R. solani* sclerotia, and hyperparasitism on microscopic slides. According to the *in vitro* experiments, several strains belonging to *T. harzianum*, *T. virens* and *T. atroviride* showed excellent biocontrol. These potential antagonist strains were further evaluated for their effectiveness in controlling sheath blight under glasshouse conditions. Among the 55 selected strains, seven significantly controlled the disease. *T. harzianum* AS12-2 was the most effective strain in controlling rice sheath blight, better even than propiconazole, the most commonly used fungicide in Iran.

Key words: biocontrol, *Oryza sativa*, *Rhizoctonia solani*, *Trichoderma harzianum, Trichoderma virens*.

Introduction

Rhizoctonia solani Kühn (teleomorph: *Thanatephorus cucumeris* [A.B. Frank) Donk.]) is a widespread soil-borne pathogen that causes economically important diseases in many crops (Adams, 1988). Rice sheath blight caused by *R. solani* is one of the most serious diseases of rice worldwide, causing considerable yield losses (Sudhakar *et al*., 1998). The widespread adoption of new, susceptible, high-yielding cultivars with large numbers of tillers, and the changes in cultural practices associated with these cultivars, favor the development of sheath blight and contribute greatly to the rapid increase in the incidence and severity of this disease in rice-producing areas throughout the world (Groth *et al*., 1991; Rush and Lee, 1992). Furthermore, environmental conditions such as low light, cloudy days, high temperature and high relative humidity also favor the disease (Ou, 1985). The pathogen overwinters as soil-borne sclerotia and mycelium in plant debris; these constitute the primary inoculum. Control of the pathogen is difficult because of its ecological behavior, its extremely

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broad host range and the high survival rate of sclerotia under various environmental conditions (Groth *et al*., 2006). So far, no rice variety completely resistant to this fungus has been found, although extensive evaluation of rice germplasm has been conducted (Oard *et al*., 2004). In the absence of a desired level of host resistance, the disease is currently managed by excessive application of chemical fungicides, which have drastic effects on the soil biota, pollute the atmosphere, and are environmentally harmful. Some potentially effective fungicides are highly phytotoxic to rice and, if the disease is not severe, these fungicides may reduce yield (Groth *et al*., 1990). It is difficult to achieve control through host resistance or fungicides, therefore, biological control may be effective in minimizing the incidence of sheath blight (Das and Hazarika, 2000).

The anamorphic fungal genus *Trichoderma* (*Hypocreales*, *Ascomycota*) contains cosmopolitan soil-inhabiting fungi that are a major component of the mycoflora in soils of various ecosystems (Harman *et al*., 2004). The genus *Trichoderma* is especially known for its antagonistic activity against several plant pathogens, including *R. solani* (Papavizas, 1985; Chet, 1987; Harman and Björkman, 1998; Harman, 2006), and some strains are already commercialized as biocontrol agents (BCAs). These are also potential agents in suppressing rice sheath blight, they are highly competitive on rice plant residue and thus exhaust the nutrient supply for the pathogen and greatly reduce its survival (Mew and Rosales, 1984, 1985; Mostafa Kamal and Shahjahan, 1995).

Sheath blight is the most serious disease of high yielding rice cultivars in Mazandaran, the largest rice-growing province in Iran, on the southern coast of the Caspian Sea, which has a Mediterranean climate. The objective of this study was to evaluate the potential of indigenous *Trichoderma* isolates recovered from paddy rice fields in controlling *R. solani*, the rice sheath blight pathogen, *in vitro* and *in vivo*.

Materials and methods

Fungal isolates

Rhizoctonia solani RBL1, isolated from naturally infected rice plants with typical symptoms of sheath blight in a paddy field of Mazandaran province, Iran, was used in all experiments. *R. solani* strain RBL1 was obtained from the culture collection of the Iranian Rice Research Institute. The fungus was purified with the hyphal tip method and maintained on potato dextrose agar (PDA, Merck, Germany). To prove pathogenicity, inoculations were done in a glasshouse on *Oryza sativa* cv. Neda by placing a 5-mm mycelial plug of *R. solani* between the junction of the basal leaf sheath and the stem above the water line at the maximum tillering stage. *R. solani* was re-isolated from characteristic lesions of sheath blight. To confirm the anastomosis group (AG) and subgroup, a nuclear rDNA region, containing the ITS1 and 2 as well as the 5.8S gene (accession No. HM211085) was subjected to a BLAST search (Altschul *et al*., 1997) to find out the most similar sequences in the NCBI GenBank.

For the isolation of *Trichoderma* strains, soil samples were collected from rice fields located all over Mazandaran province (Figure 1), on the southern coast of the Caspian Sea. Soil was taken with an auger from a depth of 15 cm. Samples were air dried for 3–5 days at room temperature. *Trichoderma* isolates were obtained by the dilution plate method (Dhingra and Sinclair, 1995) on McFadden & Sutton's RB-S-F *Trichoderma* selective medium (Davet and Rouxel, 2000). Sieved soil samples (10 g) were shaken in 90 mL sterile water for 10 minutes. For the isolation of *Trichoderma* from the rice phyllosphere, the leaves and stems were cut into small pieces (1 cm^2), transferred to 500 mL Erlenmeyer flask with 100 mL sterile distilled water and placed on a shaker for one hour. A dilution series up to 10^{-6} was made from the samples. Aliquots (1 mL) were spread on Petri plates containing a selective medium and were then incubated at 25°C in the dark. *Trichoderma* isolates were also purified directly from fungal masses on rice debris. Putative *Trichoderma* colonies (from soil and foliage samples) were purified on PDA plates by the single spore method and deposited in the Microbiological Collection of the University of Szeged (SzMC).

DNA extraction and PCR conditions

For DNA preparation, a mycelium plug of each strain was placed on a cellophane disk sterilized by autoclaving in water and placed on the surface

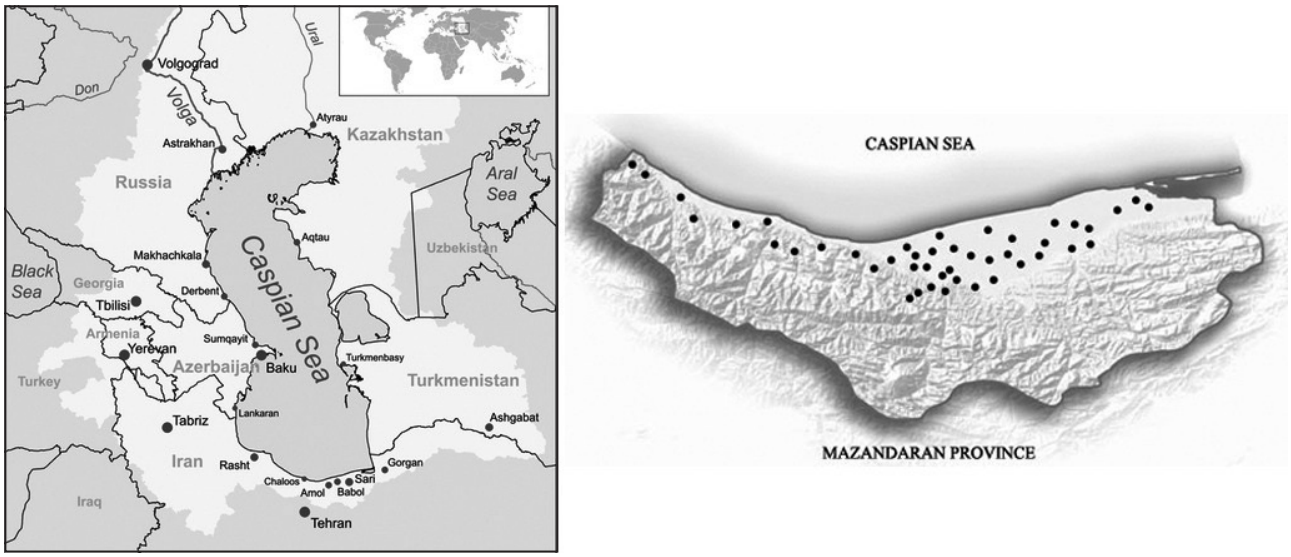

Figure 1. Sampling locations in Mazandaran province, northern Iran.

of yeast extract agar (5 g yeast extract, 5 g dextrose and 20 g agar L^{-1}) for 2–3 days at room temperature. The fresh mycelium was scraped off and ground with a mortar and pestle in liquid nitrogen. Total DNA was extracted using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, USA) according to manufacturer's instructions.

A nuclear rDNA region containing the internal transcribed spacer (ITS) regions 1 and 2 and the 5.8S rRNA gene was amplified using the primers ITS1 and ITS4 (White *et al*., 1990). PCR amplifications were performed as described previously (Hermosa *et al*., 2000). Amplicons were purified with the GenElute PCR Clean-up Kit (Sigma-Aldrich) and sequenced at Macrogen Inc., Seoul, Korea. ITS sequences representing different ITS genotypes were submitted to the NCBI GenBank database (Table 1).

Species identification

A combination of morphological and molecular analysis was used for the identification of *Trichoderma* isolates. For morphological identification, strains were grown on 2% malt extract agar and on PDA under ambient laboratory conditions of light and temperature (about 21°C). Microscopic observations and measurements were made from preparations mounted in lactic acid. Individual isolates were identified at species level using standard mycological key (Gams and Bissett, 1998), species descriptions (Bissett, 1992; Samuels *et al*., 1999; Kraus *et al*., 2004), and *Trich*OKEY 2.0, an online method (http://www.isth.info/tools/molkey/index.php) for the quick and reliable molecular identification of *Hypocrea* and *Trichoderma* at the genus, clade, and species levels based on ITS1 and 2 sequences (Druzhinina *et al*., 2005). *Trich*OKEY identifies most species unequivocally, although some species have identical or very similar ITS1 and ITS2 sequences and are therefore indistinguishable by this method (Druzhinina *et al*., 2005, 2006). An accurate identification of species in the Rufa Clade is sometimes problematic as the species of this clade are currently under revision, and both *T. viride* and *T. koningii* are being divided into several new species (Druzhinina *et al*., 2005). In such cases the preferred means of identification is to supplement the barcode method (*Trich*OKEY) with the sequence analysis of other loci by *Trich*oBLAST (Jaklitsch *et al*., 2006; Kubicek *et al*., 2008).

***In vitro* antagonism experiments**

For the dual culture tests, mycelial disks 5 mm in diameter were cut from the edges of acti-

Table 1. *Trichoderma* strains isolated during this study.

Species	Isolate code[a]	Substrate[b]	GenBank accession number[c]
Trichoderma harzianum	AD1-1	Rice debris	EU821780
	AS3-3, AS3-5, AS4-1, AS4-2, AS4-3, AS5, AS15-3, AS19-1, AS19-2, AS19-3, AS19-4, AS20-2, AS20-3, AS20-4, AS20-5, AS21-1, AS21-2, AS22-1, AS22-2, AS22-3, AS22-4	Soil	EU821780
	AS2-1, AS2-2, AS2-3, AS17-2	Soil	EU821781
	AD7	Rice debris	EU821782
	AS15-1, AS15-4, AS15-6, AS15-7, AS16-1, AS17-3	Soil	EU821782
	AD1-2, AD6	Rice debris	EU821784
	AS12-1, AS12-2, AS12-3, AS12-4	Soil	EU821789
	AS16-3, AS16-4	Soil	EU821791
T. virens	AD1-3, AD1-5	Rice debris	EU821794
	AS1-1, AS1-2, AS3-1, AS3-2, AS3-4, AS1-1, AS6-1, AS6-2, AS6-3, AS6-4, AS10-1, AS10-2, AS10-3, AS10-4, AS10-5, AS10-6, AS10-7, AS11-1, AS11-2, AS11-3, AS11-4, AS14-1, AS14-2, AS14-3	Soil	EU821794
	AS15-2, AS15-5, AS16-2, AS16-22, AS18-1, AS18-2, AS18-4	Soil	EU821795
	AS17-1, AS17-4	Soil	EU821796
T. atroviride	AS8, AS18-5	Soil	EU821797
T. hamatum	SS11-2	Soil	EU821798
T. asperellum	BS3-8	Soil	EU821799
T. brevicompactum	DS701	Soil	EU821800

[a] Isolates were grouped according to their ITS genotypes.
[b] All isolates obtained from rice fields in Mazandaran province, Iran.
[c] Identical GenBank accession numbers indicate that the sequences of the corresponding isolates are identical.

vely growing colonies of the pathogen and the *Trichoderma* isolates, and were placed opposite each other, 1.5 cm from the edge of 9 cm Petri dishes containing PDA. Petri dishes inoculated with *R. solani* alone served as controls. Each pair was replicated three times and incubated for five days at 26°C in darkness, then scored for degree of antagonism using the 1–5 scale of Bell *et al*. (1982): 1, *Trichoderma* completely overgrew the pathogen and covered the entire Petri dish; 2, *Trichoderma* overgrew at least two thirds of the Petri dish; 3, *Trichoderma* and *Rhizoctonia* each colonized 50% of the medium surface and neither organism appeared to dominate the other; 4, *Rhizoctonia* colonized at least two-thirds of the medium surface and appeared to withstand the encroachment of *Trichoderma*; 5, *Rhizoctonia* completely overgrew the entire Petri dish. An isolate of *Trichoderma* was considered to be antagonistic to the pathogen if the mean score for a given comparison was ≤2, but not highly antagonistic if the score was ≥3. The plates were maintained for one more week

and the number of sclerotia formed was counted and recorded.

The effect of *Trichoderma* spp. on the germination of *R. solani* sclerotia was determined following Mukherjee *et al*. (1999). Briefly, sclerotia of *R. solani* of uniform size were placed on six-day-old cultures of *Trichoderma* isolates. The cultures were incubated at 26°C in darkness for up to 30 days. Untreated sclerotia served as the control. The viability of *R. solani* sclerotia was evaluated by placing them on water agar plates for 24 hours at 26°C and inspecting hyphal growth with a stereomicroscope. Sclerotia were considered viable when typical *Rhizoctonia*-like hyphae grew from them.

The effect of volatile metabolites from *Trichoderma* species on *R. solani* was tested in the assemblage described by Dennis and Webster (1971). Two bottoms of Petri dishes containing PDA were individually inoculated with a disk of the pathogen and *Trichoderma*, and the bottoms were adjusted and sealed by Parafilm. The control sets contained the pathogen alone. The cultures were incubated at 26ºC (12 h light/12 h darkness). Growth rates were recorded daily by measuring the colony diameter, and the inhibition percent was obtained using the formula: I (%)=[(C2–C1)/C2)]×100, where C1 is the growth of *R. solani* in the presence of *Trichoderma* volatiles, and C2 is the growth of the control (*R. solani* alone).

For the mycoparasitism tests, a small amount of molten water agar was poured onto and evenly spread over the sterile slide to form a thin agar film. One end of the slide was kept free of the medium to facilitate handling. Mycelial disks of *Trichoderma* isolates and the pathogen were placed on the slide 1 cm apart from each other. All paired cultures on the slides were placed on 2% water agar in Petri dishes and incubated at 28°C for 5 days. At the end of the incubation period, the zones, where the hyphae of *Trichoderma* isolates met the hyphae of the pathogen were inspected under a light microscope for coiling structures in *Trichoderma* hyphae and wall disintegration in the hyphae of *R. solani*. The frequencies of coiling or wall disintegration were estimated by counting the coils or areas of disintegration in three different microscopic fields (Sivakumar *et al*., 2000).

Greenhouse assays

The efficacy of the 55 *Trichoderma* isolates selected by the *in vitro* screening tests was further evaluated for their control of sheath blight of rice *in vivo*. The selected isolates belonged to three species, *T. harzianum*, *T. virens* and *T. atroviride*. Twenty-day-old rice seedlings (cv. Neda, high yield and susceptible to sheath blight), were transplanted to plastic pots of 20 cm diameter, each with 4.5 kg of rice field soil (three seedlings per pot). Pots were arranged 25 cm apart on glasshouse benches. Seedlings were fertilized by NPK applied to the soil (100:60:40 kg ha^{-1}) and watered regularly to keep them submerged. Plants were kept at 26 to 34°C and 85 to 90% relative humidity. The pots were arranged in a completely randomized design with three replications per treatment. The rice plants were inoculated with *R. solani* as described above (five tillers per hill). Spores of *Trichoderma* isolates were harvested from 10-day-old cultures grown on PDA by adding sterile water to the plates and scraping the culture with a scalpel. The resulting suspensions were filtered through a double layer of cheesecloth to separate large mycelial fragments from conidia. The concentration of spores was measured with a Neubauer Improved haemocytometer (Precicolor, HBG, Giessen, Germany) and adjusted to 10^7 conidia per mL. Tween 20 was added at 0.1% to ensure adhesion of spores to plant surfaces. Rice plants were evenly sprayed with spore suspensions of *Trichoderma* (50 mL per replicate), using a hand sprayer, at two stages: 24 h after inoculation of the pathogen and seven days after the first spray. Plants inoculated with *R. solani* alone served as control. Propiconazole (TILT®, 250 EC, Syngenta Crop Protection Inc.), a commonly used fungicide for controlling sheath blight in Mazandaran, was used for comparison. Disease severity was recorded 30 days after pathogen inoculation as relative lesion height (RLH) following the International Rice Research Institute's standard evaluation procedure for rice (IRRI, 1996). The relative lesion height of each tiller was calculated using the formula: RLH (%) = 100× [Highest point a lesion occurred (cm)/ Plant height (cm)]. Besides the RLH, which was the main parameter recorded to indicate disease intensity, the number of infected tillers per replicate and the number of sclerotia produced on each rice plant were also counted after 30 days.

Statistical analysis

All experiments were conducted as completely randomized designs with three replications. Analysis of variance (ANOVA) was performed for each experiment. If significant differences between treatments were detected, the mean differences were separated by Fisher's protected least significant differences (LSD) test at $P \leq 0.05$. Arcsine and square root transformations were used for percentage values and count data, respectively to correct for uneven distribution or homogeneity of variance prior to analysis. All data transformed for analysis are presented as non-transformed values. All analyses were done using the SAS statistics software program (SAS Institute Inc., 1989).

Results

Species identification

The pathogenicity test in the glasshouse indicated that *R. solani* strain RBL1 was a virulent pathogen on rice. The BLAST search results revealed that this isolate showed the highest similarity to six *R. solani* AG1-IA isolates (GenBank accession numbers: AB000017, AB000016, AB000010, AF354097, AY154301, FJ492099) (E-value = 0.0 and max. identity = 99%).

A total of 202 *Trichoderma* isolates was obtained as described in the subsection Fungal Isolates of Materials and Methods. The recovered species comprised *Trichoderma harzianum* Rifai, *T. virens* (Miller, Giddens & Foster) von Arx, *T. atroviride* P. Karst, *T. hamatum* (Bonord.) Bainier, *T. brevicompactum* Kraus, Kubicek & Gams and *T. asperellum* Samuels, Lieckf. & Nirenberg (Table 1). One hundred and sixteen isolates belonged to the *T. harzianum* species complex, 70 were identified as *T. virens* and 11 as *T. atroviride*. Three isolates of *T. hamatum*, one of *T. asperellum* and one of *T. brevicompactum* were also obtained. *T. harzianum* proved to be the dominant *Trichoderma* species in rice phyllosphere, while *T. virens* was more abundant in the soil than in the phyllosphere. None of the representatives of the four remaining species were isolated from phyllosphere (Figure 2). In addition, all the *T. atroviride* isolates except one originated in the eastern part of Mazandaran province. More species were isolated from the soil than from the phyllosphere; from the phyllosphere only *T. harzianum* (31 isolates) and *T. virens* (three isolates) were recovered. The *Trichoderma* spp. ranged from 0.5×10^2 to 1×10^3 cfu g^{-1} of dry soil in paddy rice fields.

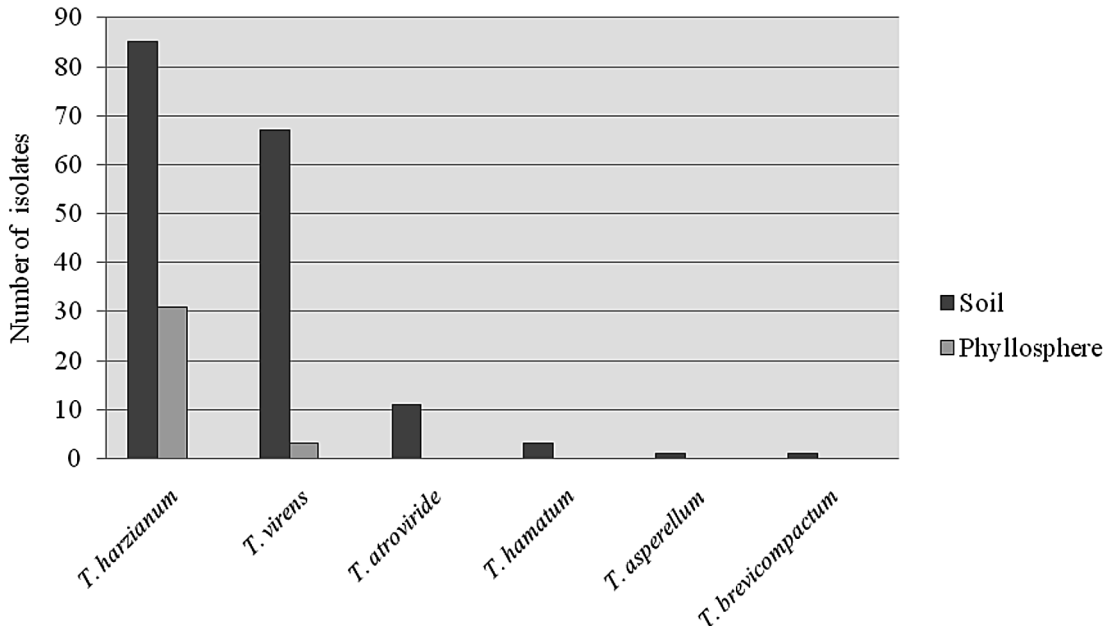

Figure 2. Proportional frequency of *Trichoderma* species isolated from rice fields in Mazandaran province, northern Iran.

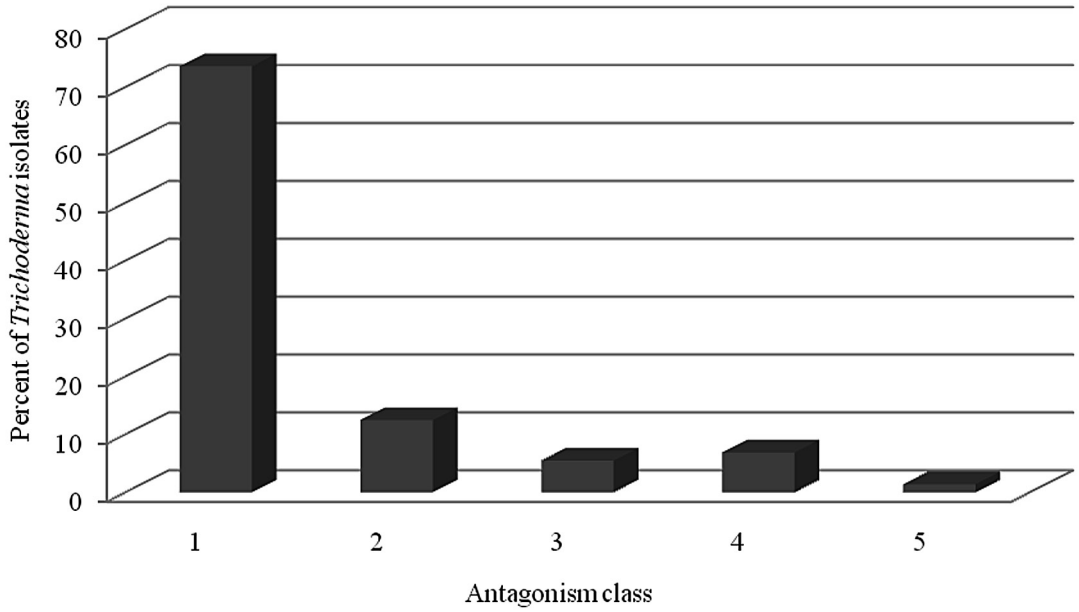

Figure 3. Antagonistic reactions of *Trichoderma* spp. with *Rhizoctonia solani* in dual culture tests. 1, *Trichoderma* completely overgrows the pathogen and covers the entire medium surface; 2, *Trichoderma* overgrows at least two thirds of the medium surface; 3, *Trichoderma* and *Rhizoctonia* each colonize 50% of the medium surface and neither organism appears to dominate the other; 4, *Rhizoctonia* colonizes at least two-thirds of the medium surface and appears to withstand encroachment by Trichoderma; 5 = *Rhizoctonia* completely overgrows the medium surface.

Dual culture tests between *R. solani* and *Trichoderma* strains

The antagonism of 78 strains of *Trichoderma* against *R. solani* was tested by the dual culture method. None of the *Trichoderma* isolates caused an inhibition zone in a colony of *R. solani*, but most of them overgrew the mycelium of the pathogen and sporulated abundantly on its surface. Of the examined *Trichoderma* isolates, 83.3% were antagonistic to *R. solani in vitro* (Figure 3). Sixty two percent of the antagonists belonged to *T. harzianum* and 37 percent to *T. virens*. All *T. harzianum* isolates had a rating of 1 or 2, i.e. they were antagonistic.

Sclerotial development of the pathogen was prevented with many *Trichoderma* isolates or the number of sclerotia formed significantly reduced (Table 2). Maximum inhibition occurred when *T. harzianum* AS15-1 and *T. harzianum* AS15-4 were paired with *R. solani*. With these no sclerotia were produced at all. There was no correlation between the antagonism of isolates in dual culture and their inhibition of sclerotia production *in vitro*. For example, *T. virens* AD1-5 was a class 3 (non-antagonist) isolate, but it strongly inhibited sclerotial development.

Effect of *Trichoderma* isolates on the viability of *R. solani* sclerotia

Seventeen percent of *Trichoderma* isolates completely inhibited sclerotial germination of *R. solani* after 30 days; 23% did not altogether inhibit germination but slowed down the growth rate of colonies derived from the sclerotia that formed (by 5.5 to 54%; data not shown). Surprisingly, 72% of the *T. harzianum* isolates completely suppressed the germination of sclerotia. Also, 80% of the isolates belonging to this species decreased the colony development from sclerotia.

Effect of *Trichoderma* volatile metabolites on *R. solani*

The growth rate of *R. solani* was significantly reduced by volatile metabolites of *Trichoderma* isolates ($P \leq 0.05$). Isolate *T. harzianum* AD6 was the most effective with an inhibition of 65.18%, followed by *T. harzianum* AS12-2 and *T. harzianum*

Table 2. Effect of *Trichoderma* isolates on the production of sclerotia by *Rhizoctonia solani in vitro*.

Isolate	No. of sclerotia[a, b]	Isolate	No. of sclerotia [a, b]
Trichoderma harzianum AD1-1	4.33	*T. virens* AS1-1	6.67
T. harzianum AD1-2	14.00	*T. virens* AS1-2	9.00
T. harzianum AS2-2	18.67	*T. virens* AD1-3	4.00
T. harzianum AS2-3	25.00	*T. virens* AD1-5	1.00
T. harzianum AS3-3	5.67	*T. virens* AS3-1	13.33
T. harzianum AS3-5	11.00	*T. virens* AS3-2	18.00
T. harzianum AS4-1	11.67	*T. virens* AS3-4	1.67
T. harzianum AS4-2	18.00	*T. virens* AS6-1	13.33
T. harzianum AS16-4	15.67	*T. virens* AS6-2	13.33
T. harzianum AS17-2	16.33	*T. virens* AS6-3	40.67
T. harzianum AS17-3	12.00	*T. virens* AS6-4	25.33
T. harzianum AS19-1	1.33	*T. virens* AS10-1	6.33
T. harzianum AS19-2	24.33	*T. virens* AS10-2	19.67
T. harzianum AS19-3	0.00	*T. virens* AS10-3	18.00
T. harzianum AS19-4	9.67	*T. virens* AS10-4	8.00
T. harzianum AS20-2	0.00	*T. virens* AS10-5	8.67
T. harzianum AS20-3	14.33	*T. virens* AS10-6	11.00
T. harzianum AS20-4	17.33	*T. virens* AS10-7	17.33
T. harzianum AS20-5	17.67	*T. virens* AS11-1	12.00
T. harzianum AS21-1	19.67	*T. virens* AS11-2	6.67
T. harzianum AS21-2	14.67	*T. virens* AS11-3	32.00
T. harzianum AS22-1	26.67	*T. virens* AS11-4	31.67
T. harzianum AS22-2	8.67	*T. virens* AS14-1	8.00
T. harzianum AS22-3	7.67	*T. virens* AS14-2	7.33
T. harzianum AS22-4	9.33	*T. virens* AS14-3	5.00
T. harzianum AS16-4	9.0	*T. virens* AS15-2	13.67
T. harzianum AS17-2	9.33	*T. virens* AS15-5	12.33
T. harzianum AS17-3	28.67	*T. virens* AS16-2	13.00
T. harzianum AS19-1	25.67	*T. virens* AS16-22	14.67
T. harzianum AS19-2	14.33	*T. virens* AS17-1	40.67
T. harzianum AS19-3	21	*T. virens* AS17-4	16.33
T. harzianum AS19-4	17.33	*T. virens* AS18-1	33.33
T. harzianum AS20-2	5.67	*T. virens* AS18-2	37.00
T. harzianum AS20-3	8.67	*T. virens* AS18-4	34.33
T. harzianum AS20-4	5.67	*T. atroviride* AS8	46.00
T. harzianum AS20-5	2.33	*T. atroviride*-	39.67
T. harzianum AS21-1	16.33	Control	79.67

[a] LSD at $P \leq 0.05 = 0.855$.

[b] The values are the means of three replications.

Table 3. Effect of volatile metabolites of *Trichoderma* isolates on the growth rate of *Rhizoctonia solani*.

Isolate	Colony diameter of *R. solani* after 48 h (cm)[a]	Inhibition of *R. solani* mycelial growth (%)[a]
Trichoderma harzianum AD1-1	5.73	36.29
T. harzianum AS2-1	6.13	31.85
T. harzianum AS3-3	6.73	25.18
T. harzianum AS3-5	4.00	55.55
T. harzianum AS4-2	4.76	47.03
T. harzianum AD6	3.13	65.18
T. harzianum AD7	5.90	34.44
T. harzianum AS12-2	3.90	56.66
T. harzianum AS15-1	7.60	15.55
T. harzianum AS15-6	4.03	55.18
T. harzianum AS17-3	5.73	36.29
T. harzianum AS19-1	7.43	17.40
T. harzianum AS20-3	8.03	10.37
T. harzianum AS22-1	6.01	32.22
T. virens AS1-1	4.13	54.07
T. virens AD1-3	5.73	36.29
T. virens AS6-1	6.13	31.85
T. virens AS6-2	6.23	30.74
T. virens AS10-1	7.13	20.74
T. virens AS10-7	9.00	0.00
T. virens AS11-1	6.83	24.07
T. virens AS14-1	7.46	17.03
T. virens AS16-22	5.90	34.44
T. virens AS17-1	9.00	0.00
T. atroviride AS8	7.63	15.18
T. atroviride AS18-5	5.96	33.70
Control	9.00	–
LSD at $P \leq 0.05$	0.738	8.342

[a]Data are the means of three replications.

AS3-5 with an inhibition of 56.66% and 55.55% respectively (Table 3). All *T. harzianum* isolates showed good inhibition of *R. solani* mycelium. Ineffective isolates, on the other hand, which constituted a statistical group together with the control, belonged to *T. virens*.

Mycoparasitism test

Microscopic inspection of the interaction zone in the slide cultures clearly showed that at an early state (two days after inoculation), 93% of the *Trichoderma* isolates established close contact with the pathogen by coiling around its mycelium. The coils were usually very dense and appeared to tightly encircle the hyphae of the pathogen. However at this stage the host cell surface was still mostly intact. When *Trichoderma* made contact with the host hyphae, it usually formed appressorium-like structures or hook-shaped contact branches. After contact with the

host was established, several types of interactions occurred. In one of these, *Trichoderma* frequently grew parallel to the host and in contact with it. In another common type of interaction, *Trichoderma* coiled around the host. This coiling was either condensed or loose. Pronounced collapse of *R. solani* and loss of turgor were typical alteration about 10 days after inoculation. Breakdown of the cell wall of *R. solani* and hyphal disintegration were also occasionally seen. Later, the hyphae showed extreme shrinkage and shriveling. *T. virens* AS16-22 was the isolate that most strongly disintegrated the host hyphae. There was however no correlation between the intensity of *Trichoderma* coiling and hyphal disintegration.

Greenhouse assays

Trichoderma isolates led to significant differences in the RLH, the number of infected tillers per plant and the number of sclerotia produced in the rice phyllosphere by *R. solani* (Table 4).

The RLH was significantly reduced by the antagonists as compared with the control. Among the isolates, *T. harzianum* AS12-2 resulted in the lowest RLH and disease severity, and was therefore the most effective, as effective as propiconazole. Nine other *T. harzianum* and three *T. virens* isolates also restricted disease lesions to less than 30% of plant height (scale 3). The single *T. atroviride* isolate was ineffective in this assay. Twenty other *Trichoderma* isolates formed part of the same group as the control inoculated with the pathogen, and were therefore also completely ineffective.

The numbers of infected tillers were last recorded one month after inoculation. They ranged from 1 to 13. *T. harzianum* AS12-2, with the lowest number of infected tillers, was more effective than any *Trichoderma* isolate in restricting the horizontal development of *R. solani*, even more effective than propiconazole, and formed a group by itself. In the case of *T. harzianum* AS3-5 and *T. virens* strains AD1-3 and AS16-22, the number of infected tillers was less than five, and these strains formed a statistical group being as effective as the fungicide.

The number of sclerotia per plant varied from isolate to isolate but there was no correlation between the number of sclerotia and disease severity. However, in cases when the disease severity was 3 or less, the number of sclerotia did not exceed six.

Discussion

Species of *Trichoderma* are very common in the soil and the aerial parts of plants. All soil samples examined contained *Trichoderma* with an average population of 4.5×10^2 cfu g^{-1}. More than 90% of the *Trichoderma* isolates from paddy rice fields belonged to *T. harzianum* and *T. virens*, and these were also the only species that were isolated from the above-ground parts of rice plants in the field. Rice field soil may be a favored habitat for these two *Trichoderma* species. This finding is consistent with Nagamani and Mew (1987), who found that *T. harzianum* is the dominant species in upland rice and a common species in lowland irrigated and rain-fed rice in the Philippines. Mostafa Kamal and Shahjahan (1995) isolated 360 *Trichoderma* strains from rice fields in different locations in Bangladesh and reported that *T. harzianum* was prevalent in all areas and all soil types. Also, Cumagun and Lübeck (2000) obtained 42 *Trichoderma* isolates from rice fields in the Philippines of which 40 were identified as *T. harzianum*. Interestingly, those most frequently detected in this study (*T. harzianum*, *T. virens*, *T. atroviride*) were similar to the most abundant *Trichoderma* spp. found among 69 biocontrol isolates obtained from different geographical locations and culture collections (Hermosa *et al.*, 2004).

In our study, *Trichoderma* strains effectively reduced the growth of *R. solani* under *in vitro* conditions. Several mechanisms may explain the biocontrol activity of these strains (Elad, 1996). Hyperparasitism and volatile metabolites may be involved in the inhibition of *R. solani*, as shown in our study. Cell wall degrading enzymes (CWDEs) such as chitinase, glucanase and proteases are thought to be closely related to the mycoparasitism of *Trichoderma* strains (Chet 1987; Harman, 2006). Inhibitory volatile substances such as alkyl pyrons may also contribute to the biocontrol activity of some *Trichoderma* strains (Claydon *et al.*, 1987).

To be successful as a BCA, a mycoparasite should be effective against the resistant survival structures of plant pathogens (Baker and Cook, 1974). The fact that mycoparasitic fungi attack sclerotia, thus reducing pathogen inoculum in the soil, makes them potential biocontrol agents (Knudsen and Eschen, 1991). In the present study some *Trichoderma* strains prevented sclerotia

Table 4. Effect of selected *Trichoderma* isolates on the control of rice sheath blight in the greenhouse[a].

Isolate	RLH[b] (%)	Score (0–9)	No. of infected tillers	No. of sclerotia
Trichoderma harzianum AD1-1	98.87	9	9.67	11.67
T. harzianum AD1-2	34.7	5	5.67	0.67
T. harzianum AS2-1	77.37	9	6.33	2.33
T. harzianum AS2-2	80.63	9	5	2
T. harzianum AS3-3	63.83	7	7	0
T. harzianum AS3-5	25.47	3	3.33	0.33
T. harzianum AS4-2	28	3	5	4
T. harzianum AS4-3	68.13	9	5	1
T. harzianum AD6	48.27	7	5	1
T. harzianum AD7	51.83	5	10.67	0
T. harzianum AS12-2	18.97	1	1.33	0
T. harzianum AS15-1	44.03	5	6	0
T. harzianum AS15-3	93.13	9	8.33	0
T. harzianum AS15-4	85.3	9	7.33	0
T. harzianum AS15-6	29.07	3	5	0.33
T. harzianum AS15-7	77.8	9	6	0
T. harzianum AS16-1	24.9	3	5	0
T. harzianum AS16-3	43.93	5	11	4.33
T. harzianum AS16-4	45.47	7	10.33	7.33
T. harzianum AS17-3	52.17	7	7.67	1.33
T. harzianum AS20-2	42.57	5	5	0
T. harzianum AS20-3	24.87	3	5	0
T. harzianum AS20-4	52.13	7	10.33	1.67
T. harzianum AS20-5	48.57	7	8	5
T. harzianum AS21-1	23.37	3	6.33	1
T. harzianum AS21-2	22	3	10.33	0
T. harzianum AS22-1	35.87	5	11.67	0
T. harzianum AS22-2	54.63	7	11.67	8
T. harzianum AS22-3	29.4	3	7.67	0
T. virens AS1-1	100	9	8.33	4.67
T. virens AS1-2	75.97	9	5	2.33
T. virens AD1-3	25.3	3	3	0
T. virens AD1-5	89.97	9	9.33	0
T. virens AS3-1	87.3	9	5.33	2.33
T. virens AS3-4	23.6	3	6.33	0
T. virens AS6-1	34.3	5	5	3.67
T. virens AS6-2	81.33	9	6.67	13.67
T. virens AS6-3	93.43	9	5.67	12
T. virens AS10-1	100	9	8.33	16
T. virens AS10-3	97.5	9	6.33	11.33
T. virens AS10-5	100	9	8	17.67
T. virens AS10-7	63.8	7	5	7.33
T. virens AS11-1	100	9	8.67	22.67
T. virens AS11-2	42.17	5	10	8
T. virens AS11-3	56.73	7	8	7.33
T. virens AS11-4	96.83	9	10.67	3.33
T. virens AS14-1	42.3	5	7.33	2.33
T. virens AS14-2	63.	7	10.33	3
T. virens AS15-5	38.73	5	6.33	2.33
T. virens AS16-22	28.67	3	3.33	0
T. virens AS17-1	44.23	5	10.33	13.33
T. virens AS18-1	41.63	5	11	5.67
T. virens AS18-2	85.87	9	10	0.33
T. virens AS18-4	40.5	5	9	1
T. atroviride AS18-5	69.87	9	8	6
Propiconazole	19.63	1	3.67	0
Control (pathogen alone)	100	9	12.67	36.67
Uninoculated control	–	0	0	0
LSD at $P \leq 0.05$	4.99	0	0.281	0.684

[a] Data are the means of three replications.
[b] Relative lesion height.

production by *R. solani in vitro* and *in planta* and inhibited the germination and growth of sclerotia that developed. This is an encouraging finding for the management of sheath blight, because sclerotia are the main primary inoculum that initiates the disease in the following season.

The 7 *Trichoderma* strains most effective in the greenhouse assays were all antagonistic in the dual culture tests carried out following Bell *et al*. (1982). In contrast, there were no correlations between some of the *in vitro* biocontrol activities of these strains and their effectiveness in controlling rice sheath blight in the greenhouse. For example, *T. harzianum* AS12-2 did not inhibit sclerotia formation *in vitro* at all, but it was the most effective isolate controlling rice sheath blight in the greenhouse. In addition, among the 7 biocontrol candidate strains, only *T. harzianum* AS12-2 suppressed sclerotia germination. Moreover, *T. harzianum* AS12-2 and *T. harzianum* AS3-5, though they controlled the disease, did not coil around the hyphae of the pathogen in the slide culture test.

Spore suspensions of *Trichoderma* isolates significantly reduced disease severity and infected tillers/hill (disease incidence) compared with the control. The present study suggests that foliar sprays are an effective delivery system to control rice sheath blight. A similar trend was observed by Tewari and Singh (2005), who reported that a spore suspension sprayed on the leaves significantly reduced disease severity and incidence and was more effective than soil treatment or seedling root dip. Furthermore, Rosales and Mew (1982) reported that lesion development was significantly reduced when spore suspensions of *Trichoderma* spp. were sprayed on rice plants inoculated with *R. solani*. Verma *et al*. (2007) reported that *Trichoderma* spp. can be applied very well as spores (especially conidia), which are more tolerant to adverse environmental conditions during product formulation and field use, unlike the mycelial and chlamydospore forms of these species applied as microbial propagules. This technique for spraying *Trichoderma* in the greenhouse proved satisfactory and enabled the fungus to establish itself in the phyllosphere and reduce the impact of *R. solani*.

Most isolates of the genus *Trichoderma* that act as BCAs have been classified as *T. harzianum*, so that *T. harzianum* is generally considered synonymous with BCA (Kubicek, 2004). *T. harzianum* is also the active ingredient of many commercial biological control products. The present investigation also showed that most of the *Trichoderma* strains that were effective both *in vitro* and *in vivo* belonged to *T. harzianum*. This is likewise consistent with Mew and Rosales (1984), Das and Hazarika (2000) and Tewari and Singh (2005) who all found that *T. harzianum* was an effective BCA in controlling rice sheath blight. Moreover, *T. harzianum* was the most abundant *Trichoderma* species in rice fields of Mazandaran province and it became established on rice phyllosphere better than other species. We therefore suggest that of the *Trichoderma* species examined, *T. harzianum* merits further study as a potential biological control agent of *R. solani* and other important pathogens on rice in northern Iran.

In the present study, *T. harzianum* AS12-2 was the most effective *Trichoderma* strain with great potential to control sheath blight of rice in the field.

It is also possible to state that the signs that BCAs will be able to control sheath blight are good. Supplementing biological control with other, non-chemical control methods will improve disease control still more. On the other hand, biological control with the antagonists will lower the dependency on synthetic will it is hoped lead to a cleaner environment and healthier foods.

Further studies on these promising antagonists are needed to identify potential compounds produced, to evaluate possible modes of action related to biocontrol and to test the efficacy of the strains under field conditions.

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Literature cited

Adams G.C., 1988. *Thanatephorus cucumeris* (*Rhizoctonia solani*) a species complex of wide host range. In: *Advances in Plant Pathology*, vol. 6. (G.S. Sidhu, ed.), Academic Press, New York, NY, USA, 535–552.

Altschul S.F., T.L. Madden, A.A. Schaffer, J.H. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

Baker K.F. and R.J. Cook, 1974. *Biological Control of Plant Pathogens*. W.H. Freeman and Co., San Francisco, CA, USA.

Bell D.K., H.D. Wells and C.R. Markham, 1982. *In vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. *Phytopathology* 72, 379–382.

Bissett J., 1992. *Trichoderma atroviride*. *Canadian Journal of Botany* 70, 639–641.

Chet I., 1987. *Trichoderma*-application, mode of action, and potential as a biocontrol agent of soil-borne plant pathogenic fungi. In: *Innovative Approaches to Plant Disease Control* (I. Chet, ed.), John Wiley and Sons, New York, NY, USA, 137–160.

Claydon N., M. Allan, I.R. Hanson and A.G. Avont, 1987. Antifungal alkyl pyrones of *Trichoderma harzianum*. *Transactions of the British Mycological Society* 88, 503–513.

Cumagun C.J.R. and M. Lübeck, 2000. Characterization of *Trichoderma* isolates from Philippine rice fields by UP-PCR and rDNA-ITS1 analysis: Identification of UP-PCR markers. *Journal of Phytopathology* 148, 109–115.

Das B.C. and D.K. Hazarika, 2000. Biological management of sheath blight of rice. *Indian Phytopathology* 53, 433–435.

Davet P. and F. Rouxel, 2000. *Detection and Isolation of Soil Fungi*. Science Publishers Inc., Enfield, NH, USA.

Dennis C. and J. Webster, 1971. Antagonistic properties of species-groups of *Trichoderma*. II. Production of volatile antibiotics. *Transactions of the British Mycological Society* 57, 41–48.

Dhingra O.D. and J.B. Sinclair, 1995. *Basic Plant Pathology Methods*. CRC Press, Boca Raton, FL, USA.

Elad Y., 1996. Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. *European Journal of Plant Pathology* 102, 719–732.

Druzhinina I., A. Koptchinski, M. Komon, J. Bissett, G. Szakacs and C.P. Kubicek, 2005. An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genetics and Biology* 42, 813–828.

Druzhinina I., A. Koptchinski, M. Komon and C.P. Kubicek, 2006. The first 100 *Trichoderma* species characterized by molecular data. *Mycoscience* 47, 55–64.

Gams W. and J. Bissett, 1998. Morphology and identification of *Trichoderma*. In: Trichoderma *and* Gliocladium. vol. 1. *Basic Biology, Taxonomy and Genetics* (C.P. Kubicek, G.E. Harman, ed.), Taylor and Francis Ltd., London, UK, 3–34.

Groth D.E., M.C. Rush and G.D. Lindberg, 1990. Foliar fungicides for control of rice diseases in the United States. In: *Pest Management in Rice* (B.T. Grayson, M.B. Green, L.G. Copping, ed.), Elsevier, London, UK, 31–52.

Groth D.E., M.C. Rush and C.A. Hollier, 1991. Rice diseases and disorders in Louisiana. *Louisiana State University, Agricultural Center, Louisiana Agricultural Experiment Station, Bulletin* No. 828. Baton Rouge, LA, USA

Groth D.E. and J.A. Bond, 2006. Initiation of rice sheath blight epidemics and effects of application timing of azoxystrobin on disease incidence, severity, yield and milling yields. *Plant Disease* 90, 1073–1078.

Harman G.E., 2006. Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* 96, 190–194.

Harman G.E. and T. Björkman, 1998. Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: Trichoderma *and* Gliocladium vol. 2. *Enzymes, Biological Control and Commercial Applications* (C.P. Kubicek, G.E. Harman, ed.), Taylor and Francis Ltd., London, UK, 229–265.

Harman G.E., C.R. Howell, A. Viterbo, I. Chet and M. Lorito, 2004. *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nature Review of Microbiology* 2, 43–56.

Hermosa M.R., I. Grondona, E.A. Iturriaga, J.M. Diaz--Minguez, C. Castro, E. Monte and I. Garcia-Acha, 2000. Molecular characterization of biocontrol isolates of *Trichoderma*. *Applied and* ***Environmental Microbiology*** 66, 1890–1898.

Hermosa M.R., E. Keck, I. Chamorro, B. Rubio, L. Sanz, J.A. Vizcaíno, I. Grondona and E. Monte, 2004. Genetic diversity shown in *Trichoderma* biocontrol isolates. *Mycological Research* 108, 897–906.

IRRI, 1996. *Standard Evaluation System for Rice*. 4th edition. International Rice Research Institute, Manila, Philippines.

Jaklitsch W.M., G.J. Samuels, S.L. Dodd, B. Lu and I. Druzhinina, 2006. *Hypocrea rufa*/*Trichoderma viride*: a reassessment, and description of five closely related species with and without warted conidia. *Studies in Mycology* 56, 135–177.

Knudsen G.R. and D.J. Eschen, 1991. Potential for biocontrol of *Sclerotinia sclerotiorum* through colonization of sclerotia by *Trichoderma harzianum*. *Plant Disease* 75, 466–480.

Kraus G., I. Druzhinina, J. Bissett, D. Zafari, H.J. Prillinger, G. Szakacs, R. Zare, W. Gams and C.P. Kubicek, 2004. *Trichoderma brevicompactum* sp. nov. *Mycologia* 96, 1057–1071.

Kubicek C.P., 2004. Molecular biology of biocontrol *Trichoderma*. In: *Fungal Biotechnology in Agricultural, Food & Environmental Applications* (Arora D.K., P.D. Bridge, D. Bhatnagar, ed.), CRC Press, Boca Raton, FL, USA, 135–145.

Kubicek C.P., M. Komon- Zelazowska and I. Druzhinina, 2008. Fungal genus *Hypocrea*/ *Trichoderma*: from barcodes to biodiversity. *Journal of Zhejiang University SCIENCE B*. 9, 753–763.

Mew T.W. and A.M. Rosales, 1984. Relationship of soil

microorganisms to rice sheath blight development in irrigated and dryland rice cultures. *Technical Bulletin ASPAC Food and Fertilizer Technology Center,* Taipei City, Taiwan. No. 79, 11 pp.

Mew T.W. and A.M. Rosales, 1985. Influence of *Trichoderma* on survival of *Thanatephorus cucumeris* in association with rice in the tropics. In: *Ecology and Management of Soil-borne Pathogens* (C.A. Parker, A.D. Rovira, K.J. Moore, P.T.W. Wong, J.K. Kollmorgen, ed.), American Phytopathological Society, St. Paul, MN, USA, 117–120.

Mostafa Kamal M.D. and A.K.M. Shahjahan, 1995. *Trichoderma* in rice field soils and their effect on *Rhizoctonia solani*. *Bangladesh Journal of Botany* 24, 75–79.

Mukherjee P.K., A.N. Mukhopadhyay, D.K. Sarmah and S.M. Shrestha, 1999. Comparative antagonistic properties of *Gliocladium virens* and *Trichoderma harzianum* on *Sclerotium rolfsii* and *Rhizoctonia solani* – its relevance to understanding the mechanisms of biocontrol. *Journal of Phytopathology* 143, 275–279.

Nagamani A. and T.W. Mew, 1987. *Trichoderma* in Philippine rice field soils. *International Rice Research Newsletter* 12(4), 25.

Oard S., M.C. Rush and J.H. Oard, 2004. Characterization of antimicrobial peptides against a US strain of the rice pathogen *Rhizoctonia solani*. *Journal of Applied Microbiology* 97, 169–180.

Ou S.H., 1985. *Rice Diseases*. 2nd ed. Commonwealth Mycological Institute, Kew, UK, 380 pp.

Papavizas G.C., 1985. *Trichoderma* and *Gliocladium*: Biology, ecology and potential for biological control. *Annual Review of Phytopathology* 23, 23–54.

Rosales A.M. and T.W. Mew, 1982. Antagonistic effects of soil microorganisms on rice sheath blight pathogen. *International Rice Research Newsletter* 7, 12–13.

Rush M.C. and F.N. Lee, 1992. Sheath blight. In: *Compendium of Rice Diseases* (R.K. Webster, P.S. Gunnell, ed.), APS Press, St. Paul, MN, USA, 22–23.

Samuels G.J., E. Lieckfeldt and H.I. Nirenberg, 1999. *Trichoderma asperellum*, a new species with warted conidia, and redescription of *Trichoderma viride*. *Sydowia* 51, 71–88.

SAS Institute Inc., 1989. *SAS/STAT User's Guide*, Version 6, 4th ed., vol. I. SAS Institute Inc., Cary, NC, USA.

Sivakumar D., R.S. Wilson Wijeratnam, R.L.C. Wijesundera, F.M.T. Marikar and M. Abeyesekere, 2000. Antagonistic effect of *Trichoderma harzianum* on postharvest pathogens of rambutan (*Nephelium lappaceum*). *Phytoparasitica* 28, 1–8.

Sudhakar R., K.C. Rao and C.S. Reddy, 1998. Studies on antagonism of *Trichoderma* and *Gliocladium* species against *Rhizoctonia solani* of rice. *Indian Journal of Plant Protection* 26, 25–29.

Tewari L. and R. Singh, 2005. Biological control of sheath blight of rice by *Trichoderma harzianum* using different delivery systems. *Indian Phytopathology* 58, 35–40.

Verma M., S.K. Brar, R.D. Tyagi, R.Y. Surampalli and J.R. Valero, 2007. Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. *Biochemical Engineering Journal* 37, 1–20.

White T.J., T. Bruns, S. Lee and J. Taylor, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In: *PCR Protocols* (M.H. Innes, D.H. Gelfand, J.J. Sninsky, T.J. White, ed.), Academic Press, San Diego, CA, USA, 315–322.

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