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Characterisation of *Rhizoctonia solani* Strains by Gas Chromatography

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Abstract

Fatty acids of the cellular wall (myristic, pentadecanoic, palmitic, 2-hydroxypalmitic, palmitoleic, stearic, oleic, linoleic and nonodecanoic) of four isolates of *Rhizoctonia solani* obtained from *Cucumis sativus*, *Fragaria* sp., *Raphanus sativus* and *Solanum tuberosum* have been used to characterize these isolates. Liquid cultures and different times of incubation for the extraction of the fatty acids were employed. The incubation times suggest that this parameter should be taken into account in comparative tests. The predominant fatty acids (linoleic, oleic and palmitic) show significant differences between isolates.

KEYWORDS: *Cucumis sativus*, Fatty acids, *Fragaria* spp., *Rhizoctonia solani, Solanum tuberosum*.

Resum

Caracterització de soques de *Rhizoctonia solani* per cromatografia de gasos

S'han caracteritzat els àcids grassos de la paret cel·lular (mirístic, pentadecanoic, palmític, 2-hidroxipalmític, palmitoleic, esteàric, oleic, linoleic i nonodecanoic) de quatre aïllats de *Rhizoctonia solani* obtinguts de *Cucumis sativus*, *Fragaria* spp., *Raphanus sativus* i *Solanum tuberosum*. Per a l'extracció dels àcids grassos s'empraren cultius líquids amb diferents temps d' incubació; els resultats de l'experiència suggereixen que aquest paràmetre hauria de tenir-se en compte en els assajos comparatius. Els àcids grassos predominants (linoleic, oleic i palmític) presenten diferències significatives entre els aïllats.

MOTS CLAU: Àcids grassos, *Cucumis sativus*, *Fragaria* spp., *Rhizoctonia solani, Solanum tuberosum*.

Resumen

Caracterización de cepas de *Rhizoctonia solani* mediante cromatografía de gases

Se han caracterizado los ácidos grasos de la pared celular (mirístico, pentadecanoico, palmítico, 2-hidroxipalmítico, palmitoleico, esteárico, oleico, linoleico y nonodecanoico) de cuatro aislados de *Rhizoctonia solani* obtenidos de *Cucumis sativus*, *Fragaria* spp., *Raphanus sativus* i *Solanum tuberosum*. Para la extracción de los ácidos grasos se utilizaron cultivos líquidos con diferentes tiempos de incubación; los resultados de la experiencia sugieren que dicho parámetro debería tenerse en cuenta en los ensayos comparativos. Los ácidos grasos predominantes (linoleico, oleico y palmítico) presentan diferencias significativas entre los aislados.

PALABRAS CLAVE: Ácidos grasos, *Cucumis sativus*, *Fragaria* spp., *Rhizoctonia solani, Solanum tuberosum*.

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Introduction

Rhizoctonia solani Kühn., anamorph of *Thanaethophorus cucumeris* (A.B. Frank) Donk includes various strains of fungi that show a wide range of pathogenicity to plants (Bains & Bisht; 1995; Caesar, 1994; Leiner & Carling, 1994; Rush *et al.*, 1994; Sneh *et al.*, 1991). *Rhizoctonia* diseases are prevalent throughout the world, including Catalonia. This fungus has a wide host range, inclosing many economically important vegetable, ornamental and field crops. Rapid and accurate identification of the aggressive strains is essential to the evaluation of potential damage from infection and to the developement of rapid and effective control measures.

The various strains are catalogued in «Anastomosis Groups» (Carling *et al.*, 1994; Engelkes & Windels, 1994; Jonhk & Johns, 1993; Vico, 1994). In general, ten main groups are recognised, though some authors propose sub-groups, based mainly on the degree of pathogenesis and, in some cases, on the appearance of the colony.

Analysis of fatty acid methyl esters (FAME) has been used in identifying various microorganisms, especially bacteria (De Boer & Sasser, 1986; Graham *et al.*, 1990). Nevertheless, the determination of fungoid species using this method is still limited (Nadal *et al.*, 1996), as the qualitative composition of fatty acids is homogeneous in this group and only quantitative variations can be assessed.

Recently, Stevens Johnk *et al.* (1994) have reported the identification and differentiation of *Rh. solani* strains. Their findings were mainly based on the profile of cellular fatty acids, and they recommend this method for distinguishing between the various groups of intraspecific anastomosis.

This study describes experiments carried out with isolates of *Rh. solani* from severals hosts to explore in depth the viability of this method for strain charac-terization atending to their morphology, virulence or procedence and the influence of the incubation time on the determination of intraspecific differences.

Material and methods

Source of isolates

We compared fatty acid composition and proportion of four isolates of *Rhizoctonia solani* collected from *Fragaria* spp., *Solanum tuberosum*, *Raphanus sativus* and *Cucumis sativus*. These isolates were grown in PDA (potato dextrose agar) and they presented different morphological, colour characteristics and microsclerotia production. The strains of *Rh. solani* were obtained from diseased plants and their main characteristics are listed:

Rh. F: from strawberries (*Fragaria* sp.). Thick, clear, whitish mycelium.

Rh. S: from potatoes (*Solanum tuberosum*). Clear, brown mycelium; microsclerotium.

Rh. R: from radishes (*Raphanus sativus*). Dark mycelium; abundant sclerotium.

Rh. C: from marrow (*Cucumis sativus*). Thick, whitish mycelium.

Cellular fatty acid analyses.

FAME compositions were determined for 5 replicates in each isolate and individual values were calculated from the mean of two determinations. Before fatty acid extraction *Rhizoctonia* strains were grown in a continuously stirred liquid medium (200 ml) for 3, 5 and 7 days (6) at 20 ºC (2). Each extraction and analysis was conducted on 5-10 liquid cultures per run. Following incubation, the mycelial fraction was recovered by centrifuge and filtration. It was then rinsed in sterile water and drained, after which a 100-500 mg portion was processed using the same technique as that employed in the MIS system (Microbial Identification System; Microbial ID, Inc., Newark, DE): Saponification by heating to 100 ^{0}C with 2 ml Na OH(1.67 % in H_2O + Met OH 1:1); methylation with 4 ml HCl 6N + Met OH (1.18:1) at 80 ºC; extraction of the FAME fraction with 1.5 ml hexane + methyl tert-butyleter (1:1); recovery of the upper phase (hydrophobe); gas chromatography.

Results

The percentatges of each fatty acid for every strain are expressed as mean and standard deviation for 5 replicates each strain (Tables 1, 2, 3, 4). The fatty acids obtained were as follows:

Analysis of variance (ANOVA) was performed on percentage FAME compositions. Mean separation was accomplished by the Duncan test (p=0.05). The first three principal components were calculated on 3-day-old cultures. The Euclidean distance coefficient was computed and a dendrogarm was constructed by the unweighted pair-group method with arithmetic averages (UPGMA). A discriminant analysis provided the canonical discriminant function.

Although the fatty acid compositions of the isolates were qualitatively similar, quantitative differences were observed. Because ANOVA showed no differences between transformed and untransformed data (Stevens & Jones, 1992), untransformed data were used in statistical analyses.

In all strains the proportion of the various fatty acids changed markedly with incubation time. This is shown by a significant decrease in saturated fatty acids, with the exception of C 15 : O and monunsaturates, and, at the same time, an increase in linoleic (C 18 : 2 CIS 9, 12) and the appearance of hydroxypalmitic (C 16 : 02 OH), which was not present before 3 days of incubation. The first three principal components (PC1, PC2, PC3) are plotted in Fig 1, they accounted for 90.5 % of the variation data. PC1 accounted for 45.3 % of the variability, PC2 accounted for 31.9 % and PC3 for 13.4 %. The first three principal components show the separation of isolates: *Rh.* C and *Rh.* F in front of *Rh.* R or *Rh.* S. A dendrogram (Fig 3) showed similar results. The discriminant analysis also showed three differentiated groups in the strains studied (Fig 2).

Discussion

The profiles obtained differ markedly, both in the number of fatty acids and in their proportions, from those reported by Stevens Johnk *et al.* (1992, 1993, 1994), which dealt with mycelium deriving from cultures in an agarized medium. They obtained twelve FAMES, seven of which corresponded to the most characteristic found in the present study. On the other hand, the results noted after 7 days' incubation in a liquid medium, which is equivalent to an older culture, bore

TABLE 1. Percentages of each fatty acid for *Rh*. C: strain obtained from marrow (*Cucumis sativus*) expressed as mean ± standard deviation.

Percentatge de cada àcid gras de la soca *Rh*. C aïllada de cogombre (*Cucumis sativus*) expressat com a mitjana ± desviació estàndard.

ECL	14.000	15.000	15.817	16.000	17.235	17.720	17.769	18.000	18.633
Formula	14:0	15:0	16:1cis 7	16:0	16:0 2OH	18:2 cis 9,12	18:1 cis 9	18:0	19: 0 iso
3 DAYS	1.389	0.751	5.293	15.012	-	43.191	30.462	3.902	-
	± 0.576	± 0.534	± 1.211	± 2.013	-	± 7.701	± 5.679	± 1.160	-
5 DAYS	0.542	1.009	1.836	12.014	-	46.704	34.935	2.776	-
	±0.111	± 0.442	± 1.084	± 1.111	-	± 4.323	± 4.579	± 0.630	-
7 DAYS	1.254	0.869	3.717	11.048	0.606	58.550	21.370	2.587	-
	±0.047	± 0.069	± 0.644	± 0.453	± 0.021	± 2.397	± 1.758	± 0.596	-

TABLE 2. Percentages of each fatty acid for *Rh*. F strain obtained from strawberry (*Fragaria* spp.) expressed as mean ± standard deviation.

Percentatges de cada àcid gras de la soca *Rh*. F aïllada de maduixa (*Fragaria* spp.) expressat com a mitjana ± desviació estàndard.

ECL	14.000	15.000	15.817	16.000	17.235	17.720	17.769	18.000	18.633
Formula	14:0	15:0	16:1cis 7	16:0	16:0 2OH	18:2 cis 9,12	18:1 cis 9	18:0	19: 0 iso
3 DAYS	1.476	0.598	5.665	12.865	-	45.717	29.391	4.289	-
	± 0.777	± 0.009	± 2.331	± 0.374	-	± 0.736	± 2.628	± 0.639	-
5 DAYS	0.932	0.741	3.993	10.198	0.493	54.704	26.294	2.646	-
	± 0.302	± 0.031	± 0.011	± 0.017	± 0.008	± 0.297	± 0.126	± 0.709	-
7 DAYS	0.593	2.004	1.371	7.806	0.800	68.150	18.373	0.903	-
	± 0.048	± 0.150	± 0.181	± 0.697	± 0.011	± 0.998	± 0.631	± 0.038	-

greater resemblance to the values obtained by the same authors with an agarized medium. Nevertheless, the liquid culture offers the advantage of being able to isolate and clean the resultant mycelium, thereby shedding non-structural fatty acids.

Comparing the values of the predominant fatty acids (linoleic, oleic (C 18 : 1 CIS) and palmitic (C 16 : 0)) in a deviation test, significant differences ($p < 0.05$) between the strains are seen, especially that which separates the *Rh.* R from the rest. The fact that *Rh.* R behaved aggressively towards various hosts suggests that this technique can provide valuable information on classifying strains. However, further experiments will be required if this method is to be adopted as a complement to other identification tests.

TABLE 3. Percentages of each fatty acid for *Rh*. R: strain obtained from radish (*Raphanus sativus*) expressed as mean ± standard deviation.

Percentatge de cada àcid gras de la soca *Rh.* R aïllada de rave (*Raphanus sativus*) expressat com a mitjana ± desviació estàndard.

ECL	14.000	15.000	15.817	16.000	17.235	17.720	17.769	18.000	18.633
Formula	14:0	15:0	16:1cis 7	16:0	16:0 2OH	18:2 cis 9,12	18:1 cis 9	18:0	19: 0 iso
3 DAYS	2.456	2.786	5.671	16.341	-	38.312	28.525	5.908	-
	± 0.229	± 0.178	±0.611	± 0.906	-	± 0.521	± 0.037	± 0.728	-
5 DAYS	1.682	2.124	4.016	11.001	-	55.233	22.030	3.913	-
	± 0.178	± 0.110	± 0.226	± 0.786	-	± 1.012	± 0.826	± 0.893	-
7 DAYS	0.487	0.712	1.038	11.284	0.502	64.573	19.548	1.775	0.082
	± 0.047	± 0.011	± 0.135	± 0.010	± 0.158	± 0.916	± 0.489	± 0.148	± 0.082

TABLE 4**.** Percentages of each fatty acid for *Rh*. S: strain obtained from potato (*Solanum tuberosum*) expressed as mean ± standard deviation.

Percentatges de cada àcid gras de la soca *Rh*. S aïllada de patata (*Solanum tuberosum*) expressat com a mitjana ± desviació estàndard.

ECL	14.000	15.000	15.817	16.000	17.235	17.720	17.769	18.000	18.633
Formula	14:0	15:0	16:1cis 7	16:0	16:0 2OH	18:2 cis 9,12	18:1 cis 9	18:0	19: 0 iso
3 DAYS	7.425	-	14.508	12.920	-	29.458	30.139	5.246	-
	± 0.727	-	± 3.174	± 0.319	-	± 2.995	± 1.880	± 1.596	-
5 DAYS	1.154	0.893	4.407	10.979	0.251	51.824	28.284	2.209	-
	± 0.080	± 0.183	± 0.425	± 0.764	± 0.251	± 3.177	±1.846	± 0.272	-
7 DAYS	0.410	0.935	1.115	11.097	0.255	59.216	24.722	2.068	0.181
	± 0.004	± 0.003	± 0.055	± 0.674	± 0.062	± 0.129	± 1.209	± 0.845	± 0.056

TABLE 5. Mean separation by the Duncan test (p= 0.05) of composition of cellular fatty acids in 3-day-old culture of the different isolates. (ND = No differences).

Separació de mitjanes pel test de Duncan (p=0.05) de la composició d' àcids grassos cel·lulars en cultius de 3 dies dels diferents aïllats. (ND = No diferències).

Isolate	14:0	15:0	16:1c7	16:0	18:2 c9,12	18:1c9	18:0
Rh. C	a	c	a	a	b	ND	a
Rh. F	a	b	a	a	c	ND	a
Rh. S	c	a	c	a	a	ND	a
Rh. R	d	d	a	c	b	ND	c

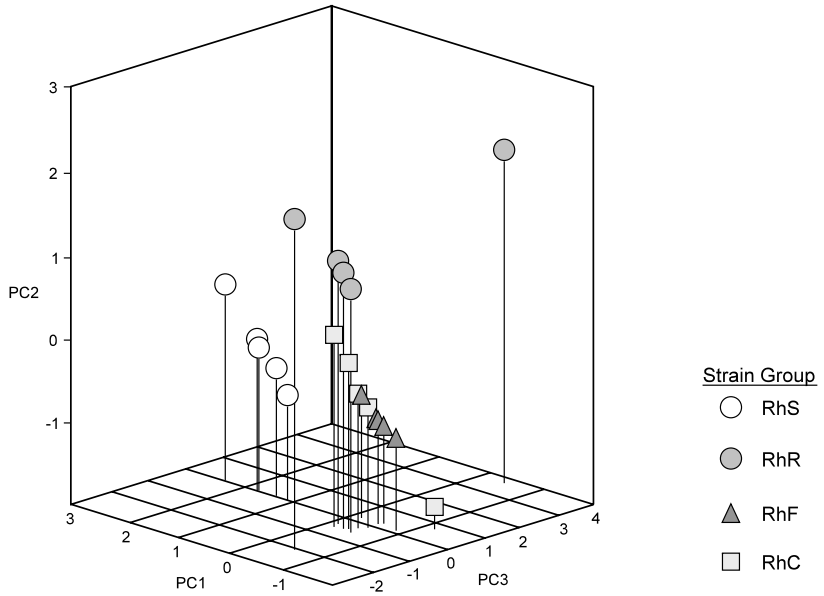

FIG. 1: Plot of the first three principal components (PC1, PC2 and PC3) from the FAME percentage composition of the *Rhizoctonia solani* isolates.

Gràfic dels tres primers components principals (PC1, PC2 i PC3) obtingut a partir dels percentatges dels àcids grassos metilats dels aïllats de *Rhizoctonia solani*

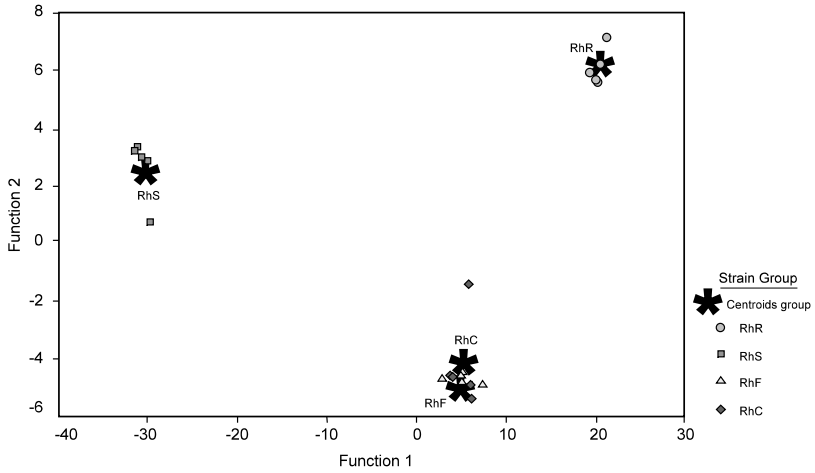

FIG. 2: Plot of the canonical discriminant function of the *Rhizoctonia solani* isolates.

Gràfic de la funció discriminant canònica dels aïllats de *Rhizoctonia solani*

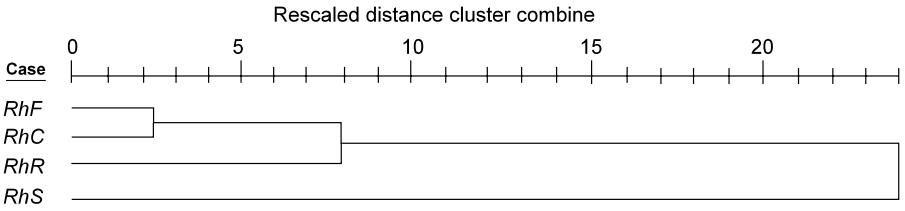

FIG. 3: Dendrogram of *Rhizoctonia* strains (*Rh.*C, *Rh.*F, *Rh.*R and *Rh.*S) based on the analysis of percentage fatty acid composition of 3-day-old cultures.

Dendrograma de les soques de *Rhizoctonia* (*Rh.* C, *Rh.* F, *Rh.* R i *Rh.* S) basat en l'anàlisi del percentatge de la composició d'àcids grassos de cultius de 3 dies.

The variations between the profiles with incubation time suggest that this parameter should be taken into account in comparative tests.

The fatty acid composition of the isolates studied do not differ qualitatively although the ANOVA indicates that there are significant quantitative differences. These aspects reported by other authors was related in the anastomosis groups (Stevens & Jones, 1993, 1994; Vico, 1994). Our experiences reveal that the differences can also be found between strains of different morphology and host.

Liquid culture allows separation of the mycelial fraction by centrifugation or filtration of the culture medium. On the other hand, it allows uniform aging in all the colony, in contrast to the agar culture, in which the central zone of the colony is older than the peripheral zone. The results as a function of the time indicate variations in the percentages of fatty acids; there was a clear increase in the values of C18:2cis9, 12 in all the isolates, while C18:1cis9 and C18:0 decreased and others such as the C16:0 2OH appear later. The presence of this fatty acid, also reported by others authors in solid culture (Stevens & Jones, 1993, 1994; Vico, 1994), can be associated with the high mycelium concentration. On the other hand it has been observed that at longer cultivation times, the differences between the strains decreases. In the case of *Rhizoctonia solani*, this type of studies has been of accomplishing in 2-4-day old culture, to avoid aging effects.

The grouping revealed here between isolates studied in 3-day-old cultures allow us to relate the strains with whitish mycelium (*Rh.*C and *Rh.*F), while the isolate from potato (*Rh.*S) is far from the others, and not particularly virulent.

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