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Ecophysiology and saprophytic ability of *Trichoderma* spp.

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Abstract — The outcome of mycelial interactions between *Sclerotium rolfsii* and several *Trichoderma* strains and the antagonistic activity of trichorzianines produced by some of them was evidenced in previous work. The aim of this study was to characterize some ecophysiological properties and saprophytic ability of those *Trichoderma* strains. Accordingly, the effect of different water potentials, the ability to grow and degrade cellulose and the inhibitory activity of diffusible metabolites were evaluated. The growth rate of all strains decreased as the water availability was reduced. Only at $\varphi = -0.138$ MPa the growth rate of the majority of *T. hamatum* strains differed statistically from those of *T. harziamum* and *T. koningii*. However, it was not possible to separate among species of *Trichoderma* for their water requirements. The ability to grow on carboxy methyl cellulose (CMC) was strains related and not species related. In general, the diameter of clearance was directly related with growth rate. The strains with highest growth rate on CMC corresponded to *T. hamatum* and *T. harzianum*. Difusible metabolites produced by all *Trichoderma* strains significantly reduce the growth rate and the number of sclerotia differentiation. No significant differences were observed between species but the most efficient strains corresponded to *T. koningii*. The different abilities found among the strains suggest that several of them must be selected for biological control.

diffusible metabolites / water potential / cellulolytic activity

Résumé — L'existence d'interactions mycéliennes entre *Sclerotium rolfsii* et plusieurs souches de *Trichoderma* a été mise en évidence au cours de travaux antérieurs de même que l'activité antagoniste des trichorzianines produites par certaines souches. L'objectif du travail présenté dans cet article était de caractériser certaines propriétés écophysiologiques de ces souches et d'évaluer leur aptitude saprophytique. Ainsi, l'influence de différentes activités en eau du substrat, l'aptitude à se développer et à dégrader la cellulose et l'activité inhibitrice des métabolites diffusibles produits ont été évaluées. Plus la disponibilité en eau du substrat est réduite, plus la croissance est affectée. Ce n'est qu'à $\varphi = -0.138$ Mpa qu'une différence de croissance est observée entre la majorité des souches de *T. hamatum* et celles des deux autres espèces testées *T. harzianum* et *T. koningii*. Malgré cela il n'a pas été possible de distinguer les espèces en fonction de leurs exigences hydriques. L'aptitude à croître sur carboxy-méthyl cellulose (CMC) dépend des souches et non des espèces. En général, le diamètre de lyse est directement corrélé au taux de croissance. Les meilleurs taux de croissance sur CMC ont été observés chez *T. hamatum* et *T. harzianum*. La production de métabolites diffusibles par *Trichoderma* réduit de

façon significative le taux de croissance et la différenciation de sclérotes chez *S. rolfsii*. Les meilleurs résultats ont été observés avec *T. koningii*. Les différentes aptitudes mises en évidence chez certaines souches permettent d'envisager leur utilisation pour le contrôle biologique.

métabolites diffusibles / potentiel hydrique / activité cellulolytique

INTRODUCTION

The use of beneficial organisms to combat pests on economic crops is being aggressively pursued world-wide in an attempt to eliminate or reduce the application of pesticides. Several species of *Trichoderma* has been extensively studied as a biological control of soil-borne diseases and are among promising biocontrol agents (Papavizas, 1985; Chet, 1987; Jensen & Wolffhechel, 1995).

The ubiquitous nature of *Trichoderma* spp. over a wide range of soil conditions and as primary colonizers of freshly felled timber is a testament to their competitiveness and probably accounts for the interest that they have received as biological control agents of plant pathogens (Chet, 1987). Several factors contribute to determine the competitive success of *Trichoderma* and their maintenance in the soil. The production of antimicrobial metabolites, that may provide a broader niche, the low sensitivity to the inhibitory effect of bacteria and the high growth rate and the high cellulolytic ability are conditions that contribute to increase the performance of *Trichoderma* strains when colonizing soils with plant residue content (Davet & Camporota, 1986; Naár & Kecskés, 1998) as occur in agricultural soil. A high index of clearance of a strain (clearance/growth rate) indicates a high saprophytic ability. The possible mechanisms operating in any one instance of biological control can be diverse and can be grouped as resource colonization and competition for nutrients; antibiotic production, including soluble, no volatile and volatile components, mycoparasitism and production of extracellular enzymes.

Several *Trichoderma* strains have been found as antagonists of *S. rolfsii*. It appears, however, that considering its variability and that of pathogens, soils and temperatures it must be necessary to test the effectiveness of more *Trichoderma* strains for different conditions. *Trichoderma* strains and *S. rolfsii* were isolated from and adapted to Uruguayan soils. Uruguay is characterized by temperate humid climate but with rains irregularly distributed along the year. Consequently, it is necessary to know more about the ecology of *Trichoderma* strains in order to use them as biocontrol agents (Davet & Camporota, 1986; Naá & Kecskés, 1998).

In previous work (Lupo, 1992) it was evidenced that several *Trichoderma* strains could antagonise to *S. rolfsii* adapted to Uruguayan soils. Moreover, the antagonistic activity of trichorzianines on *S. rolfsii* produced by some of them was also evaluated (Correa, 1995). The aim of this work was to characterize some ecophysiological properties and saprophytic ability of these *Trichoderma* strains.

MATERIAL AND METHODS

Strains and fungal cultures

Trichodema spp. strains obtained from Uruguayan soil used in this work were *T. hamatum*: MVHC 5920, MVHC 6011, MVHC 5676, MVHC 6039, MVHC 6037, MVHC 5755, MVHC 5907; *T. harzianum*: MVHC 5670, MVHC 5654, MVHC 5888, MVHC 5636, MVHC 5877, MVHC 5650; *T. koningii*: MVHC 5917, MVHC 5674, MVHC 5901, MVHC 5900, MVHC 6141, MVHC 5136, MVHC 5683 and *T. saturnisporum*: MVHC 5665, MVHC 5666, MVHC 5667, MVHC 5681. The pathogen *Sclerotium rolfsii* (MVHC 5407) was isolated from infected onions. All isolates were maintained on 2% agar-malt (AM) slants at 4 °C.

Growth of *Trichoderma spp.* at different water potentials (φ)

Agar malt 2% plus glycerol at different concentration were used to obtain φ: 1, – 0.138 MP, – 2.78 MP and – 4.19 MP water potentials (Gervais *et al.*, 1988). Plates were centrally inoculated with 5 mm mycelial discs cut from the margin of 2-day old cultures and incubated at 25 °C for 7 days in sealed polyethylene bags along with beakers containing glycerol-water solutions for each φ as the plates in order to create an atmosphere with the same equilibrium relative humidity. Three replicates for each strain incubated at each condition were used.

Cellulolytic activity

Congo-red staining technique was utilized to evaluate the cellulolytic activity (Theather & Wood, 1982). Agar plates with Eggins & Pugh (1961) medium and 5% carboxymethyl cellulose were inoculated with a disc cut from the edge of a growing colony of each strain. Diameter of clearance and growth rate ratio was determined after 48 h (Theather & Wood, 1982; Neirotti & Azevedo, 1988). Five replicates for each strain were used.

Assay of fungal inhibition in vitro

Diffusible metabolites

The ability of *Trichoderma* spp. to inhibit the mycelial growth of *S. rolfsii* by the diffusible metabolites was tested according to the method of Dennis and Webster (Dennis & Webster, 1971) with some modifications (Bettucci *et al.*, 1988). Petri dishes containing 18 ml of 2% malt-agar were utilized. An autoclaved cellophane paper membrane was placed upon the culture medium. A mycelial disc of 5 mm cut from the margin of each *Trichoderma* strain colony was placed on the cellophane. After 48 h the cellophane with the antagonist was removed and a disc from the margin of *S. rolfsii* colony was placed in the centre of the Petri dish. The growth rate of the pathogen was evaluated daily and the number of sclerotia produced was evaluated at 9 days. Three replicates were performed. The growth inhibition was expressed as the percentage of growth relative to the control (Bettucci *et al.*, 1988): $I = (t - r)\ 100/\ t$; being t the mean growth diameters of the control colonies and r, the mean growth diameters of the *S. rolfsii* tested with the antagonist.

The inhibition of sclerotia production was expressed as: Is = $(s - ts)$ 100/ t, being s the mean number of sclerotia produced by control and ts, the mean number of sclerotia by *S. rolfsii* tested with the antagonist.

Biocontrol potential on sclerotia

To evaluate the ability of *Trichoderma* strains to degrade and to suppress sclerotia germination twenty sclerotia were inoculated on the margin of 3-days-old cultures of all *Trichoderma* strains and incubated at 25 °C during 7 days. Then, ten sclerotia were surface sterilized with 0.4% hypochlorite solution during 4 min., rinsed three times with sterile water, dried on sterile filter paper and inoculated on 2% malt-agar. The inhibition of sclerotia germination was expressed as the percentage of sclerotia germination relative to the control: $Ig = (g\text{-}tg)100/tg$; being g the mean number of sclerotia geminated in the control and tg, the mean number of sclerotia germinated by *S. rolfsii* tested with the antagonist.

Analysis of the data

An ANOVA was carried out to establish if water potential produced significant differences on the growth rate of each *Trichoderma* strain. Differences among the strains at each water potential was evaluated by means of the t-test.

The statistical multiple comparisons *S*-method (Shefee, 1959) was carried out to establish the ordination of the strains in relation to: 1) the growth rate on CMC and cellulolytic ability, 2) the antibiotic effect on the mycelial growth of *S. rolfsii* and inhibition of sclerotia production and 3) the ability of degradation of sclerotia and its germination restriction.

RESULTS

Growth of Trichoderma strains at different φ

The ANOVA showed significant statistical differences of the strains growth rate at different water potential (P > 95%). As the water availability was reduced, the growth rate of all strains decreased. However, it was not possible to separate among species of *Trichoderma* for their water requirements. The growth rate of the strains of each species did not differ at any water potential (P > 95%). Only at $\varphi = -0.138$ MPa the growth rate of the majority of strains of *T. hamatum* differ statistically from those of *T. harziamum* (except MVHC 5977) and *T. koningii* according to t-test. *T. hamatum* showed the lowest growth rate at the lower water potential (Tab. 1).

CMC decomposition ability

The statistical *S*-method showed that all the strains could be ordinate in 6 groups in relation to the ability to grow on CMC and to the ability to degrade it. This ability was strain related and each group contained strains of all species. The strains with highest growth rate on CMC corresponded to *T. hamatum* and *T. harzianum* (Tab. 2).

Tab. 1. Growth rate of *Trichoderma* strains at different water potential.

Strains	*1MPa*	*−0.138 MPa*	*−278 MPa*	*−4.19 MPa*
5,907 ha	9	7.93 [a]	5.4	3.35
6,037 ha	9	8.03 [a]	4.96	2.73
6,039 ha	9	8.46	4.91	2.76
5,920 ha	9	7.75 [a]	4.81	2.83
6,011 ha	9	7.35 [a]	4.43	3.03
5,676 ha	9	8.27	3.85	3.5
5,755 ha	9	7.23 [a]	3.21	2.53
5,888 hz	9	9	8.5	4.76
5,654 hz	9	9	6.8	4.63
5,670 hz	9	9	6.25	4.33
5,650 hz	9	9	6.09	3.88
5,636 hz	9	9	5.86	3.86
5,877 hz	9	7.95	5.48	3.06
5,674 ko	9	9	7.48	5.06
5,901 ko	9	9	7.36	5.11
5,900 ko	9	9	7.25	5.18
6,136 ko	9	8.8	7.2	4.95
5,683 ko	9	9	7.2	3.216
5,917 ko	9	9	6.64	2.78
6,141 ko	9	9	6.48	3.81
5,681 sat	9	8.83	6.68	3.75
5,666 sat	9	8.78	6.58	3.766
5,665 sat	9	8.6	6.35	4
5,667 sat	9	8.58	6.43	3.6

Growth rate was expressed as diameter of colony (cm) after 72 hs.
ha: *T. hamatum*; hz: *T. harzianum*; ko: *T. koningii*; sat: *T. saturnisporum;* [a] indicates statisticaly significant differences.

On the other hand, some strains of *T. harzianum* (5670, 5636, 5654, 5877), and *T. hamatum* (6011) belonging to group 1 and group 2 according to *S*-method showed the highest CMC clearance. The majority of the strains of *T. saturnisporum* belong to the group 6 according to *S*-method. In general, the diameter of clearance was directly related with growth rate, except for *T. saturnisporum* (MVHC 5666 and 5667) with a clearance/growth rate greater than 1 reflecting that the cellulolitic activity was not related with the advancing zone of the colony.

Antagonism

The antagonistic activity of all *Trichoderma* strains on *S. rolfsii* was evaluated by the reduction of mycelial growth rate, the diminishing of sclerotia formation and the inhibition of sclerotia germination.

Tab. 2. Growth and CMC degradation of *Trichoderma* strains.

Strains	*Growth*	*Clareance*	*Clareance/growth*
Group 1			
5,670 hz	3.45	3.33	0.97
5,636 hz	3.43	3	0.88
Group 2			
5,877 hz	3.7	2.76	0.75
6,011 ha	3.65	2.75	0.75
5,654 hz	3.13	2.97	0.95
Group 3			
5,676 ha	2.9	2.48	0.86
5,650 hz	2.88	2.6	0.9
5,888 hz	2.54	2.42	0.95
5,755 hz	2.52	2.32	0.92
5,917 ko	2.46	2.46	1
Group 4			
6,136 ko	2.26	2.26	1
6,039 ha	2.45	2.1	0.86
Group 5			
6,037 ha	2.66	1.86	0.7
5,674 ko	2.3	2.02	0.88
5,901 ko	2.26	1.7	0.75
5,683 ko	2.02	1.84	0.91
5,667 sat	1.43	1.73	1.21
Group 6			
5,920 ha	2.64	1.4	0.53
5,681 sat	1.72	1.44	0.84
5,907 ha	1.56	1.56	1
5,666 sat	1.46	1.54	1.05
5,900 ko	1.46	1.46	1
6,141 ko	1.43	1.43	1
5,665 sat	1.42	1.36	0.96

Growth rate was expressed as diameter of colony (cm); degradation was expressed as diameter of clareance (cm); Groups delimited by S-method.
ha: *T. hamatum*; hz: *T. harzianum*; ko: *T. koningii*; sat: *T. saturnisporum*

Growth inhibition

Production of diffusible metabolites by Trichoderma was evidenced by the percentage of S. rolfsii growth inhibition. The S-method evidenced that all the strains of Trichoderma produced significant inhibition. The percent of inhibition was strain dependent and no significant differences were observed between species.

The strains of T. koningii were the most efficient to reduce the Sclerotium growth rate from 61% to 100%. All the strains of T. hamatum showed an inhibitory ability ranging from 41% to 76%, those of T. harzianum showed a large variation of the inhibitory ability from 32% to 86% and T. saturnisporum evidenced a low inhibition ability (43-51%). Several Trichoderma strains of all species had the ability to inhibit the sclerotia differentiation (Tab. 3).

Tab. 3. Inhibition of growth rate, sclerotia production and sclerotia germination of *S. rolfsii* by *Trichoderma* strains.

	Growth rate inhibition	*Sclerotia inhibition*	*Inhibition of germination*
6,011 ha	76,5	90[b]	100
5,755 ha	63,8	85[b]	95
6,039 ha	61,2	78[b]	90
5,907 ha	53,2	76[b]	90
5,920 ha	45,5	27[b]	20[b]
5,676 ha	41,7	84[b]	100
6,037 ha	41,6	72[b]	100
5,670 hz	86,4	90[b]	90
5,888 hz	80,1	83[b]	100
5,654 hz	32,6	45[b]	90
5,636 hz	64,2	60[b]	50[b]
5,877 hz	55,6	55[b]	90
5,650 hz	44,1	51[b]	80[b]
6,141 ko	100	100	100
5,901 ko	95,9	98	100
5,674 ko	91,8	97	90
5,917 ko	77,4	100	100
5,900 ko	72,3	81[b]	100
5,683 ko	71,1	96	100
6,136 ko	61,7	96	90
5,666 sat	51.4	85[b]	20[b]
5,665 sat	51,4	77[b]	30[b]
5,667 sat	44,9	92	20[b]
5,681 sat	43,6	77[b]	100

The growth rate inhibition was expressed as percentage of growth in relation to the control $I = (t - r)100/t$.
Inhibition of sclerotia production was expressed as $Is = (s\text{-}ts)100/t$
Inhibition of sclerotia germination was expressed as $Ig = (g\text{-}tg)100/tg$
b: indicates statistically no significant differences with the control.
ha: *T. hamatum*; hz: *T. harzianum*; ko: *T. koningii*; sat: *T. saturnisporum*.

Biocontrol of sclerotia

Some strains of each *Trichoderma* species have grown on sclerotia and have sporulated on them. Colonies of *S. rolfsii,* used as control showed that 100% of sclerotia were able to germinate. The *S*-method showed statistically significant differences between the control in relation to the 18 strains that produced 90-100% of germination inhibition being the strains of *T. koningii* (6141, 5917, 5901, 5900, 5683), *T. harzianum* (5888) and *T. hamatum* (6011, 5676, 6037) the most active (Tab. 3).

Trichoderma was recuperated when those colonized sclerotia were transferred to fresh media. Moreover, all strains of *T. koningii* and *T. harzianum* produced the medula degradation of some sclerotia remaining only the rind intact.

DISCUSION

The decreasing water potential plays an important role in decreasing *Trichoderma* growth rate. At lower water potential all strains of each species evidenced the same ability to grow except at $\varphi = -0.138$MP some strains of *T. harzianum* evidenced a better adaptation. Gervais *et al.* (1988) showed that some species of *Trichoderma* had optimum growth rate at $\varphi = -0.138$MP. In grassland soils in Uruguay the species found increased their frequency and density during the most humid month (Bettucci *et al.*, 1989). Conversely, Widden and Abitbol (1980) pointed out that differences in soil water availability determine the distribution of *Trichoderma* species. However, under experimental conditions it is possible to select strains with improved tolerance to water stress (Jin *et al.*, 1992; Kredics *et al.*, 2000).

All *Trichoderma* strains have shown the ability to degrade CMC. This ability showed by several strains could improve the saphrophytic competitive ability in relation to other species (Aneja & Mehrotra, 1980; Garret, 1970). A correlation between CMC degradation and cellulose and straw colonization by several *Trichoderma* strains was shown by Davet and Camporota (1986). Then, a strain with both clearance index equal to 1 and a high growth rate could be considered to posses a good saprophytic ability.

Diffusible metabolites produced by all strains reduced *S. rolfsii* growth rate being those of *T. koningii* the most active for reducing sclerotia formation than for growth inhibition.

The fact that some strains of *T. koningii* degrade sclerotia medulla leaving the rind intact could indicate that these strains are able to mycoparasitized *S. rolfsii*. Mycoparasitism is widely considered to be a major contributing factor to the biocontrol of a range of important plant pathogens by *Trichodema* spp. The results presented here would corroborate the view that mycoparasitism may be a significant mode of antagonism of some *Trichoderma* strains but this ability was not showed by *T. harzianum* nor *T. hamatum* as it was frequently observed (Elad *et al.*, 1984). Unfortunately *T. koningii* was an infrequent specie in grassland soils of Uruguay (Bettucci *et al.*, 1989).

Lupo (1992) has observed that *T. harzianum* (5888) and *T. koningii* (6141) have the ability to overgrow and to replace *S. rolfsii* in dual cultures and mycoparasitism was also produced by both strains of *Trichoderma*. In the interaction zone several modifications of *S. rolfsii* hyphal morphology occur such as vacuolation, wall thickening, pigmentation, citoplasmic degeneration and finally producing the diameter of the pathogen. These modifications were similar to those observed when trichorzianines produced by one uruguayan strain of *T. harzianum* tested on *S. rolfsii* and *Sclerotium cepivorum* (Correa *et al.*, 1995; 1996).

All characteristics which determine the saprophytic ability such as the inhibitory activity, the ability to degrade CMC and the effect of water potential on mycelial growth of *Trichoderma* is strain specific and it is probable that within each species several strains have different saprophytic ability (Schoeman *et al.*, 1999; Lupo, 1992). Moreover, there is not one strain which has all optimum characteristics for biological control and consequently there is no correlation between them (Lupo, 1992). Thus, as it was observed here, *T. koningii* (6141) was the most active for growth inhibition, sclerotial formation and germination due to the effect of diffusible metabolites ; *T. hamatum* (6011) and *T. harzianum* (5888) inhibited germination and mycoparasite sclerotia and the latter had also the highest growth rate at lowest water potential. *T. harzianum* 5670 and 5636 evidenced the highest

CMC degradation ability. *T. saturnisporum* an unfrequent species, not previously tested as antogonistic, has also low saprophytic ability.

The different abilities found among the strains suggest that several of them must be selected for soil pathogen biological control present under different environmental conditions, cultural type and field practice. However only the strains 6141 and 5674 were the most active for growth inhibiton. Another set of strains of the same species were more active to prevent sclerotia differentiation than to growth inhibition. These different abilities suggest that several strains must be selected for soil pathogens biological control, conversely to which several utilized formulations of *Trichoderma* are commonly composed by only one strain.

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