

WHOLE-CELL FATTY ACID PROFILES - A TOOL FOR SPECIES AND SUBSPECIES CLASSIFICATION IN THE *Puccinia recondita* COMPLEX

I.S. Ben-Ze'ev¹, E. Levy¹, T. Eilam² and Y. Anikster²

¹ Plant Protection and Inspection Services, Ministry of Agriculture and Rural Development, Bet Dagan Agricultural Center, Box 78, Bet Dagan 50250, Israel

² Department of Plant Sciences and the Institute for Cereal Crops Improvement, Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel

SUMMARY

The fatty acid profiles of 145 telial isolates of *Puccinia recondita* sensu lato (*Uredinales: Pucciniaceae*), from different host species and different geographical origins, were determined by gas chromatography (GC). The isolates clustered into three different GC groups, according to consistent quantitative differences in their fatty acid compositions. Isolates collected from bread, durum and wild emmer wheats, with *Thalictrum speciosissimum* (*Ranunculaceae*) as aecial host, regardless of their geographical origin, formed one group. The second group consists of isolates collected on *Aegilops* spp. and some from durum wheat, all alternating on Boraginaceous species. The third group contains isolates collected on rye, alternating on *Boraginaceae*. The grouping of *P. recondita* isolates by fatty acid profiles coincides with a similar one, suggested by several studies involving pathogenicity on alternate hosts, cross ability, teliospore dimensions and relative DNA content of haploid spores. It is concluded that the three groups represent two species and one subspecies: *Puccinia triticina* (on wheats, group 1), *P. recondita* subsp. *recondita* (on rye, group 3) and *P. recondita* subsp. *aegilopsis* (on *Aegilops* spp. and durum wheat, group 2).

Key words: *Aegilops*, leaf rust, *Puccinia triticina*, rye, wheat.

INTRODUCTION

The taxonomy of cereal leaf rusts (*Uredinales: Pucciniaceae*) attacking the genera *Aegilops*, *Secale*, *Triticum*, and related genera of grasses suffers from a great deal of confusion, uncertainty and disagreement. Chester (1946) reviewed the three main conflicting taxonomic concepts around the species responsible for cereal leaf rusts. He preferred the concept of species limited to one or very few hosts, with *Puccinia triticina* Eriks.

as the one responsible for wheat leaf rust and some closely related grasses, with aecial hosts: *Thalictrum* and *Isopyrum* (*Ranunculaceae*). The second concept was that of a species causing leaf rust of wheat and various grasses, with aecia on various *Ranunculaceae*: *P. elymi* Westd. The third concept grouped together leaf rusts of more than 90 species in 16 genera of grasses, with aecial stages on 36 species of plants in 25 genera of 4 unrelated families, into the composite species *P. rubigo-vera* (D.C.) Wint.

Cummins and Caldwell (1956) introduced some nomenclatural order by setting *P. elymi* as a distinct species and by pointing out that the oldest valid name applicable for the *P. rubigo-vera* complex was *Puccinia recondita* Rob. ex Desm. This revision of the third concept became accepted worldwide by most phytopathologists, apparently based on Cummins' authority.

The use of the binomial *P. recondita* for the entire complex of cereal leaf rusts does not answer major practical questions as: Is there any gene flow among the various rust types within this complex? Are cultivars of wheat and rye susceptible to any of the grass attacking rusts included in the complex? Unfortunately, many of the cereal leaf rust fungi are quite similar in teliospore morphology and have also an overlapping main-host range. Experimental inoculations and crossability tests for the elucidation of the alternate-host range and for a more clear-cut taxonomy were not practiced often enough due to difficulties to induce teliospore germination under controlled conditions (Anikster *et al.*, 1997).

Anikster *et al.* (1996, 1997) and Eilam *et al.* (1992; 1994), in their comprehensive studies of the leaf rusts attacking wheat, rye and *Aegilops* species, used an image-analysis technique to measure spore dimensions and flow cytometry to estimate the nuclear DNA content of basidiospores and pycniospores. They conducted crossability tests among isolates secured from different hosts. The conclusion of their studies was that two main groups can be distinguished within *P. recondita* sensu Cummins and Caldwell (1956): one group (I) that alternates on *Thalictrum speciosissimum* and whose main host is wheat, and a second group (II) which alternates on boraginaceous species and whose main hosts are rye and *Aegilops* spp., although one subgroup within the

second group attacks durum wheat in Morocco, Spain and Portugal. These two groups are not crossable and thus appear to be separate biological entities. Each group is further divisible into subgroups slightly differing in their range of main and alternate hosts and in morphological traits (Anikster, 1986; Anikster *et al.*, 1997).

Fatty acid profiles are used increasingly as a chemotaxonomic tool for the identification and classification of bacteria (O'Donnell, 1994). There are still fewer publications on the use of this tool for fungal taxonomy, although it has proved to be useful with fungi that can be grown in carefully standardized axenic culture conditions (Stevens Johnk and Jones, 1993, 1994), as well as for lichens (Sasaki *et al.*, 2001), arbuscular-mycorrhizal fungi (Madan *et al.*, 2002) and Oomycetes (Larkin and Groves, 2003). Tulloch and Ledingham (1960, 1962) tried this technique when computer software and extraction procedures were not as user-friendly as today, and concluded against its taxonomic value.

One goal of the present study was to find out whether this chemotaxonomic technique can be used in a group of obligate parasitic fungi, which can not be grown in axenic conditions. Another goal was to find out whether fatty acid profiles of *P. recondita* groups could be used in order to elucidate further the taxonomic complexity of this group.

MATERIALS AND METHODS

Isolates. The 145 isolates used in this study consisted of *P. recondita* (*sensu lato*) teliospores, collected in different continents and countries, stored at 4°C, as part of the Tel Aviv University rust collection. The identities of isolates, at the generic, specific and lower levels, were established by microscopic examination of the teliospores and by artificial inoculations onto telial and aecial hosts. Isolate ID details are given in Table 1.

Fatty acid methyl esters extraction and analysis. Approximately 30 mg of teliospores per isolate were processed in a 100 × 13 mm borosilicate test tube with teflon-coated, bakelite screw cap. Saponification and methylation were carried out as specified by MIDI (1992a) for aerobic bacteria. Extraction included a modification for fungi (MIDI, 1993). Isolates were processed in batches of 20-60 at a time.

Fatty acid methyl esters (FAs), with carbon chain lengths between C9 to C20, were identified by gas chromatography, using the Microbial Identification System "MIS" (MIDI, Microbial ID, Inc., Newark, DE, USA). This is a computerized gas chromatography (Hewlett-Packard GC-5890 / MIDI) system, equipped with automatic sampler, fused silica capillary column cross-linked 5% phenyl methyl silicone 25 m × 0.2 mm, film thick-

ness 0.33 mm (HP 19091J-102) and FID detector, controlled by an HP Vectra 586 work station, using MIDI's Library Generating Software (LGS version 4.15). From this program, the method "FUNGI", version 3.8 was used to control the GC parameters and the sample and calibration injection sequences. GC parameters: injector temperature 250°C, detector temperature 300°C, initial oven temperature 170°C, temperature rate 5°C min⁻¹, run time 20 min, final temperature 270°C. Column evacuation was by raising the temperature (ramp A) rate to 30°C min⁻¹ to a final 310°C for 2.5 min.

Temperature stabilization at 170°C was for 3 min preceding the next injection. Carrier and auxiliary gases (99.999% pure) were H₂ and N₂ respectively, dry and free of hydrocarbon residues. Gas pressures and flows were as specified by MIDI (1992a). Fifteen samples were analyzed using MIDI's "EUKARY" method. The initial GC parameters of this method are identical with those of "FUNGI", but the temperature continues to rise at 5°C min⁻¹ to 300°C and the carbon chain lengths detectable are between C9 to C30.

Calibrations and controls. The system was programmed to inject two repeats of calibration mix (a precise mixture of straight chain, saturated FAs, hydroxy acids included, in the ranges of C9→C20, or C9→C30 for method EUKARY, at the beginning of each (batch of samples) run, and single repeats at intervals of 11 samples. This served both for calibration purposes and as a control with a known FA profile. Next to calibrations were: a "blank" (prepared along with the analyzed batch, using reagents but no fungus), injected as a negative control and test of the reagents' purity, and an extract from a culture of *Alternaria alternata* injected as "positive control".

Fatty acid profiles of isolates. The MIS automatically compared detected peaks with the "Peak naming tables" of the methods "FUNGI" or "EUKARY" respectively, and named them by conventional abbreviations of FAs and derivatives between C9→C20 or between C9→C30.

At the end of each sample analysis, the MIS computed a profile of the sample's FAs and compared it with profiles of species found in the library FUNGI. A run succeeded if the MIS recognized the calibration mix, characterized the blank by "area = 0" (no peaks), and identified the positive control correctly as *A. alternata*.

To test the method's reproducibility and to find the expected degrees of dissimilarity between separate analyses of the same isolate (i.e., the laboratory variability), a few isolates were sampled, extracted and analyzed 2-7 times.

Fatty acid profiles of groups: building library entries. Library entries were prepared according to MIDI

Table 1. List of *Puccinia recondita* isolates analyzed and profiled for fatty acids, classified into GC-subgroups according to a library built from fatty acids profiles.

Isolate code name, Telial + Aecial Hosts, Country of Origin and Collection No.	Library subgroup
Puc-rec-aeg (var-isr) <i>Aegilops variabilis</i> + <i>Anchusa aggregata</i> , Israel: T-9283; T-9297; T-9307; T-9315; T-9378; T-9464; T-9465; T-9466; T-9479; T-9485; T-9495; T-9495; T-9497; T-9514 / 509 ; T-9519; T-9520; T-9521 / 485 and 507; T-9525 / 468 and 508; T-9529; T-9552; T-9564 T-9540;	aegilopis - “ - - “ - 'aeg'
T-9514 / 482 ; T-9350 / 411 and 447;	—
Puc-rec-aeg (kot isr) <i>Ae. kotschyi</i> + <i>A. strigosa</i> , Israel: T-9500; T-9501; T-9502; T-9504; T-9506	aegilopis
Puc-rec-aeg (lon-isr) <i>Ae. longissima</i> + <i>A. aggregata</i> , Israel: T-9186; T-9196; T-9249; T-9254; T-9311; T-9376; T-9516 / 465 and 481; T-9550;	aegilopis - “ -
Puc-rec-aeg (ita-mor) <i>Triticum turgidum</i> var. <i>durum</i> + <i>Anchusa italica</i> , Morocco: T-2719; T-2721; T-2843; T-2767;	aegilopis - “ -
T-2845; T-2846;	'aeg'
T-2722; T-2731; T-2537;	—
Puc-rec-aeg (ova-isr) <i>Ae. ovata</i> + <i>Echium glomeratum</i> , Israel: T-9187; T-9190; T-9206; T-9210; T-9211; T-9220; T-9284; T-9295; T-9370 / 517 ; T-9436; T-9463; T-9475 / 080, / 086, / 087, / 088, / 089, / 093 and 306; T-9484 / 300 and 322; T-9494 and 513; T-9511 / 483 and 510; T-9370 / 437 ;	aegilopis - “ - - “ - 'aeg'
T-9203 / 451 and 522;	—
Puc-rec-aeg (sea-isr) <i>Ae. searsii</i> + <i>A. strigosa</i> , Israel: T-9509;	aegilopis
Puc-rec-aeg (sha-isr) <i>Ae. sharonensis</i> + <i>Anchusa undulata</i> , Israel: T-9461;	'aeg'
Puc-rec-aeg (stg-isr) <i>Ae. variabilis</i> + <i>Anchusa strigosa</i> , Israel: T-9427 / 094 and 304;	aegilopis
Puc-rec-aeg (tca-tur) <i>Ae. triuncialis</i> + unknown, Turkey: T-2823;	aegilopis
Puc-rec-rec (sec-chs) <i>Secale montanum</i> + <i>A. undulata</i> , Czech Republic: T-2645; T-2887; T-2889;	recondita 'rec'
Puc-rec-rec (sec-isr) - - -, Israel: T-9258; T-9487 / 000 and 400; T-9488 / 000 and 324; T-9489 / 432 ; T-9536; T-9537; T-9545; T-9489 / 000 ; T-9535;	recondita - “ - 'rec'
T-9361; T-9486;	—
Puc-rec-rec (sec-usa) - - -, U.S.A.: T-2709; T-2805; T-9362;	recondita
Puc-rec-rec (sec-pol) - - -, Poland: T-2893;	—
Puc-tri (dic-isr) <i>T. turgidum</i> var. <i>dicoccoides</i> + <i>Thalictrum speciosissimum</i> , Israel: T-9177; T-9213 / 452 and 537; T-9261; T-9396 / 541 ; T-9399; T-9430 / 417 and 540; T-9512 / 470 and 532; T-9538;	tritricina - “ - 'trit'
T-9396 / 415 ; T-9490;	—
Puc-tri (dur-eth) <i>T. turgidum</i> var. <i>durum</i> + <i>Th. speciosissimum</i> , Ethiopia: T-2664; T-7545; T-7546 / 435 ;	tritricina 'trit'
T-7546 / 453 ;	—
Puc-tri (dur-isr) - - -, Israel: T-7607; T-9409 / 096 and 305;	tritricina
Puc-tri (dur-mex) - - -, Mexico: T-2837 / 478;	'trit'
Puc-tri (aes-aus) <i>Triticum aestivum</i> + <i>Th. speciosissimum</i> , Australia: T-2853 / 420 and 458;	tritricina
Puc-tri (aes-chi) - - -, China: T-2834; T-2627; T-2666;	tritricina 'trit'

Puc-tri (aes-eth) - - -, Ethiopia: T-2811 / 105 ; T-2812; T-2743 / 542 ; T-2810 / 455 ; T-2811 / 456 ;	triticultura
T-2743 / 475 ; T-2810 / 423 ;	'trit'
Puc-tri (aes-hol) - - -, Holland: T-2796;	—
Puc-tri (aes-isr) - - -, Israel: T-7872; T-9254; T-9277; T-9288; T-9358; T-9373; T-9390;	triticultura
T-9447 / 092 and 605; T-9457; T-9469; T-9472 / 471 and 601; T-9482; T-9483 / 122 and 302;	- " -
T-9491 / 449 and 602; T-9524; T-9573;	- " -
T-2601; T-9271;	'trit'
T-9453; T-9455;	—
Puc-tri (aes-mex) - - -, Mexico: T-2799; T-2801; T-2838; T-2856 / 419 and 611;	triticultura
T-2857 / 418 and 457; T-2876 / 477 and 613; T-2877 / 476 and 612;	
Puc-tri (aes-pol) - - -, Poland: T-2842;	triticultura
Puc-tri (aes-usa) - - -, U.S.A., T-2750 / 427 and 473; T-2752 / 099 and 474;	- " -
T-2753; T-2767 / 426 and 472; T-2803; T-2804 / 454 ;	triticultura
T-2751; T-2804 / 424 ;	'trit'
T-2804 / 104 ;	—
Puc-tri (trc-mex) " <i>Triticale</i> " + <i>Tb. speciosissimum</i> ; Mexico, T-2798;	'trit'
Puc-tri (trc-pol) - - -, Poland, T-2836;	'trit'

Library subgroup in full bold = isolates included in Library profile; Library subgroup 'abbreviated' = isolates correctly identified (with similarity <0.500) but not included in Library profile; Library subgroup - = not identified and not included in Library profile. Bold collection numbers = 2 or more duplicates of the same isolate differing in identification robustness.

(1992b). Graphic illustrations of the relationships among the profiles of individual isolates were computed using the 'Dendrogram' and the '2-D Plot' programs of the MIDI software, based on covariance and cluster analyses (MIDI, 1992a,b; Stevens Johnk and Jones, 1994). Well-defined clusters of profiles (isolates) were processed together, using the 'Create entry' option of the 'Create Library' program, resulting in profiles of groups (library entries). A library entry profile is a list of the FAs characteristic for a given group of isolates or a taxon. Each FA in the profile is characterized by its minimum and maximum values and its mean percentage relative to the total FA composition of the group. A fatty acid was used (included) in the profile if its quality threshold (QT = mean % of a given FA in a profile, multiplied by the profile's ratio of isolates having it) was ≥ 0.25 . Otherwise it was excluded (Stevens Johnk and Jones, 1993; 1994).

Library entries were refined through several stages, each time excluding some unfit isolates and adding others. Isolates that departed more than 3 SD units from the mean of the group were excluded using the 'Histogram distance' option. After having created a new library, "RUST", with several entries, all *P. recondita* isolates in the database were reclassified (compared with the entries in the new library), using the 'Classify file' program. Isolates with similarity indices lower than 0.5 to their own group profile and isolates with high simi-

larity indices to 2 different group profiles were marked. The library entries (group profiles) were refined, using the 'retrain entry' option, by excluding marked (unfit) isolates and by adding new ones (analyzed since the last entry creation, with similarity ≥ 0.5 to the entry). Fitness of isolates in each entry was reassessed using the 'Histogram distance' option and the 'Library validation' program (Mendala, 1990; MIDI, 1992b).

RESULTS

The standard library FUNGI available from MIDI contains only fungi grown in axenic culture, in standard conditions; it has no entries of rust fungi or any other obligate parasites.

This library recognized none of the *P. recondita* isolates analyzed in this study.

A database of 186 fatty acid profiles from teliospores of *P. recondita* was gathered during 1994-1996, including 145 isolates and 41 replicates (isolates extracted and analyzed more than once, at different dates). The telial and aecial hosts, countries of origin and classification by FAs of all of these database entries are listed in Table 1.

Isolates extracted and analyzed twice or more (e.g. stg-isr T-9427a and b; lon-isr T-9515a and b; aes-isr T-9447a and b; aes-isr T-9491a and b; and aes-isr T-9472a and b, see dendrogram, Fig. 1) produced highly similar

profiles, with 1.0-2.0 Euclidean distances between replicates 'a' and 'b' of the same isolate. This very satisfactory repeatability indicated that the resulting profiles could be used to build library entries (Ben-Ze'ev *et al.*, 1997).

A tentative "*Puccinia recondita sensu lato*" library profile was built after having analyzed the first 60 profiles (not shown). Three FAs not used in this profile, pentadecanoic (15:0), palmitoleic (16:1 ω 9c), and 2-hydroxystearic (18:0 3OH), were present in substantial numbers of: 24, 20 and 18 isolates, respectively. A dendrogram of the 60 profiles was prepared (not shown) and the three FAs were marked beside the isolates containing them.

This revealed that 15:0 and 16:1 ω 9c were frequent in isolates from *T. aestivum*, *T. turgidum* var. *durum* and

wild emmer, but very rare in the other isolates, while 18:0 3OH was not noticeably frequent in any group. The next step was to prepare a profile including only isolates from *Triticum*. This profile, tentatively named "*P. recondita* GC-subgroup *triticina*" (Table 2), included as used features both 15:0 and 16:1 ω 9c, indicating these two FAs to be rather characteristic for isolates having *T. aestivum* and *T. turgidum* (vars *durum* and *dicoccoides*) as telial hosts and *Thalictrum* as aecial host. The remaining isolates, from *Secale*, *Aegilops* and other telial hosts, having aecial hosts in the *Boraginaceae*, formed a different, rather loose cluster in the dendrogram. Their FA profile was computed (not shown) and named tentatively "*P. recondita* GC-subgroup *recondita*". In this profile 15:0 and 16:1 ω 9c were indeed too rare (QT <0.25) to be used.

Table 2. Fatty acid profile of *Puccinia triticina* (GC-subgroup "*triticina*").

Index ^a	Feature (abbreviated)	Count ^b	Mean (%) ^c	Minimum (%) ^c	Maximum (%) ^c	Feature status ^d
21	12:0	3	0.02	0.00	0.52	
25	C9 Dicarboxylic acid	6	0.06	0.00	1.34	
48	14:0	59	1.40	0.00	3.43	USED
65	15:0	31	0.26	0.00	0.70	
70	16:1 cis ω 7 Alcohol	13	0.13	0.00	1.26	
76	16:0 Anteiso	4	0.04	0.00	0.82	
78	16:1 ω 9 cis	33	0.65	0.00	3.13	USED
79	16:1 ω 7 cis	1	0.03	0.00	1.70	
80	16:1 ω 5 cis	1	0.01	0.00	0.53	
82	16:0	61	22.05	17.41	26.28	USED
104	16:0 Iso 3OH	5	0.03	0.00	0.49	
109	18:1 trans ω 9 Alcohol	9	0.12	0.00	0.96	
117	18:2 cis 9,12/ 18:0 Ante	61	7.27	5.63	8.75	USED
122	18:0	61	4.75	3.34	6.73	USED
132	19:0 N Alcohol	2	0.03	0.00	1.12	
138	19:1 ω 9 trans	2	0.03	0.00	1.05	
146	20:1 cis ω 9 Alcohol	2	0.02	0.00	0.87	
150	Unknown 19.521 ^f	1	0.01	0.00	0.73	
151	18:0 3OH	16	0.16	0.00	1.11	
152	C20 N Alcohol	1	0.01	0.00	0.86	
161	18:0 12OH	2	0.06	0.00	1.96	
162	20:0	55	1.75	0.00	2.93	USED
170	Summed Feature 8 ^e	61	60.14	54.91	65.09	USED
172	Summed Feature 10 ^e	50	0.95	0.00	1.57	USED

Telial isolates from *Triticum aestivum*, *T. turgidum* var. *durum* and *T. turgidum* var. *dicoccoides*, with *Thalictrum speciosissimum* as aecial host: 45 isolates + 16 duplicates = 61 samples in the profile.

^a Serial no. of feature in the Peak naming table of method "FUNGI". ^b The number of isolates sharing a certain feature. ^c Relative quantity (in %) of a feature across the profile. ^d Used or not used for the profile. ^e A peak shared by 2 or more molecules with retention times too close to be separated by the current method. Summed feature 8 = 18:1 ω 9c and/or 18:1 ω 8c. Summed feature 10 = Unknown 18.218^f and/or 18:1 Cis 9 DMA. ^f Peak of unknown molecule, with chromatographic properties indicating an equivalent carbon length (i.e., 18.218) known to occur in fungi.

In continuation, more batches of isolates were analyzed and their profiles were added to the database. The FA profiles of the aforementioned groups were 're-trained' time and again, with dendrograms and 2-D plots computed in order to visualize the clustering of isolates.

Profile "triticina" grew to contain 61 samples, by which time 15:0 was excluded from the profile as it was found in only 31 samples (QT <0.25). Many more profiles of isolates from *Aegilops* accumulated in the database and clustered together, separating from the *Secale* cluster. Their profile was tentatively named "*P. recondita* GC-subgroup *aegilopsis*".

The three final subgroup profiles, making up the entire database shown in Table 1, are shown in Tables 2, 3 and 4. A dendrogram (Fig. 1) built from 67 database entries illustrates the clusters from which the library profiles were built. Fig. 2 shows the clustering of 142 entries of the library. Subgroup "triticina" (Table 2) is a li-

brary entry made of 61 samples: 45 telial isolates and 16 duplicates from *T. aestivum* and *T. turgidum* (var. *durum* and var. *dicoccoides*), with *Thalictrum speciosissimum* (*Ranunculaceae*) as aecial host. Subgroup "aegilopsis" (Table 3) is a library entry made of 69 samples: 54 telial isolates and 15 duplicates and replicates mainly from *Aegilops* species, but also from *T. turgidum* var. *durum*, with species of *Anchusa* and *Echium* (*Boraginaceae*) as aecial hosts. Subgroup "recondita" (Table 4) is the smallest library entry, made of 14 samples only: 12 telial isolates and 2 duplicates from *Secale montanum*, with *Anchusa undulata* as aecial host.

The ultimate test of a new library is a comparison of all profiles obtained by GC analysis with the profiles of the library entries, using the 'classify file' option. All the samples (isolates and replicates) in each of the three library entries were classified correctly by the RUST library, each having a higher similarity index to its own entry than to any other. Of 23 samples from the "triticina" subgroup, not included in the library due to questioned analyses or low similarity, 14 were identified correctly, as were 5 of 13 such samples of the "aegilopsis" and 3 of 6 samples of the "recondita" subgroups (Table 1).

DISCUSSION

The fatty acid compositions of subgroups in *Puccinia recondita*. Qualitatively, the profiles of the three subgroups found in *Puccinia recondita* are very similar, containing the same seven fatty acids: myristic (14:0), palmitic (16:0), linoleic (18:2 cis 9,12), stearic (18:0), oleic (18:1 ω 9c and/or ω 8c), unknown 18.218 and/or 18:1 cis9 dimethylacetal, and eicosanoic (20:0). An eighth component, palmitoleic acid (16:1 ω 9c) is found in subgroup "triticina", but only as traces in the others (Table 5). Tulloch and Ledingham (1962) found a similar composition for *P. recondita* aeciospores, using different extraction and chromatography procedures. Quantitatively, however, there are differences among the subgroups in the mean percentage of all components. These quantitative differences become quite substantial when certain acids are considered (Table 5). Oleic and palmitic acids, in this order, are the two main components in the profiles of all 3 subgroups, accounting together for 82-88% of the total FAs. Their distribution is almost inversely proportional, increasing for oleic from 60.1% to 63.9% and to 67.1% in the "triticina", "aegilopsis" and "recondita" profiles, respectively, and decreasing for palmitic from 23.8% in "aegilopsis" to 22.0% in "triticina" and to 19.7% in "recondita".

Next in quantity but perhaps more prominent as a dividing character is the peak shared in the Peak Naming Table of method "FUNGI" by linoleic and anteisostearic acids (18:2 cis 9,12 / 18:0 a). Tulloch and Ledingham (1962) found in aeciospores of *P. recondita*

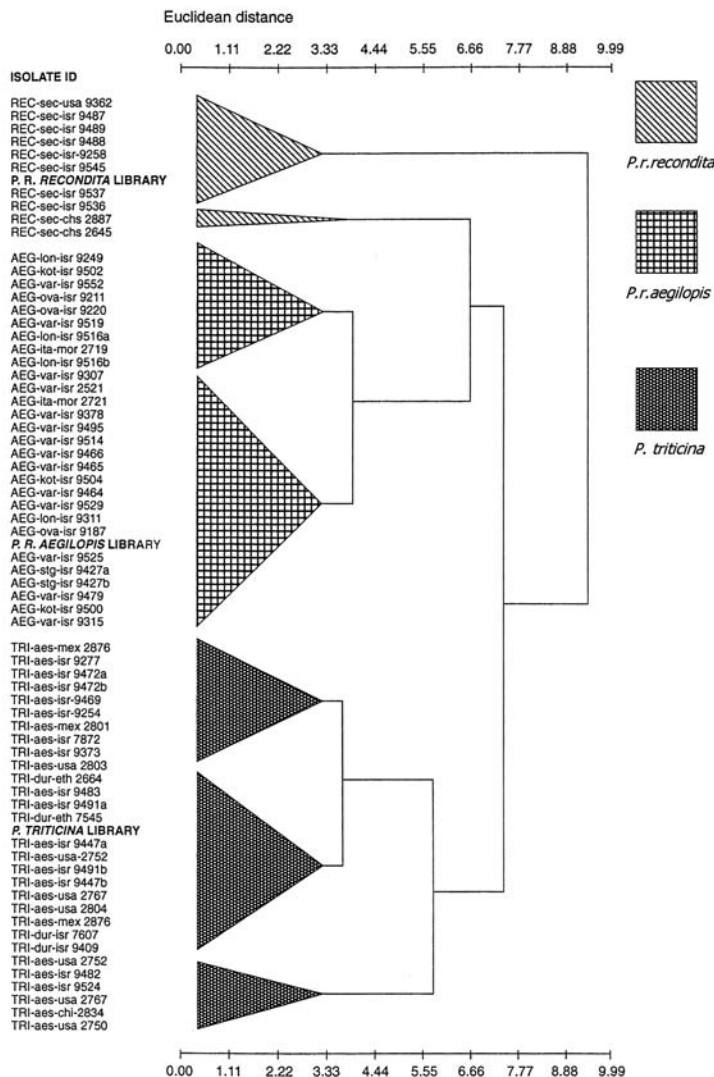


Fig. 1. Dendrogram showing three subgroups within *Puccinia recondita* as determined by fatty acid profiles. Isolate IDs preceded by REC are classified by library RUST in subgroup "recondita"; AEG in subgroup "aegilopsis", TRI in "triticina".

Table 3. Fatty acid profile of *Puccinia recondita* GC-subgroup "aegilopsis".

Index ^a	Feature (abbreviated)	Count ^b	Mean (%) ^c	Minimum (%) ^c	Maximum (%) ^c	Feature status ^d
21	12:0	1	0.00	0.00	0.26	
48	14:0	44	0.52	0.00	1.34	USED
65	15:0	10	0.06	0.00	0.61	
73	C16 N Alcohol	2	0.01	0.00	0.39	
78	16:1 ω9 cis	3	0.02	0.00	0.43	
79	16:1 ω7 cis	1	0.01	0.00	0.51	
82	16:0	69	23.84	21.74	26.79	USED
105	16:0 2OH	1	0.01	0.00	0.53	
117	18:2 cis 9,12/ 18:0 Ante	69	3.76	1.77	6.02	USED
122	18:0	69	4.71	2.85	7.69	USED
124	17:0 Iso 3OH	1	0.00	0.00	0.33	
132	19:0 N Alcohol	10	0.12	0.00	1.46	
138	19:1 ω9 trans	8	0.12	0.00	1.91	
150	Unknown 19.521 ^f	2	0.03	0.00	1.25	
151	18:0 3OH	19	0.16	0.00	0.85	
161	18:0 12OH	13	0.33	0.00	1.94	
162	20:0	48	1.36	0.00	2.54	USED
170	Summed feature 8 ^e	69	63.92	60.02	67.11	USED
172	Summed Feature 10 ^e	54	1.02	0.00	1.93	USED

Isolates from *Aegilops* spp. and *Triticum turgidum* var. *durum*, with species of *Anchusa* and *Echium* as aecial hosts: 54 isolates + 15 duplicates and replicates = 69 samples in the profile (for abbreviations see Table 2).

5.6% linoleic acid, which is similar to our findings. Subgroup "triticina" is characterized by having almost twice as much (7.3%) of this acid, as subgroups "recondita" and "aegilopsis" (4.6 and 3.8% respectively).

Myristic acid is found in similar quantities in subgroups "recondita" and "triticina", 1.6% and 1.4% respectively, but only 0.52% is found in subgroup "aegilopsis". The other 3 FAs included in the three profiles, stearic, 18:1 c9 dimethylacetal and eicosanoic are found in similar percentages in the three subgroups and have therefore no value as discriminant characters. The ω9c isomer of palmitoleic acid is the only FA used in one profile, "triticina" (QT = 0.35), but not in the other two (Table 5).

The most substantial difference between the findings of Tulloch and Ledingham (1960, 1962) and those presented here is their finding of rather large percentages of 9,10 epoxy octadecanoic acid (18:0 ω9,10 epoxy), which was not detected with the method used here.

This may be explained by differences in both the recovery and detection sensitivity of different methods for particular acids (Müller *et al.*, 1994; Ben-Ze'ev *et al.*, 1997); 18:0 ω9,10 epoxy is not listed in the peak naming tables of MIDI's methods, but was detected as a ~1% trace in *Puccinia* by GC-MS (Ben-Ze'ev *et al.*, 1997).

Are fatty acid profiles of taxonomic value in *Puccinia*? The FA profiles of bacterial congeneric species are qualitatively similar, but quantitatively different, distinguishing well among species and lower level taxa (O'Donnell, 1994). The similarities and differences of the FA profiles of the three *P. recondita* subgroups are consistent with those found for bacteria and should therefore be considered as having a rather similar taxonomic value.

Euclidean distance is used as a measure of relatedness among bacterial isolates. Experience indicates that FA profiles of the same isolate, analyzed twice or more, usually link within 2 Euclidean units (EU). Isolates related at the subspecific, specific and generic levels usually link within ~6, ~10 and ~25 EU respectively. This EU scale is approximate and depends upon the current state of taxonomy of the analyzed taxa (Sasser, 1990). The results obtained during this study are again consistent with the experience accumulated in studies with bacteria. Two analyses of the same isolate usually linked within 2 EU, all the isolates in two of the three subgroups are located within 5-6 EU ("recondita" within ~9.4) and the three subgroups link within ~10 EU. In Fig. 1 it appears that two of the "recondita" isolates link closer to the "aegilopsis" subgroup, however, the RUST

Table 4. Fatty acid profile of *Puccinia recondita* "GC-subgroup *recondita*".

Index ^a	Feature (abbreviated)	Count ^b	Mean (%) ^c	Minimum (%) ^c	Maximum (%) ^c	Feature status ^d
48	14:0	12	1.63	0.00	6.06	USED
65	15:0	4	0.12	0.00	0.44	
70	16:1 cis Alcohol ω7	1	0.03	0.00	0.40	
73	C16 N Alcohol	1	0.05	0.00	0.65	
78	16:1 ω9 cis	4	0.22	0.00	1.35	
82	16:0	14	19.73	17.90	23.23	USED
104	16:0 Iso 3OH	1	0.03	0.00	0.37	
109	18:1 Trans ω9 Alcohol	1	0.05	0.00	0.77	
117	18:2 cis 9,12/ 18:0 Ante	14	4.60	3.27	5.84	USED
122	18:0	14	3.63	2.91	4.84	USED
132	19:0 N Alcohol	1	0.12	0.00	1.73	
146	20:1 cis ω9 Alcohol	1	0.03	0.00	0.48	
151	18:0 3OH	4	0.14	0.00	0.62	
161	18:0 12OH	3	0.38	0.00	2.13	
162	20:0	9	1.17	0.00	2.30	USED
170	Summed Feature 8 ^(e)	14	67.14	60.45	72.18	USED
172	Summed Feature 10 ^(e)	10	0.93	0.00	1.61	USED

Isolates from *Secale montanum* and *S. cereale*, aecial hosts - *Anchusa undulata*, *Lycopsis arvensis*, *Boraginaceae*: 12 isolates + 2 duplicates = 14 samples in the profile (for abbreviations see Table 2).

library, identifies them clearly as *P.r. recondita*.

It would be premature to conclude that the value of fatty acid profiles for fungal taxonomy is as high as the well documented one for bacteria, but the results for *Puccinia* spp. are encouraging. Another new finding is the possibility to identify and to classify certain obligate parasites, without having to grow them in carefully monitored conditions. Rust teliospores can be easily collected in nature in sufficient quantity for FA analysis, as described here. Spores stored in collections for 10-20 years were found to have maintained their characteristic FA profiles. The MIDI system is relatively simple to operate for personnel who are not analytical chemists, is automated and allows processing of large numbers of samples at a time.

Tulloch and Ledingham (1962) have commented on the apparent lack of host influence on the fatty acid composition of rust fungi. Our results show that conspecific *Puccinia* isolates from different telial hosts (e.g. "aegilopsis" isolates from *Aegilops* and from *Triticum*) and from different countries have very similar FA profiles. This indicates that the FA composition is determined by the genetic make-up of the fungus, with little if any influence contributed by the plant host or by the environment.

The FA profiles of several other species of *Puccinia* are being analyzed (Anikster *et al.*, in preparation), and

some have been included in our RUST library besides the *P. recondita* subgroups. Isolates belonging to all entries (species and subspecies) of this library, tested against the library, have been classified correctly in a great majority of cases, proving the taxonomic value of *Puccinia* FA profiles and the diagnostic value of the library.

Taxonomic conclusions. Anikster *et al.* (1997) have shown that *P. recondita* isolates from cultivated wheat, wild wheat and rye belong to two groups, I and II (see Introduction), with possible exchange of genes within each group, but not between members of the two.

These two groups fit therefore the definition of separate biological species. A number of other characters have taxonomic value for the classification of isolates into one of these groups: (a) virulence to aecial hosts (*Ranunculaceae* for group I, *Boraginaceae* for II), (b) teliospore dimensions, (c) the relative amount of DNA in haploid nuclei (Eilam *et al.*, 1994; Anikster *et al.*, 1997), and (d) the morphology of the substomatal vesicles of uredospore germlings (Swertz, 1994). Specialization on cereal hosts and some other morphological and quantitative nuclear-DNA differences, indicate the existence of subgroups within each of the main groups (Anikster *et al.*, 1997), as indicated here by the FA profiles and as illustrated in the dendrogram (Fig. 1).

The present study was carried out with the same collection of isolates used by Anikster *et al.* (1997) and Eilam *et al.* (1994). The FA profiles obtained match perfectly with the two main groups and one subgroup of Anikster *et al.* (1997): group I (there) = GC-subgroup “*tritricina*” here; group II = GC-subgroups “*recondita*” + “*aegilopsis*”, where “*recondita*” consists of isolates from *Secale*, while “*aegilopsis*” includes isolates from *Aegilops* spp. and from *T. turgidum* var. *durum* associated with *Anchusa italica*.

Judging by the Euclidean units scale, as used for bacterial taxa, the “*recondita*”, “*aegilopsis*” and “*tritricina*” FA profiles seem to fit as three subspecific groups of one species (Fig. 1). However, the fact that “*tritricina*” (group I) isolates cannot exchange genetic information with isolates of “*recondita*” and “*aegilopsis*” (group II) demarcates the 2 groups as separate species. Within group II, the “*recondita*” and “*aegilopsis*” entities are crossable and should therefore be considered as conspecific subgroups. This conclusion, put in other words, means that the fungus causing the leaf rust of wheat is *Puccinia tritricina* Eriksson, 1899 (*Ann. Sci. Nat.* 8 ser. 9: 273) [syn. *P. dispersa* f.sp. *tritici* Eriks. and Henn., 1894 (*Z. Pfl.-Krankh* 4: 259); *P. recondita* f.sp. *tritici* Eriks. and Henn. in Wilson and Henderson (1966, pp. 289-290)], with aecial stage on *Thalictrum* as described by Jackson and Mains (1921) and on *Isopyrum* (Brizgalova, 1935, cited in Chester, 1946), characterized as described by Anikster *et al.* (1997) and identifiable by the fatty acid profile shown here (Table 2).

The leaf rusts of *Secale* and *Aegilops* are caused by *Puccinia recondita* Roberge ex Desmazieres 1857 (*Bull. Soc. Bot. Fr.* 4: 798). This species is a complex of subgroups, two of which are shown by the FA profiles “*aegilopsis*” and “*recondita*” (Tables 3 and 4, respectively). We regard these two subgroups as two subspecies, “*aegilopsis*” having to be redescribed, since *Puccinia aegilopsis* Maire 1914 (*Bull. Soc. Bot. Fr.* 61: 14-24) is a *nomen nudum*, without type designation and Latin description (although Latin descriptions are only required for *taxa* described since 1935):

Puccinia recondita ROBERGE ex DESMAZIERES 1857, *Bull. Soc. Bot. Fr.* 4: 798 subsp. ***aegilopsis*** ANIKSTER, EILAM, BEN-ZE'EV & LEVY, **subsp. nov.** Spermagoniis amphigenis, flavo-aurantiis. Aeciis hypophyllis, aliquando epiphyllis. Pseudoperidium hyalinum, aeciosporiis auranticis, $22.7 \pm 0.7 \times 19.3 \pm 1.2 \mu\text{m}$, membrana hyalina, dense verruculosa. Urediniis amphigenis, plerumque epiphyllis, regulariter dispersi, diametro 0.2-0.6 mm, ferrugineo-brunneis. Uredinosporis echinulatis, flavo-brunneis, magnitudine $26.1 \pm 0.2 \times 23.3 \pm 0.6 \mu\text{m}$. Teliis ovalibus, plerumque hypophyllis, atro-brunneis, 1-10 mm longis, epidermide pertinaciter tectis; paraphysibus brunneis. Teliosporiis brunneis, $47.2 \pm 2.0 \times 28.0 \pm 0.9 \mu\text{m}$ (Fig. 3).

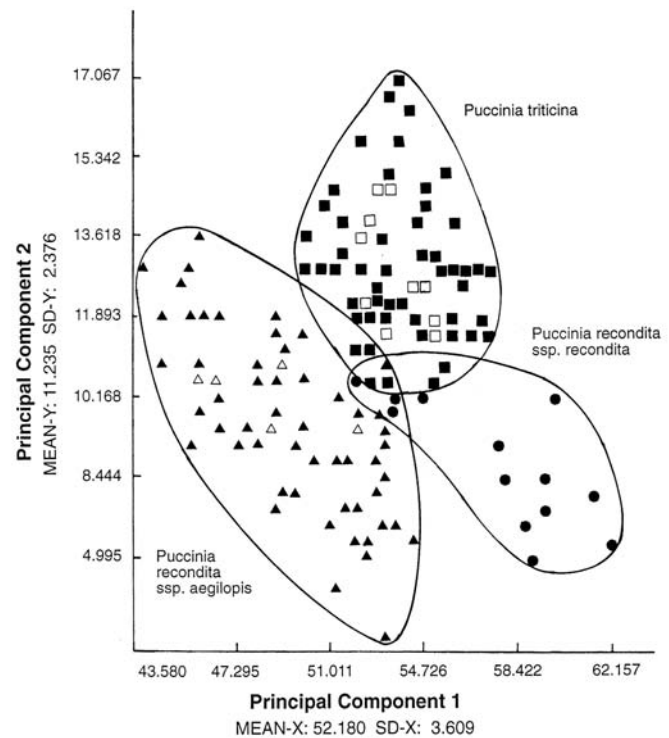


Fig. 2. Two-dimensional plot representing the multi-dimensional relations among all the isolates in library RUST.

Spermagonia amphigenous, yellow-orange. Aecia hypophyllous, sometimes epiphyllous. Pseudoperidium hyaline, aeciospores orange, $22.7 \pm 0.7 \times 19.3 \pm 1.2 \mu\text{m}$, wall hyaline, densely verrucose. Uredia amphigenous, mostly epiphyllous, uniformly scattered, 0.2-0.6 mm in diameter, rusty-brown. Uredospores echinulate, yellow-brown, $26.1 \pm 0.2 \times 23.3 \pm 0.6 \mu\text{m}$. Telia oval, mostly abaxial, dark brown, 1-10 mm long, covered by host epidermis, with brown paraphyses. Teliospores brown, $47.2 \pm 2.0 \times 28.0 \pm 0.9 \mu\text{m}$ (Fig. 3).



Fig. 3. Teliospores of *Puccinia recondita* subsp. *Aegilopsis*, bar (lower right corner) = 20 μm .

Table 5. Fatty acids distinguishing between *Puccinia triticina* and the GC-subgroups *Puccinia recondita* ssp. *recondita* and *P. r.* ssp. *aegilopsis*.

Feature (Fatty acid)	<i>P. triticina</i>		<i>P. recondita</i> ssp. <i>aegilopsis</i>		<i>P.r.</i> ssp. <i>recondita</i>	
	av.% (min. - max.)% ^a Count ^b	USED / not used ^c	av.% (min. - max.)% Count	USED / not used	av.% (min. - max.)% Count	USED / not used ^c
Myristic (14:0)	1.40 (0.00 - 3.43) 59/61	USED	0.52 (0.00 - 1.34) 44/69	USED	1.63 (0.00 - 6.06) 12/14	USED
Palmitic (16:0)	22.05 (17.41 - 26.28) 61/61	USED	23.84 (21.74 - 26.79) 69/69	USED	19.73 (17.90 - 23.23) 14/14	USED
Palmitoleic (16:1 ω9c)	0.65 (0.00 - 3.13) 33/61	USED	0.02 (0.00 - 0.43) 3/69	not used	0.22 (0.00 - 1.35) 4/14	not used
Linoleic and/or... (18:2 cis 9,12 and/ or 18:0 Anteiso)	7.27 (5.63 - 8.75) 61/61	USED	3.76 (1.77 - 6.02) 69/69	USED	4.60 (3.27 - 5.84) 14/14	USED
Summed feature 8 ^(d) = Oleic (isomers 18:1 ω9c and/or 18:1 ω8c)	60.14 (54.91 - 65.09) 61/61	USED	63.92 (60.02 - 67.11) 69/69	USED	67.14 (60.45 - 72.18) 14/14	USED
Eicosanoic (20:0)	1.76 (0.00 - 2.93) 55/61	USED	1.36 (0.00 - 2.54) 48/69	USED	1.17 (0.00 - 2.30) 9/14	USED

^a Relative quantity (in %) of a feature across the profile; ^b Ratio of isolates having a certain feature / total isolates in the profile; ^c Used or not used for the profile; ^d A peak shared by 2 or more molecules with retention times too close to be separated by the current method.

Main + alternate hosts (distribution in Israel in descending order): *Aegilops longissima* + *Anchusa aggregata*, *Ae. ovata* + *Echium glomeratum*, *Ae. Variabilis* + *A. aggregata* (+*A. strigosa*), *Ae. Searsii* + *A. strigosa*, *Ae. kotschyi* + *A. strigosa*, *Ae. sharonensis* + *A. undulata*. The main host recorded by Maire (1914) was *Aegilops ovata*, with experimental alternate host: *Lithospermum arvense*. More hosts are listed by Urban and Markova (1995).

Holotype. *Aegilops ovata*, Mount Carmel, Israel, May 2000, det. Y. Anikster; 3 teliospore preparates and 5 herbarium specimens each, deposited at FH; and TELA (isotype).

According to Art. 26 of the International Code of Botanical Nomenclature, the agent of the leaf rust of rye, which is the type of *P. recondita*, becomes an autonym: ***Puccinia recondita* Rob. ex Desm. subsp. *recondita*.**

The *P. recondita recondita* subgroup in our dendrogram (Fig. 1) and 2-D plot (Fig. 2) is considerably smaller than the other two subgroups due to the scarcity of isolates in our collection. Moreover, most of the isolates in this library profile originate in the Golan Heights, while Central Europe, the center of origin of rye leaf rust, is represented by only two isolates, which appear to be a subgroup within a group. It is conceivable that a profile made with more isolates would have shown several smaller subgroups, like in the "*triticina*" and "*aegilopsis*" profiles.

The subspecies *P.r. recondita* and *P.r. aegilopsis* de-

scribed here are characterized and identifiable as shown by Anikster *et al.* (1997) and by their FA profiles shown in Tables 3 and 4. The systematic position advocated here, with *Puccinia triticina* and *P. recondita sensu stricto* as individual species and with "*P. aegilopsis*" as a subspecies of *P. recondita*, is quite similar to that proposed by Urban and Markova (1995), where "*P. aegilopsis*" was regarded as *P. recondita* f.sp. *graminicola*. We, however, prefer the use of subspecies to that of varieties or of ***formae speciales*** (e.g.: Urban and Markova, 1995; 1997), as the latter are phytopathological definitions, without standing under the International Code. The various *taxa* listed under *Puccinia recondita* as synonyms (e.g., in Cummins, 1971) or as *formae speciales* (e.g., in Wilson and Henderson, 1966) will have to be investigated regarding their relatedness to *P. recondita* as subspecies or as separate species. Until then, they are best referred to under their original specific names.

ACKNOWLEDGMENTS

We thank Eileen Wozek of the Farlow Herbarium, Harvard University, for critical suggestions regarding the description of *Puccinia recondita* subsp. *Aegilopsis*. Paulina Goldshlag, Osnat Ben-Gal, Evgenia Elkind, Bella Kuchuk-Sigal and Faina Zlatzin, of the Plant Protection and Inspection Services, Bet Dagan, are acknowledged for their technical assistance with GC analysis.

REFERENCES

- Anikster Y., 1986. Teliospore germination in some rust fungi. *Phytopathology* **74**: 1061-1064.
- Anikster Y., Bushnell W.R., Eilam T., Manisterski J., Roelfs A.P., 1997. *Puccinia recondita* causing leaf rust on cultivated wheats, wild wheats and rye. *Canadian Journal of Botany* **75**: 2082-2096.
- Anikster Y., Eilam T., Bushnell W.R., 1996. The use of image-analysis for spore measurements in cereal leaf rust fungi. In: Kema G.H.S., Niks R.E., Luntheren D.R.A. (eds.). *Proceedings of 9th Cereal Rust and Mildews Conference, Lunteren 1996*, 128-129.
- Anonimous, 1992a. MIDI - Microbial identification system - Operating manual, Version 4., Microbial ID, Inc. (MIDI), Newark, Delaware, USA.
- Anonimous, 1992b. MIDI - Microbial identification system - Library generation system - User's manual. Microbial ID, Inc. (MIDI), Newark, Delaware, USA.
- Anonimous, 1993. MIS software update, February 1993. An introduction to the fungal database Microbial ID, Inc. (MIDI), Newark, Delaware, USA.
- Ben-Ze'ev I.S., Levy E., Goldshlag P., Eilam T., Anikster Y., 1997. Characterization of *Puccinia* species by teliospore fatty acids. *Phytoparasitica* **25**: 265-266.
- Chester K.S., 1946. The nature and prevention of the cereal rusts as exemplified in the leaf rust of wheat. In: F. Verdoorn (ed.). *Annales Cryptogamici et Phytopathologici*, vol. 4, pp. 269. Chronica Botanica, Waltham, MA, USA.
- Cummins G.B., 1971. The rust fungi of cereals, grasses and bamboos. Springer Verlag, Berlin, Germany.
- Cummins G.B., Caldwell R.M., 1956. The validity of binomials in the leaf rust fungus complex of cereals and grasses. *Phytopathology* **46**: 81-82.
- Eilam T., Bushnell W.R., Anikster Y., McLaughlin D.J., 1992. Nuclear DNA content of basidiospores of selected rust fungi as estimated from fluorescence of propidium iodide-stained nuclei. *Phytopathology* **82**: 705-712.
- Eilam T., Bushnell W.R., Anikster Y., 1994. Relative nuclear DNA content of rust fungi estimated by flow cytometry of propidium iodide-stained pycniospores. *Phytopathology* **84**: 728-735.
- Jackson H.S., Mains E.B., 1921. Aecial stage of the orange leaf rust of wheat *Puccinia triticina* Erikss. *Journal of Agricultural Research* **22**: 151-172.
- Larkin R.P., Groves C.L., 2003. Identification and characterization of isolates of *Phytophthora infestans* using fatty acid methyl ester (fame) profiles. *Plant Disease* **87**: 1233-1243.
- Madan R., Pankhurst C., Hawke B., Smith S., 2002. Use of fatty acids for identification of AM fungi and estimation of the biomass of AM spores in soil. *Soil Biology and Biochemistry* **34**: 125-128.
- Maire R., 1914. Deuxieme contribution a l'étude de la flore mycologique de la Tunisie. *Bulletin de la Société Botanique de France* **61**: 14-24.
- Mendala B., 1990. A user generated "custom" library for the MIS. MIDI Technical Note No. 103, pp. 4. Microbial ID, Inc. (MIDI), Newark, Delaware, USA.
- Müller M.M., Kantola R., Kitunen V., 1994. Combining sterol and fatty acid profiles for the characterization of fungi. *Mycological Research* **98**: 593-603.
- O'Donnell A.G., 1994. Quantitative and qualitative analysis of fatty acids in the classification and identification of microorganisms. In: Hawksworth D.L. (ed.). *The identification and characterization of pest organisms*, pp. 323-335. CAB International, Wallingford, UK.
- Sasaki G.L., Cruz L.M., Gorin P.A.J., Iacomini M., 2001. Fatty acid composition of lipids present in selected lichenized fungi: a chemotyping study. *Lipids* **36**: 167-174.
- Sasser M., 1990. Tracking a strain using the Microbial Identification System. MIDI Technical Note No. 102, pp. 4. Microbial ID, Inc. (MIDI), Newark, Delaware, USA.
- Stevens Johnk J., Jones R.K., 1993. Differentiation of populations of AG-2-2 of *Rhizoctonia solani* by analysis of cellular fatty acids. *Phytopathology* **83**: 278-283.
- Stevens Johnk J., Jones R.K., 1994. Comparison of whole-cell fatty acid compositions in intraspecific groups of *Rhizoctonia solani* AG-1. *Phytopathology* **84**: 271-275.
- Swertz C.A., 1994. Morphology of germlings of urediniospores and its value for the identification and classification of grass rust fungi. *Studies in Mycology* **36**: 1-152.
- Tulloch A.P., Ledingham G.A., 1960. The component fatty acids of oils found in spores of rusts and other fungi. *Canadian Journal of Microbiology* **6**: 425-435.
- Tulloch A.P., Ledingham G.A., 1962. The component fatty acids of oils found in spores of plant rusts and other fungi. *Canadian Journal of Microbiology* **8**: 379-387.
- Urban Z., Markova J., 1995. The rust fungi of grasses in Europe. *Puccinia recondita* Rob. ex Desm. S. str.. *Acta Universitatis Carolinae Biologica* **39**: 59-83.
- Urban Z., Markova J., 1997. The rust fungi of grasses in Europe. *Puccinia persistens* Plow., *P. perplexans* Plow., and *P. elymi* Westend. *Acta Universitatis Carolinae Biologica* **41**: 329-402.
- Wilson M., Henderson D.M., 1966. British rust fungi. Cambridge University Press, UK.