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Selection of *Trichoderma* spp. isolates antagonistic to *Rosellinia necatrix*

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Abstract

Fifty-six bulk isolates of *Trichoderma* spp. from avocado (*Persea americana* Mill.), carnation (*Dianthus caryophyllus* L.), litchi (*Litchi chinensis* Sonn), rice (*Oryza sativa* L.) and sugar beet (*Beta vulgaris* L.) crops located in southern Spain were evaluated for antagonism against one isolate of *Rosellinia necatrix* Prill. Isolates of both types of fungi were tested in dual and cellophane culture. The origin, cultural characteristics, overgrowth sporulation and staining of growth medium were recorded. As a result, 21 *Trichoderma* bulk isolates were selected and their corresponding monoconidial isolates were evaluated as above. Next eight monoconidial *Trichoderma* isolates with the largest *in vitro* antagonism were selected, and they were additionally tested against nine representative isolates of *R. necatrix* from nine virulence groups. These were established after pathogenicity tests on 57 isolates of *R. necatrix* from diseased avocado orchards in southern Spain. These monoconidial *Trichoderma* isolates were considered as potential biological control agents with a high potential for effective control of white root rot of avocado.

Additional key words: avocado white root rot; biocontrol; cellophane culture; dual culture.

Resumen

Selección de aislados de *Trichoderma* spp. antagonistas a *Rosellinia necatrix*

Se analizó el antagonismo de cincuenta y seis aislados masales de *Trichoderma* spp. procedentes de cultivos de aguacate (*Persea americana* Mill.), clavel (*Dianthus caryophyllus* L.), litchi (*Litchi chinensis* Sonn), arroz (*Oryza sativa* L.) y remolacha (*Beta vulgaris* L.) del Sur de España frente a un aislado de *Rosellinia necatrix* Prill. Los aislados de ambos hongos se analizaron *in vitro* en cultivos duales y de celofán, valorándose la procedencia, características culturales, sobrecrecimiento, esporulación y tinción del medio de cultivo. Se seleccionaron 21 aislados masales y se evaluaron sus correspondientes monoconídicos mediante los análisis *in vitro* citados. Posteriormente se seleccionaron los 8 aislados monoconídicos de *Trichoderma* que presentaron el mayor antagonismo *in vitro*. Estos 8 aislados se evaluaron adicionalmente mediante análisis frente a nueve aislados representativos de *R. necatrix*, procedentes de nueve grupos de virulencia. Estos se obtuvieron mediante análisis de patogenicidad de 57 aislados de *R. necatrix* procedentes de fincas de aguacate enfermas del Sur de España. Aquellos aislados monoconídicos de *Trichoderma* podrían considerarse como agentes de control biológico con un alto potencial para un efectivo control de la podredumbre blanca del aguacate.

Palabras clave adicionales: control biológico; cultivo dual; cultivo en celofán; podredumbre blanca radical del aguacate.

Introduction

White root rot (WRR; *Rosellinia necatrix* Prill.) is one of the most serious diseases affecting avocado (*Persea americana* Mill.) orchards in southern Spain (López Herrera, 1998). *R. necatrix* occurs worldwide and has a host range of 170 plant species in 63 genera (Ten Hoopen and Krauss, 2006). Disease control

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Abbreviations used: AUDPC (area under the disease progress curve), BCA (biological control agent), LSD (least significant difference), PDA (potato dextrose agar), RGI (radial growth inhibition), RH (relative humidity), RT (room temperature), TL (time lag), WRR (white root rot).

strategies include cultural practices, soil disinfestation (Guillaumin, 1986), soil solarisation (Freeman *et al.*, 1990; López Herrera *et al*., 1998, 1999) and fluazinam fungicide (López Herrera and Zea Bonilla, 2007). Concerns about environmental pollution have necessitated the development of alternative methods of disease control. Biological control strategies have been proposed for the control of WRR of avocado and apple crops using *Trichoderma* (Freeman *et al*., 1986; Sztejnberg *et al*., 1987). *Trichoderma* has been proposed as a potentially viable biological control agent for WRR because of its antagonism *i.e.* growth inhibition by antibiosis, competition, mycoparasitism, plant growth promotion and induced resistance (Benítez *et al*., 2004) to plant pathogenic fungi such as *Fusarium* (Segarra *et al*., 2010), *Pythium* (Naseby *et al*., 2000), *Pyrenophora tritici-repentis* (Perelló *et al*., 2003), *Rhizoctonia* (Hajieghrari *et al.*, 2008) and *Sclerotium cepivorum* (Clarkson *et al*., 2004).

Antibiotics produced by *Trichoderma* species have been shown to inhibit fungal pathogens. *Trichoderma* species are often able to suppress the growth of endogenous fungi on an agar medium, and this mechanism has been observed in *T. virens* suppressing *Macrophomina phaseolina*, which causes charcoal rot in a range of crops (Howell, 2003). Various authors have demonstrated that mycoparasitism is not the main mechanism by which *Trichoderma* controls fungal pathogens.

The aim of this study was to identify isolates of *Trichoderma* with efficient *in vitro* antagonism to isolates of *R. necatrix* obtained from diseased avocado orchards in southern Spain, and select these isolates as biological control agents (BCAs) against WRR.

Material and methods

The experiments of this work were carried out in the period 2001-2006.

In vitro evaluation of *Trichoderma* spp. against *R. necatrix*

A total of 56 bulk *Trichoderma* isolates were collected from avocado, carnation, garlic, litchi, rice and sugar beet crops in southern Spain (Table 1), and evaluated for *in vitro* antagonistic activity to *R. necatrix* isolate Rn 400.

Antagonism of *Trichoderma* spp. bulk isolates in dual culture

Experiment 1

Forty-eight bulks isolates of *Trichoderma* spp. were evaluated for *in vitro* antagonism to Rn 400 in dual cultures (Royse and Ries, 1978). Petri dishes (90-mm diam.) containing 20 mL of potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) were each inoculated with a 5-mm diam. mycelial disc of a 7-day-old culture of *R. necatrix* grown under chamber conditions (25°C in darkness). Three days later, Petri dishes were co-inoculated with a 5-mm diam. mycelial disc of a three-day-old culture of *Trichoderma* spp. isolate at a distance of 5 cm from the *R. necatrix* mycelial disc. *R. necatrix* colony growth was measured along two radii: R1 (between the sowing point and farthest point of the colony) and R2 (between the sowing point and the edge of the colony) from where *R. necatrix* and *Trichoderma* mycelia came into contact. Percentage of radial growth inhibition (%RGI) was calculated as $\%RGI = [(R1 - R2) / R1]*100$ (Royse and Ries, 1978). Controls comprised *R. necatrix* cultures without *Trichoderma*; and were conducted in duplicate. The profusion of growth over opposite microorganisms (*i.e.,* overgrowth) such as *R. necatrix* growth over *Trichoderma* or vice versa) was examined, as were sporulation and staining of growth medium. Thus, 25 bulk *Trichoderma* isolates with efficient antagonistic properties to *R. necatrix* were selected for further evaluation.

Experiment 2

The 25 selected *Trichoderma* isolates plus 7 new isolates were re-evaluated using the same dual culture techniques as used in experiment 1, except that the cultural characteristics were not recorded. Isolates were replicated 10 times. The 7 new isolates of *Trichoderma* from escape trees (*i.e.* healthy trees in an orchard affected by WRR) were included based on promising results from preliminary experiments in our laboratory.

The experiments were complete randomised designs and an analysis of variance (ANOVA) was applied to the average of arcsin-transformed data of 3 and 10 replicates per isolate for experiment 1 and 2, respectively. The isolate means were compared by Fisher's Least Significant Difference (LSD) test ($p < 0.05$) to separate the means (Steel and Torrie, 1985). All statistical

Table 1. *Trichoderma* bulk isolates collected in Spain (1992-2001)

Isolate	Genus/Species	Host	Year	Origin
CH 76	*T. harzianum*	Sugar beet	1992	Córdoba
CH 77	*T. harzianum*	Sugar beet	1992	Córdoba
CH 78	*Trichoderma* spp.	Garlic	1992	Córdoba
CH 80	*Trichoderma* spp.	Garlic	1992	Córdoba
CH 81	*Trichoderma* spp.	—	1992	Córdoba
CH 101	*T. atroviride*	Avocado	1993	Málaga
CH 214	*Trichoderma* spp.	Avocado	1998	Málaga
CH 215	*Trichoderma* spp.	Avocado	1998	Málaga
CH 216	*Trichoderma* spp.	Avocado	1998	Málaga
CH 217	*Trichoderma* spp.	Avocado	1998	Málaga
CH 218	*Trichoderma* spp.	Avocado	1998	Málaga
CH 219	*Trichoderma* spp.	Avocado	1998	Málaga
CH 220	*Trichoderma* spp.	Avocado	1998	Málaga
CH 221	*T. longibrachiatum*	—	1998	Salamanca
CH 222	*T. auroviride*	—	1998	Salamanca
CH 223	*T. longibrachiatum*	—	1998	Salamanca
CH 224	*T. harzianum*	—	1998	Salamanca
CH 226	*Trichoderma* spp.	Avocado	1999	—
CH 227	*Trichoderma* spp.	Avocado	1999	—
CH 228	*Trichoderma* spp.	Avocado	1999	Málaga
CH 230	*Trichoderma* spp.	Avocado	1999	Málaga
CH 231	*Trichoderma* spp.	Avocado	1999	Granada
CH 232	*Trichoderma* spp.	Carnation	1999	Sevilla
CH 233	*Trichoderma* spp.	Carnation	1999	Sevilla
CH 234	*Trichoderma* spp.	Carnation	1999	Sevilla
CH 235	*Trichoderma* spp.	Carnation	1999	Sevilla
CH 236	*Trichoderma* spp.	Carnation	1999	Sevilla
CH 237	*Trichoderma* spp.	Carnation	1999	Sevilla
CH 238	*Trichoderma* spp.	Rice	1999	Sevilla
CH 239	*Trichoderma* spp.	Rice	1999	Sevilla
CH 240	*Trichoderma* spp.	Rice	1999	Sevilla
CH 242	*Trichoderma* spp.	Avocado	2000	Málaga
CH 243	*Trichoderma* spp.	Avocado	2000	Málaga
CH 250	*Trichoderma* spp.	Carnation	2000	—
CH 251	*Trichoderma* spp.	Carnation	2000	—
CH 252	*T. harzianum*	Carnation	2000	—
CH 253	*Trichoderma* spp.	Carnation	2000	—
CH 254	*Trichoderma* spp.	Avocado	2000	Málaga
CH 255	*Trichoderma* spp.	Avocado	2000	Málaga
CH 256	*Trichoderma* spp.	Avocado	2000	Málaga
CH 262	*Trichoderma* spp.	Litchi	2000	Málaga
CH 273	*T. atroviride*	Avocado*	2001	Granada
CH 276	*Trichoderma* spp.	Avocado*	2001	Granada
CH 278	*Trichoderma* spp.	Avocado*	2001	Granada
CH 294	*Trichoderma* spp.	Avocado*	2001	Granada
CH 295	*Trichoderma* spp.	Avocado*	2001	Granada
CH 296	*T. cerinum*	Avocado*	2001	Granada
CH 298	*Trichoderma* spp.	Avocado*	2001	Granada
CH 299	*Trichoderma* spp.	Avocado*	2001	Granada
CH 300	*Trichoderma* spp.	Avocado*	2001	Granada
CH 303	*T. virens*	Avocado*	2001	Málaga
CH 304	*T. atroviride*	Avocado*	2001	Granada
CH 314	*T. atroviride*	Avocado*	2001	Granada
CH 316	*T. atroviride*	Avocado*	2001	Granada
CH 390	*Trichoderma* spp.	Avocado	2001	—
CH 391	*Trichoderma* spp.	Avocado	2001	—

* Isolate from roots of healthy avocado tree with diseased neighbours trees («escape trees»).

analyses used the software package, Statistix 9 (Analytical Software, Version 9.0, Tallahassee, FL, USA).

Antagonism of bulk *Trichoderma* isolates in cellophane culture

Bulk *Trichoderma* spp. isolates (32 in total = 25 from Experiment 1 + 7 new isolates from Experiment 2) with an additional isolate previously tested in our laboratory were evaluated in cellophane culture (Dennis and Webster, 1971) against one isolate of Rn 400. Sterile cellophane film was transferred to Petri dishes containing 20 mL PDA. Each Petri dish was inoculated with a 5-mm diam. mycelial disc from different isolates of *Trichoderma,* one isolate per Petri dish in the centre of the dishes and incubated in chamber conditions for 2 days. The cellophane film was removed, and a 5-mm diam. mycelial disc of Rn 400 transferred to the growth medium at a rate of 1 disc per Petri dish. When control mycelia of *R. necatrix* (no previous *Trichoderma* exposure) covered the Petri dish, two colony diameters of all treatments were measured; isolates were replicated five times. Thus, 21 isolates with efficient antagonism *in vitro* to *R. necatrix* were finally selected for additional evaluation.

The experiment was a complete randomised design and an ANOVA was applied to the average data from five dishes per isolate. Isolate means were compared by Fisher's LSD test ($p < 0.05$) (Steel and Torrie, 1985).

Preparation of *Trichoderma* monoconidial isolates

In order to minimise variability in the antagonistic response observed with bulk isolates of *Trichoderma*, monoconidial isolates were obtained for evaluation in dual and cellophane culture. Selected *Trichoderma* bulk isolates were grown on PDA for 7 days in chamber conditions. Spores were then removed by scraping the colony surface with 5 mL of sterile deionised water, and 0.1 mL of the spore preparation transferred to Petri dishes containing water agar and was incubated for 1 day at room temperature (RT = 24°C). A single conidium per isolate was recovered using an optical microscope, and transferred to dishes containing PDA, except for isolate CH 304 for which two conidia were recovered (described as CH 304 · 1-mc and CH 304 · 2-mc). Monoconidial cultures were maintained by serial transfer of bulk mycelium on PDA and stored at 4°C for future study.

Selection of monoconidial isolates of *Trichoderma* in dual and cellophane culture

In two experiments, 22 monoconidial isolates of *Trichoderma* were evaluated against Rn 400 in dual and cellophane culture as described previously; there were five replicates per isolate. Eight *Trichoderma* isolates were then selected based on the degree of antagonism to Rn 400 as measured in the same way as *«Antagonism of* Trichoderma *spp. bulk isolates in dual culture»* section for dual culture and *«Antagonism of bulk Trichoderma isolates in cellophane culture»* section for cellophane culture.

Experiments were complete randomised designs and an ANOVA was applied to the average of arcsin-transformed data (dual culture) and data of five replicates per isolate (cellophane culture). Isolate means were compared by Fisher's LSD test ($p < 0.05$) to separate the means.

In vitro antagonism of eight *Trichoderma* monoconidial isolates to nine isolates of *R. necatrix*

To obtain representative virulent isolates of *R. necatrix*, the pathogenicity of 57 isolates (Table 2) from infested avocado orchards of southern Spain were tested on 12-month-old avocado plants from germinated seeds of cv. Topa-Topa. The plants were grown in 1.15 L pots containing a Laura substrate consisting of peat, coconut fibre, and perlite at a ratio of 6:1:0.6 v/v/v, respectively. Inoculations were as described by Sztejnberg and Madar (1980). Specifically, 3.75 g of wheat seed per L of substrate colonised by *R. necatrix* isolates was added at different depths and distances from the plant stem. The experiment was conducted under greenhouse conditions (18-26°C and relative humidity (RH) of 56-88%).

Five replicate pots per treatment were used. Aerial symptoms were evaluated every three days on the following scale of 1-5: 1 = healthy plant; 2 = plant with first symptoms of wilt; 3 = plant wilted; 4 = plant wilted with first symptoms of leaf desiccation; and 5 = plant completely desiccated and dead. Data were calculated as the area under the disease progress curve (AUDPC)

Table 2. *Rosellinia necatrix* isolates collected in Spain from diseased avocado trees (1986-2001)

R. necatrix isolate	Origin	Year	*R. necatrix* isolate	Origin	Year	*R. necatrix* isolate	Origin	Year
Rn 10	La Herradura-Granada	1986	Rn 67	Almuñécar-Granada	1992	Rn 119	Torre del Mar-Málaga	1995
Rn 11	Fuengirola-Málaga	1986	Rn 68	Almuñécar-Granada	1992	Rn 200	Benagalbón-Málaga	1996
Rn 12	Salobreña-Granada	1988	Rn 69	Benamargosa-Málaga	1992	Rn 201	La Viñuela-Málaga	1996
Rn 13	Almuñecar-Granada	1988	Rn 70	Algarrobo-Málaga	1992	Rn 202	Vélez-Málaga	1996
Rn 15	Estepona-Málaga	1989	Rn 71	Fuengirola-Málaga	1992	Rn 203	Coín-Málaga	1996
Rn 16	Vélez-Málaga	1989	Rn 96	Estepona-Málaga	1993	Rn 204	Canillas-Málaga	1996
Rn 17	Almuñécar-Granada	1989	Rn 97	Fuengirola-Málaga	1993	Rn 205	Vélez-Málaga	1996
Rn 18	Almuñécar-Granada	1989	Rn 98	Algarrobo-Málaga	1993	Rn 244	La Mayora-Málaga	1999
Rn 19	Almuñécar-Granada	1989	Rn 99	Coín-Málaga	1993	Rn 245	Veléz-Málaga	1999
Rn 29	Vélez-Málaga	1990	Rn 100	Alhaurín el Grande-Málaga	1993	Rn 246	Coín-Málaga	1999
Rn 30	Almuñécar-Granada	1990	Rn 106	Mijas-Málaga	1994	Rn 247	Vélez-Málaga	1999
Rn 31	Motril-Granada	1990	Rn 107	Coín-Málaga	1994	Rn 268	Motril-Granada	2001
Rn 32	Motril-Granada	1990	Rn 108	Coín-Málaga	1994	Rn 269	Motril-Granada	2001
Rn 33	Jete-Granada	1990	Rn 109	Benagalbón-Málaga	1994	Rn 284	Vélez-Málaga	2001
Rn 48	Almuñécar-Granada	1991	Rn 110	Vélez-Málaga	1994	Rn 285	Churriana-Málaga	2001
Rn 49	Almuñécar-Granada	1991	Rn 111	Estepona-Málaga	1994	Rn 289	Motril-Granada	2001
Rn 50	Almuñécar-Granada	1991	Rn 116	Vélez-Málaga	1995	Rn 290	Vélez-Málaga	2001
Rn 51	Almuñécar-Granada	1991	Rn 117	Vélez-Málaga	1995	Rn 320	Coín-Málaga	2001
Rn 52	Vélez-Málaga	1991	Rn 118	Torre del Mar-Málaga	1995	Rn 400	Almuñécar-Granada	1991

(Campbell and Madden, 1990). These AUDPC data were statistically analysed as a complete randomised design comparing the means by Fisher's LSD test ($p < 0.05$). Nine bulk representative *Rosellinia necatrix* isolates, each from a different virulence group, were selected. The activity of eight isolates of *Trichoderma*, selected for their antagonism to *R. necatrix*, was again tested in cellophane and dual culture over the nine isolates of *R. necatrix*. Data describing *in vitro* antagonisms were statistically analysed using a factorial design where the main factor was the *R. necatrix* isolates and the sub-factors were the *Trichoderma* isolates. The means were compared by Fisher's LSD test ($p < 0.05$).

Results

Antagonism of *Trichoderma* bulk isolates in dual culture

Experiment 1

Of the 48 bulk isolates of *Trichoderma* spp. tested, the following isolates showed the highest inhibition (%RGI = 40.01-26.15%) of Rn 400 in dual culture with statistically similar means not significantly different among them: CH 215, CH 101, CH 314, CH 256, CH 252, CH 316, CH 220, CH 218, CH 262, CH 300, CH 238, CH 255, CH 254, CH 304, CH 230, CH 242, CH 251, CH 237, CH 231, CH 78, CH 216, CH 243, CH 77, CH 76, CH 219, CH 226, CH 234, CH 391, CH 232 and CH 227. The isolates CH 214, CH 81, CH 276, CH 222, CH 240, CH 390, CH 228, CH 221 and CH 253 had medium levels of antagonism (%RGI = 23.78-20.29%). The remaining *Trichoderma* isolates (CH 250, CH 239, CH 217, CH 233, CH 235, CH 223, CH 224, CH 80 and CH 236) gave the least inhibition (%RGI = 19.83-9.28%), although these were still significantly different from controls (Table 3).

Staining of the growth medium, possibly indicative of antibiosis, occurred in 10 isolates. Profuse sporulation of *Trichoderma* and overgrowth of *R. necatrix* by *Trichoderma* was evident in 22 and 48 isolates, respectively. Overgrowth of *R. necatrix* by *Trichoderma* occurred only with *Trichoderma* isolate CH 217.

Twenty five of the 48 isolates of *Trichoderma* spp. were selected for further study. Of these, 19 isolates (CH 215, CH 101, CH 314, CH 256, CH 252, CH 316, CH 220, CH 218, CH 262, CH 300, CH 238, CH 255, CH 254, CH 304, CH 230, CH 242, CH 251, CH 237 and CH 231) were characterised by a combination of high %RGI (40.01%-27.63%), high sporulation and overgrowth. Some selected isolates (CH 300, CH 230, CH 242, CH 251 and CH 231) showed evidence of considerable staining of the growth medium. The remaining *Trichoderma* isolates (CH 221, CH 222, CH

Table 3. Effect of different *Trichoderma* bulk isolates on radial growth inhibition (%RGI) of *R. necatrix* (Rn 400). «Dual» culture technique; Experiment 1

Trichoderma isolate	%RGI[a]	Culture characteristics[b]				*Trichoderma* isolate	%RGI[a]	Culture characteristics[b]			
CH 215	40.01[a]	S	OT	—	—	CH 226	26.44[abcdefg]	—	—	—	—
CH 101	36.49[ab]	S	OT	—	—	CH 234	26.41[abcdefg]	—	OT	—	—
CH 314	33.63[abc]	S	OT	—	—	CH 391	26.38[abcdefg]	—	—	A	—
CH 256	33.33[abc]	S	OT	—	—	CH 232	26.27[abcdefg]	—	—	—	—
CH 252	32.91[abcd]	S	OT	—	—	CH 227	26.15[abcdefg]	—	OT	—	—
CH 316	30.58[abcde]	S	OT	—	—	CH 214	23.78[bcdefgh]	—	OT	—	—
CH 220	30.53[abcde]	S	OT	—	—	CH 81	21.67[bcdefghi]	S	—	—	—
CH 218	30.27[abcde]	S	OT	—	—	CH 276	21.13[cdefghi]	S	OT	A	—
CH 262	29.72[abcde]	S	OT	—	—	CH 222	20.97[cdefghi]	—	OT	—	—
CH 300	29.56[abcde]	S	OT	A	—	CH 240	20.87[cdefghi]	—	OT	—	—
CH 238	29.54[abcde]	S	OT	—	—	CH 390	20.67[cdefghi]	S	—	—	—
CH 255	29.15[abcde]	S	OT	—	—	CH 228	20.62[cdefghi]	S	—	A	—
CH 254	28.89[abcdef]	S	OT	—	—	CH 221	20.40[cdefghi]	—	—	A	—
CH 304	28.74[abcdef]	S	OT	—	—	CH 253	20.29[cdefghi]	S	OT	—	—
CH 230	28.69[abcdef]	S	OT	A	—	CH 250	19.83[defghij]	S	—	—	—
CH 242	28.17[abcdef]	S	OT	A	—	CH 239	18.75[ghij]	S	—	—	—
CH 251	27.99[abcdef]	S	OT	A	—	CH 217	18.72[defghij]	—	—	—	OR
CH 237	27.65[abcdef]	S	OT	—	—	CH 233	16.73[fghij]	—	OT	—	—
CH 231	27.63[abcdef]	—	OT	A	—	CH 235	16.68[efghij]	—	—	—	—
CH 78	27.43[abcdef]	S	—	—	—	CH 223	13.55[ghij]	—	OT	A	—
CH 216	27.11[abcdef]	—	OT	—	—	CH 224	13.32[hij]	S	—	—	—
CH 243	26.60[abcdef]	—	OT	—	—	CH 80	10.99[ij]	—	OT	—	—
CH 77	26.56[abcdef]	S	OT	—	—	CH 236	9.28[j]	S	—	—	—
CH 76	26.45[abcdef]	S	OT	—	—						
CH 219	26.44[abcdefg]	—	OT	—	—	Control	1.88[k]	—	—	—	—

[a] Least significant difference (LSD) ($p < 0.05$) = 9.79; Multiple comparisons between means are based on arcsin-transformed values. However, mean percentages are shown. The data were means of three replicates, which were compared by Fisher's protected LSD test ($p < 0.05$). In each column, numbers followed by the same letter are not significantly different according to the LSD test.
[b] S: profuse sporulation. OT: overgrown of *Trichoderma* spp. with sporulation over *R. necatrix*. A: staining of growth medium, possible antibiosis. OR: overgrowth of *R. necatrix* over Trichoderma. —: without important characteristic.

223, CH 224, CH 228 and CH 276) were selected based on the cultural characteristics of interest, including a very high marked staining of growth medium, sporulation and overgrowth over *R. necatrix*, even though inhibition did not increase.

Experiment 2

Of the 32 *Trichoderma* isolates evaluated, the following isolates showed the highest inhibition (%RGI = 39.28-26.86%) of Rn 400 in dual culture: CH 300, CH 224, CH 262, CH 303, CH 255, CH 218, CH 238, CH 223, CH 316, CH 314, CH 222, CH 256, CH 304, CH 254 and CH 295. The following *Trichoderma* isolates remaining showed the least inhibition in their antagonism to Rn 400 (Table 4), although statistically different ($p < 0.05$) from controls: CH 231, CH 278, CH 242, CH 220, CH 237, CH 276, CH 101, CH 251, CH 296, CH 299, CH 252, CH 228, CH 298, CH 294, CH 230, CH 221 and CH 215.

In contrast to Experiment 1, the lowest inhibition occurred with isolate CH 215 in Experiment 2, and many of the *Trichoderma* isolates studied in the two experiments showed considerable variation in %RGI (*e.g.,* CH 101, CH 215, CH 222, CH 223, CH 224, CH 230, CH 252, CH 262 and CH 300).

Antagonism of bulk *Trichoderma* isolates in cellophane culture

Of the 33 *Trichoderma* bulk isolates tested, 16 showed an antagonistic effect significantly different ($p < 0.05$)

Table 4. Effect of different *Trichoderma* bulk isolates on radial growth inhibition (%RGI) of *R. necatrix* (Rn 400). «Dual» culture technique; Experiment 2

Trichoderma isolates	%RGI[a]
CH 300	39.28[a]
CH 224	35.96[ab]
CH 262	35.61[ab]
CH 303	34.47[abc]
CH 255	32.68[abcdef]
CH 218	32.37[abcd]
CH 238	31.93[abcdefg]
CH 223	31.75[abcd]
CH 316	31.32[abcd]
CH 314	31.20[abcde]
CH 222	30.82[abcde]
CH 256	29.24[abcdefg]
CH 304	29.23[abcdefg]
CH 254	28.88[abcdefg]
CH 295	26.86[abcdefgh]
CH 231	26.47[bcdefgh]
CH 278	26.36[bcdefgh]
CH 242	26.26[bcdefgh]
CH 220	26.07[bcdefgh]
CH 237	25.65[defghi]
CH 276	25.48[cdefgh]
CH 101	24.97[bcdefgh]
CH 251	24.82[bcdefgh]
CH 296	24.16[cdefgh]
CH 299	24.13[defghi]
CH 252	23.91[cdefgh]
CH 228	23.57[efghi]
CH 298	23.43[defghi]
CH 294	20.89[ghi]
CH 230	20.45[fghi]
CH 221	16.84[hi]
CH 215	14.90[i]
Control	2.02[j]

[a] LSD ($p < 0.05$) = 7.19. Multiple comparisons means are based on arcsin-transformed values. However, mean percentages are shown. The data were means of ten replicates, which values were compared by Fisher's protected LSD test ($p < 0.05$). In each column, numbers followed by the same letter are not significantly different according to LSD test.

Table 5. Effect on growth of *R. necatrix* (Rn 400) of different *Trichoderma* spp bulk isolates in cellophane culture

Trichoderma isolate	Average of diam. (cm) Rn 400[a]
CH 252	0.00[i]
CH 273	0.00[i]
CH 316	0.00[i]
CH 303	0.71[i]
CH 262	3.74[h]
CH 296	4.02[h]
CH 231	4.70[gh]
CH 251	4.81[gh]
CH 255	5.06[fgh]
CH 237	5.10[fgh]
CH 218	5.16[fgh]
CH 101	5.22[efgh]
CH 276	5.71[defg]
CH 228	6.45[cdef]
CH 278	6.57[cdef]
CH 304	6.80[bcde]
CH 298	6.88[abcd]
CH 300	8.50[a]
CH 230	7.15[abcd]
CH 242	7.44[abc]
CH 256	7.45[abc]
CH 254	7.68[abc]
CH 299	7.71[abc]
CH 220	7.99[abc]
CH 314	8.21[ab]
CH 215	8.33[ab]
CH 295	8.35[ab]
CH 294	8.40[ab]
CH 223	8.44[ab]
CH 224	8.44[ab]
Control	8.50[a]
CH 221	8.50[a]
CH 222	8.50[a]
CH 238	8.50[a]

[a] LSD ($p < 0.05$) = 1.63. The data were means of five replicates, which were compared by Fisher's protected LSD test ($p < 0.05$). In each column, numbers followed by the same letter are not significantly different according to the LSD test.

to controls. Four isolates (CH 252, CH 273, CH 316 and CH 303) were totally effective over Rn 400 (Table 5). Of the remaining isolates (CH 298, CH 230, CH 242, CH 256, CH 254, CH 299, CH 220, CH 314, CH 215, CH 295, CH 294, CH 223, CH 224, CH 221, CH 222, CH 238 and CH 300) did not differ significantly from controls. However, isolates CH 238 and CH 300 demonstrated high inhibition in dual culture but not in cellophane culture. Variability in isolate responses was high but less so than in dual culture.

Thus, 21 bulk isolates of *Trichoderm*a were selected based on high or medium %RGI in dual culture or cellophane culture, in combination with culture characteristics of overgrowth, staining and sporulation (Table 6).

Evaluation of monoconidial *Trichoderma* isolates in dual culture

Trichoderma isolate CH 296 resulted in the maximum growth inhibition of Rn 400 followed by isolates

Table 6. Level of antagonism to *R. necatrix* and cultural characteristics of selected *Trichoderma* bulk isolates

Trichoderma isolate	Inhibition in dual culture	Inhibition in cellophane culture	Culture characteristics[a]	Source
CH 303	High	High	—	Avocado «escape»
CH 316	High	High	—	Avocado «escape»
CH 255	High	High	—	Avocado
CH 218	High	—	—	Avocado
CH 314	High	—	—	Avocado «escape»
CH 222	High	—	—	Unknown
CH 231	Medium	Medium	—	Avocado «escape»
CH 251	Medium	Medium	S-OT-A	Carnation
CH 252	High[b]	Medium	—	Carnation
CH 296	Medium	Medium	—	Avocado «escape»
CH 304	Medium	Medium	—	Avocado «escape»
CH 101	Medium[b]	—	S-OT	Avocado
CH 220	Medium[b]	—	S-OT	Avocado
CH 254	Medium	—	—	Avocado
CH 215	High[b]	—	—	Avocado
CH 221	—	—	A	Unknown
CH 230	—	—	S-OT-A	Avocado
CH 242	—	—	S-OT-A	Avocado
CH 276	—	—	S-OT-A	Avocado «escape»
CH 256	High[b]	—	—	Avocado
CH 273	—	High	—	Avocado

[a] A: staining of growth medium, possible antibiosis; S: profuse sporulation; OT: overgrown of *Trichoderma* spp. with sporulation over *R. necatrix*; —: without important characteristics Avocado «escape»: healthy avocado tree with diseased neighbours. [b] In Experiment 1 only.

CH 314, CH 101, CH 273, CH 304·1, CH 304·2 and CH 303 showing statistically similar results ($p < 0.05$) (Table 7). Variability was lower in this experiment with isolates CH 304·1 and CH 304·2 from the same bulk isolate giving similar results (Table 7).

Evaluation of monoconidal isolates of *Trichoderma* in cellophane culture

Total inhibition of Rn 400 growth occurred with *Trichoderma* isolates CH 252, CH 273, CH 303 and CH 316; inhibition was less with CH 296 although the difference was not significant ($p < 0.05$) (Table 7). *Trichoderma* isolates CH 256, CH 230, CH 242, CH 220, CH 222, CH 254, CH 251 and CH 215 had little effect on the growth of Rn 400. Isolates CH 304·1 and CH 304·2, from a same bulk isolate, had different effects in cellophane culture and variability was similar to that of the bulk isolates.

Hence, isolates CH 273, CH 296 and CH 303 were selected on the basis of high or total inhibition of Rn 400 growth in both cellophane and dual culture (Table 6). In addition, isolates CH 101, CH 304·1 and CH 314 were selected for a high %RGI in dual culture, and isolates CH 252 and CH 316 were selected for their inhibition in cellophane culture (Table 7).

In vitro evaluation of eight monoconidial *Trichoderma* isolates to nine *R. necatrix* isolates in different virulence groups

The study of pathogenicity of 57 isolates of *R. necatrix* was finished 24 days after inoculation when all control plants were dead. During the experiment, plants inoculated with the isolates Rn 12 and Rn 29 did not show symptoms of wilt, while all the remaining inoculated plants showed symptoms of wilt or were dead. Nine significantly different groups of virulence were established and the isolates Rn 320, Rn 400, Rn 10, Rn 17, Rn 50, Rn 33, Rn 30, Rn 49 and Rn 12 selected (Table 8).

The average effect of each monoconidial isolate of *Trichoderma* over the nine isolates of *R. necatrix* is shown in Table 9. No positive correlation was detected

Table 7. Effect of different *Trichoderma* monoconidial isolates on radial growth inhibition (%RGI) of *R. necatrix* (Rn 400) in dual and cellophane culture

Trichoderma isolate	Dual culture[a] (%RGI)	*Trichoderma* isolate	Cellophane culture[b] Mean colony diam. (cm) Rn 400
CH 101	27.41^{ab}	CH 101	2.61^{i}
CH 215	13.11^{gh}	CH 215	8.32^{ab}
CH 218	9.78^{hi}	CH 218	5.50^{defg}
CH 220	10.45^{hi}	CH 220	7.56^{abc}
CH 221	20.40^{def}	CH 221	3.74^{ghi}
CH 222	20.96^{bcdef}	CH 222	7.90^{ab}
CH 230	11.90^{hi}	CH 230	7.22^{abcd}
CH 231	12.87^{gh}	CH 231	4.94^{efgh}
CH 242	20.39^{cdef}	CH 242	7.55^{abc}
CH 251	18.48^{fg}	CH 251	8.02^{ab}
CH 252	1.00^{j}	CH 252	0.00^{j}
CH 254	18.89^{ef}	CH 254	7.96^{ab}
CH 255	18.54^{fg}	CH 255	4.90^{fgh}
CH 256	19.24^{def}	CH 256	6.95^{abcde}
CH 273	27.03^{abc}	CH 273	0.00^{j}
CH 276	1.02^{jk}	CH 276	6.36^{bcdef}
CH 296	29.17^{a}	CH 296	1.88^{ij}
CH 303	23.47^{abcdef}	CH 303	0.00^{j}
CH 304·1	25.38^{abcd}	CH 304·1	3.34^{hi}
CH 304·2	25.29^{abcde}	CH 304·2	5.59^{cdefg}
CH 314	28.64^{a}	CH 314	3.28^{hi}
CH 316	7.30^{i}	CH 316	0.00^{j}
Control	0.00^{k}	Control	8.50^{a}

[a] LSD ($p < 0.05$) = 4.51. Multiple comparisons means are based on arcsin-transformed values. However, mean percentages values are shown. [b] LSD ($p < 0.05$) = 2.04. The data were means of five replicates, which values were compared by Fisher's protected LSD test ($p < 0.05$). In each column, numbers followed by the same letter are not significantly different according to the LSD test.

between inhibition in dual and cellophane cultures. In dual culture, isolates CH 101, CH 273, CH 304·1 and CH 303 demonstrated the highest inhibition to all *R. necatrix* isolates (%RGI = 29.2-21.8%). In contrast, isolates CH 296, CH 316, CH 252, CH 303 and CH 273 had the highest inhibition to all *R. necatrix* isolates in cellophane culture.

Discussion

The presence of *Trichoderma* spp. in the root systems of plants that survive disease may be evidence of biological control. The best method for obtaining potential BCAs might be where candidate *Trichoderma* are isolated from plants and soils in situations where they are thought to function in disease control (Howell, 1998). It is often recommended that potential BCAs should be sought from healthy plants in fields of diseased plants of the same species (Linderman *et al*., 1983). The antagonistic organisms would be expected to be able to function in the same environmental niche as the target pathogen (Knudsen *et al*., 1997). Therefore, isolates of *Trichoderma* with high antagonistic activity but from different crops might not be expected to be as effective in the rhizosphere of avocado trees. For our selection process, the origin of isolates from avocado was considered and the majority of the *Trichoderma* isolates selected were from the rhizosphere of healthy avocado trees adjacent to diseased avocado trees. In addition, some *Trichoderma* isolates were obtained from different hosts (*i.e.,* carnation) where they had been proven effective in our laboratory for controlling other soil-borne fungi (*i.e., Fusarium opxysporum* f. sp. *dianthi*) (López Herrera *et al.,* 2008).

Elad *et al*. (1982), established a positive correlation between *in vitro* degradation of cell wall lytic activity

Table 8. Pathogenicity and virulence group of 57 bulk isolates of *R. necatrix* on 12 months old avocado plants from germinated seeds under greenhouse conditions (18-26ºC and relative humidity= 56-88%).

R. necatrix isolate	AUDPCs[a]	Virulence Group	*R. necatrix* isolate	AUDPCs[a]	Virulence Group
Rn 10	2.8^{cde}	3	Rn 106	2.8^{cde}	3
Rn 11	3.2^{abc}	1	Rn 107	3.2^{abc}	1
Rn 12	1.0^{i}	9	Rn 108	3.0^{bcd}	2
Rn 13	3.4^{ab}	1	Rn 109	3.0^{bcd}	2
Rn 15	3.0^{bcd}	2	Rn 110	3.0^{bcd}	2
Rn 16	3.0^{bcd}	2	Rn 111	3.2^{abc}	1
Rn 17	2.6^{de}	4	Rn 116	3.0^{bcd}	2
Rn 18	2.8^{cde}	3	Rn 117	3.2^{abc}	1
Rn 19	2.8^{cde}	3	Rn 118	3.0^{bcd}	2
Rn 29	1.0^{i}	9	Rn 119	3.4^{ab}	1
Rn 30	1.8^{gh}	7	Rn 200	3.4^{ab}	1
Rn 31	2.6^{de}	4	Rn 201	3.2^{abc}	1
Rn 32	3.0^{bcd}	2	Rn 202	3.0^{bcd}	2
Rn 33	2.0^{fg}	6	Rn 203	3.2^{abc}	1
Rn 48	2.6^{de}	4	Rn 204	3.6^{a}	1
Rn 49	1.4^{hi}	8	Rn 205	3.2^{abc}	1
Rn 50	2.4^{ef}	5	Rn 244	3.0^{bcd}	2
Rn 51	2.8^{cde}	3	Rn 245	3.2^{abc}	1
Rn 52	2.6^{de}	4	Rn 246	3.4^{ab}	1
Rn 67	3.0^{bcd}	2	Rn 247	3.0^{bcd}	2
Rn 68	3.0^{bcd}	2	Rn 268	3.0^{bcd}	2
Rn 69	3.0^{bcd}	2	Rn 269	3.2^{abc}	1
Rn 70	3.4^{ab}	1	Rn 284	3.2^{abc}	1
Rn 71	2.8^{cde}	3	Rn 285	3.0^{bcd}	2
Rn 96	2.8^{cde}	3	Rn 289	3.2^{abc}	1
Rn 97	2.4^{ef}	5	Rn 290	3.4^{ab}	1
Rn 98	3.0^{bcd}	2	Rn 320	3.6^{a}	1
Rn 99	3.0^{bcd}	2	Rn 400	3.0^{bcd}	2
Rn 100	3.0^{bcd}	2			

[a] The area under the disease progress curve (AUDPC), obtained from data of aerial symptoms, was evaluated every 3 days on the following scale of 1-5: 1: plant healthy; 2: plant with first symptoms of wilt; 3: plant wilted; 4: plant wilted with first symptoms of leaf desiccation; 5: plant completely desiccated and dead. Data were standardised means of five replicates, which values were compared by Fisher's protected LSD test ($p < 0.05$). LSD value = 0.489. In each column, numbers followed by the same letter are not significantly different according to the LSD test.

due to large number of *T. harzianum* isolates and the degree of biological control against the pathogens *Sclerotium rolfsii*, *Rhizoctonia solani* and *Pythium aphanidermatum in vivo*. Knudsen *et al.* (1997) also reported a positive correlation between fungi antagonistic isolates *in vitro* and biological control of *Drechslera teres* and *Tilletia caries* on cereals fields.

The high variability observed within data in dual culture and cellophane experiments with the same bulk isolates of *Trichoderma* and *R. necatrix*, and also among replicates in the same experiment, was possibly associated with the bulk origin of the fungal isolates used. Bulk isolates constituted a pool of several genotypes with differing activity levels in essential aspects such as growth or sporulation, as well as in more specific factors, such as biocontrol. This high diversity may be related to activity of the different genotypes giving rise to synergisms between different isolates with variable genotypes and coexisting in bulk *Trichoderma* isolates (Harman *et al*., 1998).

Variability within strains may be due to differences in the degree of genetic variation within the genus *Trichoderma* (Hjeljord and Tronsmo, 1998). This may give strains within the genus *Trichoderma* a high

Table 9. Mean effect of monoconidial *Trichoderma* isolates on nine representative *R. necatrix* isolates of virulence groups

Trichoderma isolate	Mean of inhibition radial growth (%) of nine representative *Rosellinia necatrix*[a] in dual culture[b]	Mean diam. (cm) colony of nine representative *Rosellinia necatrix* in cellophane culture[c]
CH 101	29.16[a]	6.92[b]
CH 252	19.66[cd]	2.15[e]
CH 273	27.22[ab]	3.13[de]
CH 296	20.67[cd]	1.80[e]
CH 303	21.83[abc]	2.91[de]
CH 304·1	25.26[abc]	4.58[c]
CH 314	22.46[bc]	4.21[cd]
CH 316	13.95[d]	1.82[e]
Control	3.34[e]	8.50[a]

[a] Nine significantly different groups of virulence: Rn 320, Rn 400, Rn 10, Rn 17, Rn 50, Rn 33, Rn 30, Rn 49 and Rn 12. [b] LSD ($p < 0.05$) = 5.01 Multiple comparisons means are based on arcsin-transformed values. However, mean percentages values are shown. [c] LSD ($p < 0.05$) = 1.34. The data were means of three replicates, which values were compared by Fisher's protected LSD test ($p < 0.05$). In each column, numbers followed by the same letter are not significantly different according to LSD test.

degree of ecological adaptability as reflected in its worldwide presence in soils under a range of differing environmental conditions (Harman *et al*., 1998). There is also intra- and inter-specific variability in the intensity of *Trichoderma* response against other microorganisms (Hjeljord and Tronsmo, 1998; Clarkson *et al*., 2004).

Thalli of wild or successively transferred strains of *Trichoderma* are likely to comprise complex heterokaryons (*i.e.,* individual nuclei may differ). Therefore, the thallus of the genus *Trichoderma* may be considered a complex community of nuclei, some differing subtly and others differing markedly from their neighbours (Harman *et al*., 1998). To reduce such variability, we prepared monoconidial isolates of *Trichoderma* with conidia receiving a single nucleus from the phiallide and hence being homokaryotic. The monoconidial generation comprises individuals that each possess a single genotype that may differ to a greater or lesser extent from that of other individuals of the same population (Harman *et al*., 1998). This variation may explain differences in the responses between bulk and the corresponding monoconidial isolates. Nevertheless, Worasatit *et al.* (1994) observed considerable variation among single spore isolates of *T. koningii* and their capacity to inhibit *R. solani* growth on agar. A similar variation occurred with isolates of *T. harzianum* inhibiting the growth of *Gaeumannomices graminis* var. *triciti* (Ghisalberti *et al*., 1990). Variability observed in our experiments did not disappear with monoconidial isolates of *Trichoderma*, perhaps because *R. necatrix* is a dikaryotic homokaryon (Kanda *et al*., 2003) and a bulk isolate providing a new source of variation.

We observed that isolates with high %RGI in dual culture did not show the same response in cellophane culture (*e.g.,* isolates CH 238 and CH 300). This may be attributable to the lack of evidence of stimulation of control activity due to a lack of direct contact between pathogen and antagonist in cellophane cultures. Kubicek *et al*. (2001) reported high-levels of induction of extracellular chitinolytic enzymes when *Trichoderma* was grown on purified chitin, fungal cell walls or mycelia as the exclusive source of carbon. Similar behaviour was reported by Inbar and Chet (1995) who studied the role of recognition in the induction of specific chitinases during mycoparasitism by *T. harzianum* of *Sclerotium rolfsii*.

In our dual culture experiments, RGI did not exceed 40%, which is in contrast to an RGI of 70% reported by Dubey *et al*. (2007) for *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *ciceris* and by Royse and Ries (1978) for *Cytospora cincta* against *Alternaria alternata*, *Epicoccum purpurascens*, *Coniothyrium olivaceum* and *Aureobasidium pullulans*. This analysis of inhibition is based mainly on the competition for space in Petri dishes. For this reason, when two fungi with very different growth rates (*e.g., R. necatrix* and *Trichoderma)* are confronted, the %RGI is not high, but is still

useful for selecting isolates with the potential for biocontrol.

We have identified isolates with significantly higher rates of sporulation, although high sporulation is a common characteristic of this genus (Gams and Bissett, 1998). High sporulation would favour rapid colonisation of substrate, and hence would be of valuable property as BCA due to high reproductive activity (Benítez *et al*., 2004).

We selected antagonistic isolates that overgrew the pathogen and rejected isolates that were overgrown by the pathogen. Haran *et al.* (1996) reported dual culture experiments in which *T. harzianum* was overgrown by *R. solani* but hardly overgrown by *S. rolfsii* under the same conditions, thus demonstrating differential chitinolytic activity. Similar results were obtained by Limón *et al.* (2004) for transformed isolates of *T. harzianum* 2413 that overgrew *R. solani* and prevented overgrowth of pathogen-antagonism. Nevertheless, Mukherjee and Raghu (1997) reported a direct relationship between overgrowth and BCA of *Trichoderma* spp. and *S. rolfsii.*

Although there may be a relationship between staining of growth medium and antibiosis (Rey *et al.*, 2001), it was not always observed with our isolates. Isolates CH 300 and CH 221 showed considerable staining of growth medium, which did not correspond with antibiotic activity in cellophane cultures. This lack of corresponding activity may be due to specificity between antagonist and pathogen (Gams and Bissett, 1998), or indicative of its diversity and variability similar to *Streptomyces* spp. (Ndonde and Semu, 2001).

The high variability that exists in the virulence of isolates found in avocado orchards of southern Spain, corresponds with the high genetic diversity observed by Pérez Jiménez *et al*., (2002) and the existence of a somatic incompatibility system in isolates of *R. necatrix* of the same origin. The response differences in antagonism observed among the eight monoconidial *Trichoderma* isolates to nine *R. necatrix* isolates representative of different virulence groups in dual and cellophane cultures, suggests the existence of different antagonistic modes of action to pathogens.

In conclusion, nine groups of virulence were established for a collection of 57 isolates of *R. necatrix*, which demonstrates high variability among isolates of the pathogen infesting avocado orchards in southern Spain. The *in vitro* experiments with a high number of bulk isolates of *Trichoderma* from different hosts lead to the final selection of eight *Trichoderma* monoconidial isolates being tested over nine isolates of *R. necatrix*. These eight *Trichoderma* monoconidial isolates can be considered as BCAs with high potential for effective control of *R. necatrix*. These eight monoconidial isolates have been later evaluated in new experiments, not included in this study, as biocontrol agents against avocado white root rot, and have provided high levels of WRR control when two of them were tested singly or combined (Ruano-Rosa and López-Herrera, 2009).

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