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# Isolation, Characterization, and Avenacin Sensitivity of a Diverse Collection of Cereal-Root-Colonizing Fungi

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**A total of 161 fungal isolates were obtained from the surface-sterilized roots of field-grown oat and wheat plants in order to investigate the nature of the root-colonizing fungi supported by these two cereals. Fungi were initially grouped according to their colony morphologies and then were further characterized by ribosomal DNA sequence analysis. The collection contained a wide range of ascomycetes and also some basidiomycete fungi. The fungi were subsequently assessed for their abilities to tolerate and degrade the antifungal oat root saponin, avenacin A-1. Nearly all the fungi obtained from oat roots were avenacin A-1 resistant, while both avenacin-sensitive and avenacin-resistant fungi were isolated from the roots of the non-saponin-producing cereal, wheat. The majority of the avenacin-resistant fungi were able to degrade avenacin A-1. These experiments suggest that avenacin A-1 is likely to influence the development of fungal communities within (and possibly also around) oat roots.**

Plant health and development are affected by interactions between the plant and the microbial community which develops in and around the plant root. Many different fungal species exist in soil (13), but the factors which determine which members of this community are able to colonize living plants are complex and poorly understood. The colonization of roots by fungi may be affected by a variety of factors, including the plant species (34) and developmental stage (20), climatic conditions (45), management practices (29, 32), and chemicals present in the root (9).

Oats appear to be unusual among cereals in that they produce antifungal compounds belonging to the class of plant secondary metabolites known as saponins (28). The antifungal activity of saponins is associated with their ability to form complexes with membrane sterols (16, 30, 35) and so is relatively nonspecific. The resistance of oats to the root-infecting fungus *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all disease of wheat, has been associated with the triterpenoid avenacin saponins, which are found in oat roots (24, 38). Oat roots have been shown to attract and lyse zoospores of *Pythium* species, and this effect has also been linked with avenacins (10). The major avenacin, avenacin A-1, is localized in the root epidermis and so is likely to present a protective barrier to the infection of oats by saponin-sensitive fungi (26). Consistent with this is the demonstration that the ability of an oat-attacking variant of the take-all pathogen (*G. graminis* var. *avenae*) to infect this host is dependent on the production of the saponin-detoxifying enzyme avenacinase, which hydrolyzes D-glucose molecules from avenacin A-1 (3, 7, 25, 39, 40). The detoxification of avenacin A-1 is not unique to *G. graminis* var. *avenae* and has also been described for another oat pathogen, *Fusarium avenaceum* (7). Further evidence to indicate a protective role for avenacin A-1 comes from observations that an oat species lacking avenacin A-1 is susceptible to *G. graminis* var. *tritici* (26).

Little is known about the variety of fungi found on the roots of cereal crops or about the factors which determine colonization. In order to investigate the extent to which different cereal crops support different populations of root-colonizing fungi, a collection of fungal isolates from field-grown oat and wheat plants was established. These fungi were initially grouped according to their colony morphologies. Further characterization was carried out by ribosomal DNA (rDNA) sequence analysis, since studies of root-colonizing fungi have often been hindered by problems of classification on the basis of morphological criteria (especially for the sterile, darkly pigmented fungi which commonly occur in this habitat) (18, 42). The fungi were then assessed for their abilities to tolerate and degrade the oat root saponin avenacin A-1, to determine whether there was any relationship between the ability to colonize oat roots and resistance to avenacin A-1.

## MATERIALS AND METHODS

**Isolation of fungi from cereal roots.** Samples were collected from Bottom Holbach South Field, ADAS Rosemaund Research Centre, Preston Wynne, Hereford, United Kingdom, on 15 July and 2 August 1995 in the week before harvesting. The soil was a silty clay loam (Bromyard series) with a pH of 7.1. From 1993 to 1995 the majority of the field had been planted with winter oats (cv. Gerald) except for trial plots located in the middle of the field, which were sown on 7 October 1994 either with different cultivars of winter wheat or with winter oats.

Samples were taken from locations at least 1 m apart as shown in Table 1. The roots of each plant were rinsed in sterile water, and five random 10-cm lengths were cut and surface sterilized with sequential 1-min washes with solutions containing 1% silver nitrate, 1% sodium chloride, and sterile distilled water. These root sections were chopped into 1-cm lengths, and sections from each plant were pooled. Twenty randomly selected 1-cm root sections per plant were placed on 1/10 strength potato dextrose agar (PDA) containing streptomycin (0.1 mg/ml), and twenty were placed onto a semiselective medium (SM-GGT3) routinely used for the isolation of *G. graminis* (22). Following incubation in the dark at 20°C, filamentous fungi growing from the root sections were transferred to water agar and incubated at 20°C for 7 to 14 days, before purification by two successive rounds of hyphal tip isolation. Fungi were routinely grown on PDA at 20°C in the dark. All isolates were stored at 4°C on PDA stock plates, with long-term storage on PDA slants under light paraffin oil (Sigma Chemical Co., Poole, United Kingdom).

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TABLE 1. Origins of the fungal isolates in this study

| Crop succession[a] | Sample[b] | Cultivar | No. of fungal isolates obtained | No. of groups identified[c] |
|---|---|---|---|---|
| OOO | 1 | Gerald | 23 | 9 (OOO1–9) |
| | 2 | Gerald | 12 | 8 (OOO10–17) |
| | 3 | Gerald | 15 | 11 (OOO18–28) |
| | 4 | Gerald | 19 | 10 (OOO29–38) |
| | 5 | Gerald | 11 | 9 (OOO39–47) |
| Total | | | 80 | 47 |
| OOW | 1 | Riband | 7 | 7 (OOW1–7) |
| | 2 | Lynx | 3 | 3 (OOW8–10) |
| | 3 | Riband | 13 | 2 (OOW11–12) |
| | 4 | Zentos | 9 | 2 (OOW13–14) |
| Total | | | 32 | 14 |
| OWW | 1 | Brigadier | 5 | 3 (OWW1–3) |
| | 2 | Brigadier | 8 | 3 (OWW4–6) |
| | 3 | Brigadier | 7 | 3 (OWW7–9) |
| | 4 | Brigadier | 5 | 2 (OWW10–11) |
| | 5 | Brigadier | 8 | 4 (OWW12–15) |
| | 6 | Brigadier | 7 | 1 (OWW16) |
| | 7 | Brigadier | 8 | 2 (OWW17–18) |
| Total | | | 48 | 18 |
| Overall total | | | 161 | 79 |

[a] OOO, third successive oat crop; OOW, wheat grown after two successive oat crops; OWW, second successive wheat crop following oats.

[b] A sample consists of a group of at least five plants taken from a single site for crop successions OOO and OOW; for OWW each of the seven samples represents a single plant, each from different sites within the plot. Isolations were carried out for individual plants, and the fungi from each plant were kept separate.

[c] Each group consists of those fungi which were isolated on the same medium from the same plant and which were indistinguishable on the basis of their appearance when grown on agar plates. The groups are designated by the cereal succession abbreviation followed by the group number.

**rDNA sequence analysis.** Mycelia from colonies cultured on PDA for 7 days were prepared for PCR by a modification of the direct tissue PCR method (23) as described by Bryan et al. (4). A region of the rRNA gene cluster including the 3′ end of the 18S rRNA gene, the 5.8S rRNA gene, and the internal transcribed spacers (ITS1 and ITS2) was amplified with primers psrDNA2p (5′-GTCCAC ACACCGCCCGT-3′) (41) and pITS4 (5′-TTCTTCGCTTATTGATATGC-3′) (43). Amplification was carried out under the conditions described by Bryan et al. (4). PCR products were purified from the reaction mixture with the QIAquick PCR purification kit (Qiagen Ltd., Crawley, West Sussex, England). For reactions which failed to yield DNA of sufficient quality for sequencing, the PCR products were purified after separation on a 2% agarose gel with the Concert gel extraction system (Life Technologies, Paisley, Scotland).

PCR products were sequenced in both directions by using the ABI PRISM Big Dye terminator cycle sequencing kit (Perkin-Elmer, Warrington, Cheshire, England) primed with either of the two primers used to originally amplify the fragment. Sequencing reactions were run on an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

The 158 bases coding for the 5.8S rRNA gene and the ITS1 and ITS2 sequences were used individually to search the EMBL and GenBank databases with BLAST (1). The 5.8S rRNA gene sequences were compared to sequences present in the databases after sequence alignment by using the software contained in PHYLIP (Phylogeny Inference Package), version 3.5 (15).

**Preparation of avenacin A-1.** Avenacin A-1 was extracted from oat roots by the method of Crombie et al. (6) with the addition of a final purification step with a C18 Sep-Pak cartridge (Waters Co., Milford, Mass.). The C18 Sep-Pak cartridge was equilibrated with water and, after addition of the sample, washed with 5 volumes of water and then eluted successively with 5 volumes of 50%, 75%, and 100% methanol. The eluates were analyzed by thin-layer chromatography (TLC) as described by Osbourn et al. (25), and the fractions containing avenacin A-1 (which was eluted with 75% methanol) were pooled.

**Effects of avenacin A-1 on fungal growth.** The effects of avenacin A-1 on fungal growth were assessed either by incorporation of avenacin A-1 into PDA and measurement of inhibition of colony growth or by a filter paper disc assay (Fig. 1). Each assay was carried out at least twice.

**Avenacin A-1 degradation by fungi.** Five-millimeter agar blocks were cut from the margins of actively growing colonies and used to inoculate 5 ml of either Jermyn's medium (21) or Quaker oat medium (0.03 g of Quaker oats [Quaker

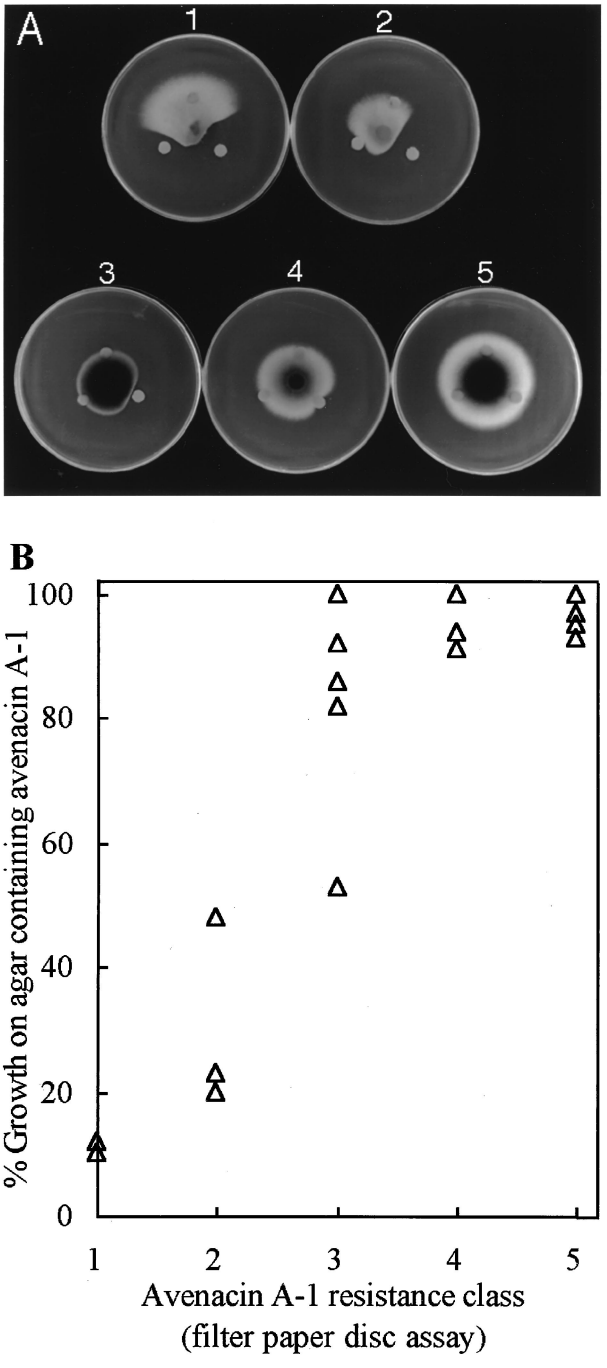


FIG. 1. (A) Assessment of the effects of avenacin A-1 on fungal growth by filter paper disc assay. Three filter paper discs containing 0, 2, and 10 μg of avenacin A-1 (counterclockwise from the top) were placed equidistantly from a mycelial plug of inoculum on PDA. Colonies were scored on a scale of 1 to 5 as follows: 1, clear inhibition of fungal growth by both the 2-μg disc and the 10-μg disc; 2, only partial inhibition around the 2-μg disc but clear inhibition around the 10-μg disc; 3, no inhibition around the 2-μg disc but clear inhibition around the 10-μg disc; 4, partial inhibition only by the 10-μg disc; 5, no inhibition. Five different fungal isolates, one representing each of these categories, are shown. (B) Comparison of the two different methods for assessing the effect of avenacin A-1 on fungal growth. Seventeen fungal isolates were tested for sensitivity or resistance. Mean values for growth on agar with and without avenacin A-1 were determined from measurements of diameters of four colonies, and the means were used to calculate the percent growth in the presence of the saponin. The standard error for these mean values was never greater than 10% of the mean.

Oats Ltd., Southall, United Kingdom]/ml of water) in 30-ml glass screw-top bottles. Fungi were grown in a static culture with the cap of the bottle loosely screwed down at 20°C for 8 days, by which time all of the fungi had produced mycelial mats. The mycelial mats were washed in sterile distilled water and incubated at 20°C in 5 ml of sterile distilled water containing 50 μg of avenacin A-1 per ml. After 6, 12, 24, and 48 h 100-μl aliquots were withdrawn, evaporated to dryness, and redissolved in methanol, and avenacin A-1 and any breakdown products were analyzed by TLC. Each of the 79 fungal isolates (representing each of the 79 groups) was tested twice.

For further investigation selected isolates were grown in Jermyn's medium with three flasks per isolate, and protein preparations were prepared from the culture filtrates as described previously (25). For the preparation of protein from mycelia, the mycelia were washed three times with ice-cold water, frozen in liquid nitrogen, and ground to a fine powder. The powder was then extracted sequentially with 2 volumes of solutions containing 50 mM Tris-HCl (pH 8.0) and 50 mM Tris-HCl–500 mM NaCl (pH 8.0) followed by 50 mM Tris-HCl–500 mM NaCl–0.25% Nonidet P-40 (Sigma), pH 8.0, with centrifugation (10,000 × *g* for 10 min at 4°C) between each extraction. Protein concentrations were measured by using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany), with bovine serum albumin as a standard. Aliquots (50 μg) of protein preparations were assayed for the ability to degrade avenacin A-1 for 16 h as described previously (25). Control reactions with boiled protein preparations were also carried out.

**Pathogenicity tests.** Fungi were tested for the ability to infect oat (cv. Image) and wheat (cv. Norman) seedlings by using an adaptation of the tube assay method of Wilkinson et al. (44) as described by Osbourn et al. (25). Seedlings were assessed individually for discoloration of the roots and for wilting and necrosis of the leaves.

## RESULTS

**Fungal isolation and characterization.** A total of 161 fungal isolates was obtained from the roots of oat and wheat plants (Table 1). The assumption was made that those cultures which were isolated on the same medium from the same plant and which were indistinguishable on the basis of colony morphology and pigmentation represented independent isolations of the same fungus, and these were classed in the same group. Fungi originating from different plants were regarded as belonging to separate groups, even if they appeared to be morphologically identical to isolates from other plants.

Sixteen different 5.8S rDNA sequences were obtained from isolates representing 76 of the 79 fungal groups shown in Table 1 (Table 2). Isolates belonging to the remaining three groups (OOO10, OOO25, and OWW18) could not be recovered from storage, and so they were not included in this analysis. Of the 16 5.8S rDNA sequences, 12 (sequences 1 to 12) grouped with ascomycete sequences with a bootstrap value of 78% after phylogenetic analysis, while the remaining four (sequences 13 to 16) were apparently more similar to basidiomycete sequences (Fig. 2). The number of different fungal groups within each 5.8S rDNA sequence type (Table 2) reflects the morphological variation within a 5.8S rDNA type and/or the number of different plants from which fungi of this 5.8S rDNA type were isolated.

The majority of the fungal isolates from the third successive oat crop group (OOO) had 5.8S rDNA sequence 1 or 12 (58 and 26%, respectively; Table 2). Fungi with these sequences were isolated from all of the OOO samples except sample 2, which did not yield fungi with 5.8S rDNA sequence 12 (Tables 1 and 2). Fungi with 5.8S rDNA sequence 12 were also isolated from two of four sample sites from the OOW crop succession (wheat grown after two successive oat crops). However, in general the fungi from the OOW crop succession showed more site-related variation than those from the OOO succession, with those from sample sites 3 and 4 consisting solely of those with 5.8S rDNA sequences 11 or 7, respectively (Tables 1 and 2). These 5.8S rDNA sequences were not obtained from isolates from the other two OOW sites. All isolates from the seven wheat plants (cv. Brigadier) sampled from different sites within the OWW crop succession (second successive wheat crop following oats) were of 5.8S rDNA sequence 7 (Tables 1 and 2). Fungi with 5.8S rDNA sequence 1, which were common among OOO isolates, were not isolated from the OOW or OWW crop successions.

The 5.8S rDNA sequence comparisons shown in Fig. 2 were generally supported by analysis of the ITS1 and ITS2 sequences. Isolates with 5.8S rDNA sequences 1, 7, 12, and 16 could be further subdivided on the basis of their ITS sequences (Table 2). Most isolates had matches of >75% identity with ITS sequences present in the databases (Table 2), and some showed greater than 95% identity with known cereal pathogens. For example, for isolates OOW2 and OOW4 (5.8S rDNA sequence 5), the ITS1 and ITS2 sequences were 100% identical to those reported for *Fusarium cerealis*; ITS1 and ITS2 sequences for isolates OOW3 and OOO19 (5.8S rDNA sequence 9) were 95 and 99% identical, respectively, to *Microdochium nivale* sequences; and all 17 OWW isolates which were sequenced had ITS sequences which were almost completely identical to those of the wheat-infecting variety of the take-all fungus, *G. graminis* var. *tritici* (Table 2). Although isolates with 5.8S rDNA sequences 10, 13, and 14 clearly grouped with the taxonomic groups shown in Fig. 2, close ITS matches (>75% identity) were not found for these isolates. No close matches to either the 5.8S rDNA or ITS sequences were found for isolates with 5.8S rDNA sequences 6, 12, 15, and 16 (Fig. 2 and Table 2).

**Effects of avenacin A-1 on fungal growth.** A collection of 17 fungal isolates known to vary in avenacin sensitivity was assessed by measuring the effects of the saponin on the growth of fungal colonies when avenacin A-1 was incorporated into the growth medium and also by using a filter paper disc assay (Fig. 1A). After growth on PDA with or without 10 μg of avenacin A-1 per ml the isolates showed levels of growth inhibition ranging from approximately 10 to 100%, as expected. According to the results of the filter paper disc assay these fungi were also scored on a scale of increasing resistance to avenacin A-1 from 1 to 5. When isolates were retested with the disc assay, the results did not differ by more than 1 point on the scale. In general, there was a good correlation between the results of the two assays (sample linear correlation coefficient, 0.83) (Fig. 1B).

The filter paper disc assay was selected for further studies with all 161 isolates. The majority (74 of 78) isolates from the OOO succession were highly resistant to avenacin A-1, with scores of 4 or 5 in this assay, while 20 of 32 OOW isolates were avenacin A-1 resistant (Table 2). All of the isolates from the OWW collection were sensitive to avenacin A-1, with scores of 1 or 2.

**Degradation of avenacin A-1.** The 61 isolates representing each of the groups within the OOO and OOW fungal collections were tested for the ability to degrade avenacin A-1 after growth of mycelial mats in Jermyn's or Quaker oat medium. Forty-four of the 47 OOO isolates were able to degrade avenacin A-1, apparently in a manner similar to that seen for the avenacinase enzyme of *G. graminis* var. *avenae* (data not shown). The exceptions were OOO12 and OOO33, which both gave scores of 2 in the disc assay, and OOO11, which scored 5 in the disc assay. Six of the 14 OOW isolates were also able to degrade avenacin A-1 in these assays. These were the isolates representing groups OOW1, -6, -9, -10, and -12 (which all scored 5 in the disc assay) and OOW2 (which scored 3 in the disc assay). Those which did not degrade avenacin A-1 were the isolates representing groups OOW3, -13, and -14 (which scored 1 in the disc assay), OOW7 (which scored 2), OOW4 and -8 (which scored 3), and OOW5 and -11 (which scored 4 and 5, respectively).

TABLE 2. rDNA typing, avenacin resistance, and pathogenicity of isolates representing fungal groups

| 5.8S rDNA type[a] | ITS subtype | Representative isolate | No. of isolates in group | Avenacin resistance score[b] | Pathogenicity[c] to: | | No. of isolates from crop successions[d] | | | Closest ITS sequence match[e] | % Nucleotide identity | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Oats | Wheat | OOO | OOW | OWW | | ITS1 | ITS2 |
| 1 | A | OOO1 | 3 | 5 | + | + | 29 (27) | 0 | 0 | *Phialophora gregata* | 84 | 95 |
| | | OOO2 | 4 | 4 | + | + | | | | | | |
| | | OOO3 | 1 | 5 | + | + | | | | | | |
| | | OOO13 | 1 | 4 | + | + | | | | | | |
| | | OOO14 | 1 | 4 | + | + | | | | | | |
| | | OOO16 | 4 | 5 | + | + | | | | | | |
| | | OOO29 | 4 | 5 | + | ++ | | | | | | |
| | | OOO33 | 1 | 2 | + | + | | | | | | |
| | | OOO35 | 3 | 4 | + | + | | | | | | |
| | | OOO37 | 1 | 2 | + | + | | | | | | |
| | | OOO39 | 1 | 4 | + | + | | | | | | |
| | | OOO40 | 1 | 5 | + | + | | | | | | |
| | | OOO41 | 1 | 4 | + | + | | | | | | |
| | | OOO42 | 2 | 4 | + | ++ | | | | | | |
| | | OOO43 | 1 | 5 | + | ++ | | | | | | |
| | B | OOO4 | 3 | 5 | + | + | 5 (5) | 0 | 0 | *Phialophora gregata* | 84 | 93 |
| | | OOO27 | 1 | 3 | + | + | | | | | | |
| | | OOO38 | 1 | 4 | + | ++ | | | | | | |
| | C | OOO6 | 1 | 4 | + | + | 10 (10) | 0 | 0 | *Phialophora gregata* | 85 | 95 |
| | | OOO9 | 2 | 4 | + | + | | | | | | |
| | | OOO22 | 1 | 3 | + | + | | | | | | |
| | | OOO24 | 3 | 5 | − | + | | | | | | |
| | | OOO30 | 3 | 4 | + | + | | | | | | |
| | D | OOO15 | 1 | 4 | + | + | 1 (1) | 0 | 0 | *Phialophora gregata* | 86 | 95 |
| | E | OOO36 | 1 | 4 | + | + | 1 (1) | 0 | 0 | *Phialophora gregata* | 85 | 95 |
| 2 | | OOW7 | 1 | 2 | + | ++ | 0 | 1 (0) | 0 | *Phomopsis* sp. | 92 | 94 |
| 3 | | OOO44 | 1 | 5 | + | + | 1 (1) | 0 | 0 | *Penicillium echinulatum* | 98 | 99 |
| 4 | | OOW5 | 1 | 5 | + | + | 0 | 1 (1) | 0 | *Corynascus sexualis* | 87 | 93 |
| 5 | | OOW2 | 1 | 3 | ++ | +++ | 0 | 2 (2) | 0 | *Fusarium cerealis* | 100 | 100 |
| | | OOW4 | 1 | 3 | ++ | +++ | | | | | | |
| 6 | | OOO28 | 1 | 4 | + | ++ | 1 (1) | 0 | 0 | None | | |
| 7 | A | OOW13 | 5 | 1 | + | ++ | 0 | 5 (0) | 0 | *Gaeumannomyces graminis* var. *tritici* | 100 | 100 |
| | B | OOW14 | 4 | 1 | + | ++ | 0 | 4 (4) | 0 | *Gaeumannomyces graminis* var. *tritici* | 100 | 100 |
| | C | OWW1 | 3 | 1 | + | ++ | 0 | 0 | 31 (0) | *Gaeumannomyces graminis* var. *tritici* | 99 | 100 |
| | | OWW2 | 1 | 1 | − | +++ | | | | | | |
| | | OWW3 | 1 | 1 | − | ++ | | | | | | |
| | | OWW4 | 2 | 1 | + | ++ | | | | | | |
| | | OWW6 | 5 | 1 | + | ++ | | | | | | |
| | | OWW7 | 5 | 1 | + | +++ | | | | | | |
| | | OWW8 | 1 | 1 | − | ++ | | | | | | |
| | | OWW9 | 1 | 1 | + | ++ | | | | | | |
| | | OWW10 | 4 | 2 | − | +++ | | | | | | |
| | | OWW11 | 1 | 1 | − | +++ | | | | | | |
| | | OWW12 | 5 | 2 | + | ++ | | | | | | |
| | | OWW14 | 1 | 1 | − | +++ | | | | | | |
| | | OWW15 | 1 | 1 | + | ++ | | | | | | |
| | D | OWW5 | 1 | 1 | + | +++ | 0 | 0 | 16 (0) | *Gaeumannomyces graminis* var. *tritici* | 99 | 100 |
| | | OWW13 | 1 | 1 | + | ++ | | | | | | |
| | | OWW16 | 7 | 1 | + | +++ | | | | | | |
| | | OWW17 | 7 | 1 | − | +++ | | | | | | |
| 8 | | OOO17 | 1 | 5 | + | + | 1 (1) | 0 | 0 | *Plectosphaerella cucumerina* | 99 | 96 |
| 9 | | OOO19 | 1 | 5 | ++ | +++ | 1 (1) | 1 (0) | 0 | *Microdochium nivale* | 95 | 99 |
| | | OOW3 | 1 | 1 | − | ++ | | | | | | |
| 10 | | OOW8 | 1 | 3 | + | ++ | 0 | 1 (1) | 0 | None | | |
| 11 | | OOW11 | 5 | 5 | + | ++ | 0 | 13 (13) | 0 | *Ophiosphaerella herpotricha* | 83 | 88 |
| | | OOW12 | 8 | 5 | + | ++ | | | | | | |
| 12 | A | OOO5 | 7 | 5 | + | ++ | 19 (19) | 4 (4) | 0 | None | | |
| | | OOO7 | 1 | 4 | + | ++ | | | | | | |
| | | OOO8 | 1 | 5 | + | ++ | | | | | | |
| | | OOO18 | 1 | 5 | ++ | ++ | | | | | | |

*Continued on following page*

TABLE 2—*Continued*

| 5.8S rDNA type[a] | ITS subtype | Representative isolate | No. of isolates in group | Avenacin resistance score[b] | Pathogenicity[c] to: | | No. of isolates from crop successions[d] | | | Closest ITS sequence match[e] | % Nucleotide identity | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Oats | Wheat | OOO | OOW | OWW | | ITS1 | ITS2 |
| | | OOO20 | 1 | 5 | + | ++ | | | | | | |
| | | OOO21 | 2 | 5 | + | ++ | | | | | | |
| | | OOO31 | 2 | 5 | + | + | | | | | | |
| | | OOO32 | 2 | 4 | + | ++ | | | | | | |
| | | OOO34 | 1 | 5 | + | ++ | | | | | | |
| | | OOO46 | 1 | 4 | + | ++ | | | | | | |
| | | OOW1 | 1 | 5 | + | ++ | | | | | | |
| | | OOW6 | 1 | 5 | + | ++ | | | | | | |
| | | OOW9 | 1 | 5 | + | ++ | | | | | | |
| | | OOW10 | 1 | 5 | + | ++ | | | | | | |
| | B | OOO23 | 2 | 5 | + | ++ | 2 (2) | 0 | 0 | | | |
| 13 | | OOO12 | 2 | 2 | + | + | 2 (0) | 0 | 0 | None | | |
| 14 | | OOO26 | 1 | 4 | + | ++ | 1 (1) | 0 | 0 | None | | |
| 15 | | OOO11 | 1 | 5 | + | + | 1 (1) | 0 | 0 | None | | |
| 16 | A | OOO45 | 2 | 4 | + | + | 2 (2) | 0 | 0 | None | | |
| | B | OOO47 | 1 | 4 | − | ++ | 1 (1) | 0 | 0 | None | | |
| Total | | | 157 (94) | | | | 78 (74) | 32 (20) | 47 (0) | | | |

[a] Isolates within each 5.8S rDNA type had identical 5.8S rDNA sequences. For some of the 5.8S rDNA groups isolates could be subdivided into different ITS subtypes on the basis of differences in their ITS sequences. EMBL database accession numbers for the sequences are as follows: 1A, AJ246140; 1B, AJ246141; 1C, AJ246142; 1D, AJ246143; 1E, AJ246144; 2, AJ246145; 3, AJ246146; 4, AJ246147; 5, AJ246148; 6, AJ246149; 7A, AJ246150; 7B, AJ246151; 7C, AJ246152; 7D, AJ246153; 8, AJ246154; 9, AJ246155; 10, AJ246156; 11, AJ246157; 12A, AJ246158; 12B, AJ246159; 13, AJ246160; 14, AJ246161; 15, AJ246162; 16A, AJ246163; 16B, AJ246164.

[b] Avenacin A-1 resistance was assessed with the filter paper disc assay. All 161 isolates were tested at least twice. Isolates within groups nearly always gave the same score in this disc assay. When differences were seen these were never by more than 1 point on the scale, and isolates belonging to one group never spanned more than 2 points on the scale; in these cases the group was assigned the resistance rating of the majority.

[c] Ten seedlings per isolate were tested, and the experiment was repeated. Results are as follows: −, no discernible symptoms; +, minor discoloration of the roots; ++, extensive root discoloration and/or lesion formation; +++, extensive lesion formation and necrosis or wilting of leaves.

[d] Numbers in parentheses are the numbers of isolates which scored 3 or higher in the filter paper disc avenacin sensitivity assay.

[e] Database accession numbers are shown in Fig. 2.

A more detailed investigation of avenacin degradation was carried out for selected avenacin-resistant and avenacin-sensitive isolates (Fig. 3). Fluorescent spots with the same mobility as the mono- and bis-deglycosylated forms of avenacin A-1 were seen for culture filtrates for eight of nine of the avenacin-resistant isolates, including OOW5, which was negative in the mycelial mat experiment (Fig. 3). Isolate OOO45 produced a spot with an $R_f$ value identical to that of the aglycone of avenacin A-1. These degradation products were observed only when avenacin A-1 was included in the assay and were not present in assays involving boiled protein preparations. Isolates OOW14 and OOO11 (avenacin A-1 sensitive and resistant, respectively), which did not degrade avenacin A-1 in the mycelial mat experiments, also failed to show avenacinase activity in these experiments. None of the protein preparations derived from mycelia were able to degrade avenacin A-1 as assessed by TLC analysis.

**Pathogenicity to oats and wheat.** Most fungi either produced minor discoloration or failed to produce discernible symptoms on the roots of oats (Table 2 and Table 3). Only four isolates (OOW2, OOW4, OOO18, and OOW19) produced extensive discoloration and/or lesions on this host. In total, around 40% of the isolates were also nonpathogenic or weakly pathogenic to wheat, while the majority produced extensive root damage, with some isolates also causing wilting and necrosis of the leaves (Table 2 and Table 3). A greater proportion of the representative fungi from the OOW collection was moderately or highly pathogenic to wheat than isolates from the OOO succession (92.9%, compared with 39.1% for OOO isolates) (Table 3). All isolates from the OWW collection were moderately or highly pathogenic to wheat, consistent with their identification as *G. graminis* var. *tritici* with little or no pathogenicity to oats (Table 2 and Table 3).

## DISCUSSION

Molecular analysis of fungal rDNA at the sequence level provides a powerful technique for assessing fungal diversity and avoids the problems associated with the identification of root-colonizing fungi on the basis of morphological criteria (8, 31). The collection of fungi described here includes a wide variety of ascomycete isolates and also some fungi which can be provisionally classed as basidiomycetes (Fig. 2). No oomycete or zygomycete fungi were isolated. Of the 5.8S rDNA sequences which we obtained, all except 5.8S rDNA sequences 6, 12, 15, and 16 were identical to sequences from the EMBL or GenBank database, from genera which have previously been isolated from soil or root material (14). Fewer of the ITS sequences gave close matches, with isolate groups with 5.8S rDNA sequences 5, 6, and 12 to 16 all failing to show relatedness to known sequences with 75% or higher identity (Table 2). The number of novel ITS sequences obtained in this study confirms that DNA sequence databases do not yet fully reflect the genetic diversity of natural habitats (31). This molecular analysis has relied on rDNA sequences to enable comparisons with other known fungal sequences for the purpose of identification. Clearly, sequence identity within the rDNA region cannot be regarded as proof of overall genetic uniformity, although rDNA sequence differences do indicate that the isolates are not the same.

For all five sampling sites for oats, the fungi which were recovered were dominated by isolates which had 5.8S rDNA sequence 1, which was identical to that of the pathogen of soybeans and mung beans, *Phialophora gregata* (5) (Fig. 2). The ITS1 and ITS2 sequences of these fungi were generally between 85 and 95% identical to those of *P. gregata*. The ITS2 sequences also showed 97% identity to those of a group of

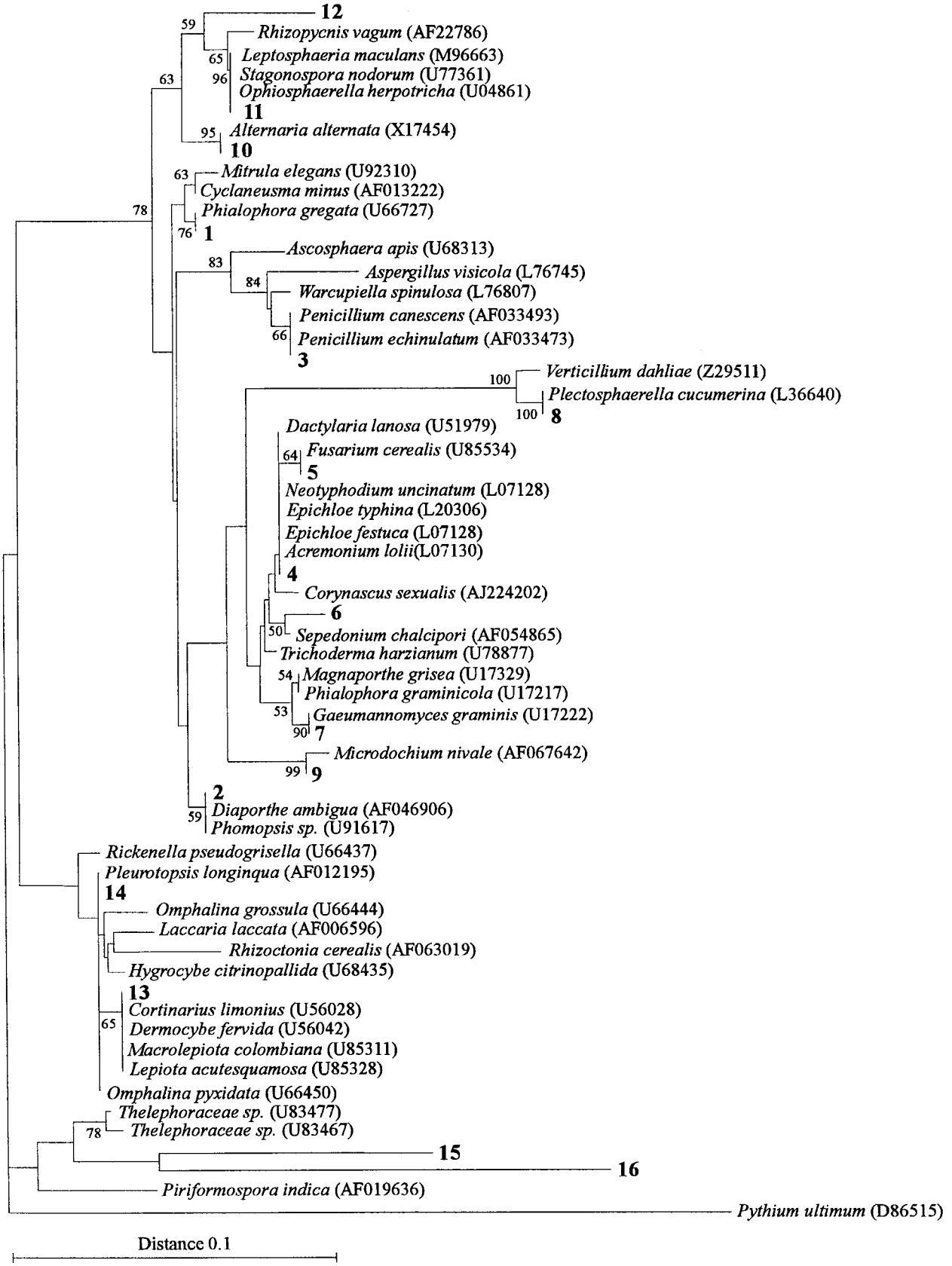


FIG. 2. Unrooted phylogenetic tree showing the relationship between the 5.8S rDNA sequences obtained in this study (numbered in bold) and 5.8S rDNA sequences obtained from the EMBL and GenBank databases. Accession numbers of database sequences are given in parentheses. After alignment, the sequences were analyzed with programs contained in PHYLIP (15). A distance matrix was produced with the DNADIST program, run with the Jukes and Cantor option. The distance matrix was converted to an unrooted tree by using the NEIGHBOR neighbor-joining program, and the unrooted tree was plotted with DRAWGRAM. A bootstrap analysis (1,000 replicates) was carried out with the SEQBOOT program, and a consensus tree was produced with CONSENSE. Bootstrap values of 50% or more are indicated.

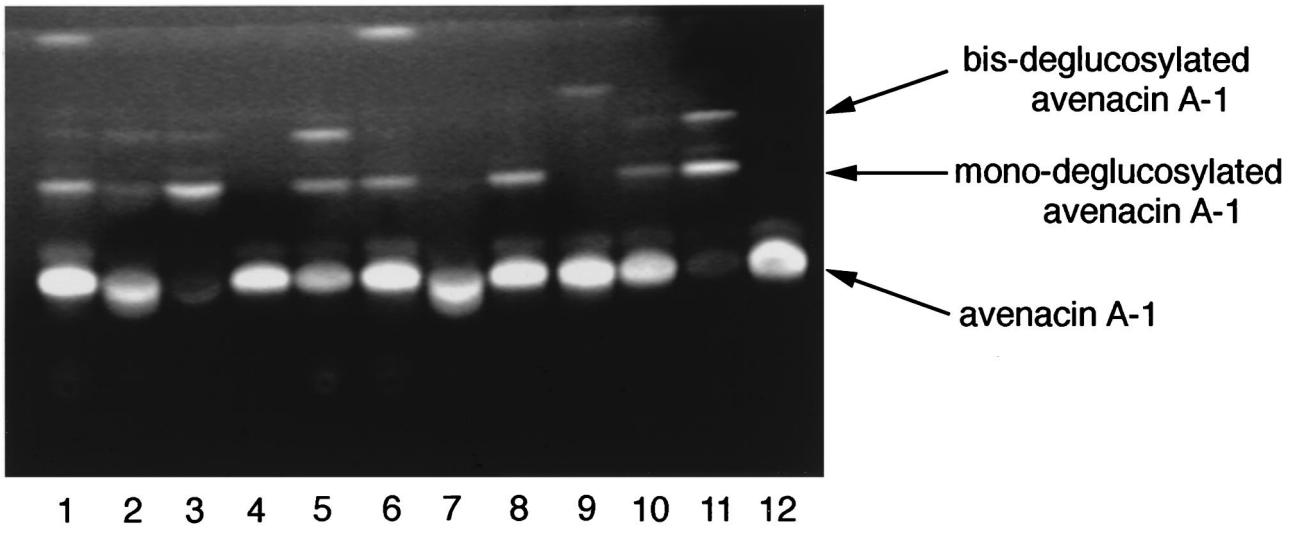


FIG. 3. Degradation of avenacin A-1 by extracellular proteins from fungal cultures. Protein preparations were incubated with avenacin A-1 for 14 h, and avenacin A-1 and any degradation products were separated by TLC and visualized under UV light. The positions of avenacin A-1 and of the mono- and bis-deglucosylated products generated by *G. graminis* var. *avenae* are indicated. Lane 1, OOW1; lane 2, OOW2; lane 3, OOW5; lane 4, OOW14; lane 5, OOO1; lane 6, OOO5; lane 7, OOO11; lane 8, OOO29; lane 9, OOO45; lane 10, OOO46; lane 11, *G. graminis* var. *avenae* isolate A3; lane 12, avenacin A-1. The spots at the solvent front in lanes 1 and 6 had a yellow fluorescence and were also present in assays carried out with boiled protein preparations; thus, they are not breakdown products of avenacin A-1. The spot which has migrated in front of the mono- and bis-deglucosylated products in lane 9 is likely the aglycone of avenacin A-1.

p-type endophytes from grass species for which 5.8S rDNA or ITS1 sequences are not yet available (2), and the ITS1 sequences showed 90 to 93% identity to those published for *Pseudoscercosporella herpotrichoides* (27) but which are not represented in either of the databases searched. Attempts to induce isolates from the groups with 5.8S rDNA sequence 1 to sporulate have so far been unsuccessful, and further work is under way to confirm the relationships between these fungi. Fungi with 5.8S rDNA sequence 1 were not recovered from the OOW or OWW samples, despite the ability of some fungi with this 5.8S rDNA sequence type to cause mild or moderate symptoms on wheat in the laboratory (Table 2). This suggests that this group of fungi are more successful colonizers of oat roots and that when wheat is grown they may be outcompeted by other fungi. Fungi with 5.8S rDNA sequence 12 were also commonly isolated from oats and were obtained from roots from four of five of the oat sample sites (Tables 1 and 2). Fungi with the same 5.8S rDNA sequence were also recovered from two of four of the OOW sample sites. The ITS1 and ITS2 sequences of these fungi did not have close sequence matches in the database (defined as >75% identity). A sporulating culture of OOO7, one of the isolates with this 5.8S rDNA sequence, has been identified according to classical morphological criteria as *Periconia macrospinosa*, a fungus which is often found in arable soils (14). *P. macrospinosa* is not generally regarded as a pathogen of gramineous plants, but the isolates in these experiments were clearly able to cause disease symptoms on wheat (Table 2).

The fungi recovered from wheat roots from the four OOW sampling sites were varied. The comparison of the variation between sites and also of the effects of cultivating wheat after oats was difficult because of the small numbers of isolates obtained from each site and because three different wheat cultivars were sampled (Table 1). The variation between sampling sites did not appear to be cultivar specific, since samples 1 and 3 yielded different fungi yet were both from the wheat cultivar Riband (Tables 1 and 2). Fungi isolated from the OOW crop succession included a number of isolates which were closely related to known cereal pathogens such as *F. cerealis*, *M. nivale*, and *G. graminis* var. *tritici* on the basis of rDNA sequence analysis (Fig. 2 and Table 2) and which were clearly pathogenic to wheat (Table 2). In contrast, only a single isolate from the OOO succession (OOO19 with 5.8S rDNA sequence 9) had rDNA sequence relatedness to cereal patho-

TABLE 3. Pathogenicities of representatives of the fungal groups isolated from the three crop successions

| Crop succession[a] | % of isolates with disease symptoms[b] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Wheat | | | | Oats | | | |
| | − | + | ++ | +++ | − | + | ++ | +++ |
| OOO | 0 | 60.9 | 37.0 | 2.1 | 4.3 | 91.4 | 4.3 | 0 |
| OOW | 0 | 7.1 | 78.6 | 14.3 | 7.1 | 78.6 | 14.3 | 0 |
| OWW[c] | 0 | 0 | 52.9 | 47.1 | 41.2 | 58.8 | 0 | 0 |
| Total | 0 | 37.7 | 48.1 | 14.2 | 13.0 | 81.8 | 5.2 | 0 |

[a] OOO, third successive oat crop; OOW, wheat grown after two successive oat crops; OWW, second successive wheat crop following oats.
[b] Results are as follows: −, no discernible symptoms; +, minor discoloration of the roots; ++, extensive root discoloration and/or lesion formation; +++, extensive lesion formation and necrosis or wilting of leaves (Table 2).
[c] These fungi were all identified as *G. graminis* var. *tritici* on the basis of morphological characteristics and rDNA sequence.

gens, in this case to *M. nivale* (Fig. 2 and Table 2). All of the isolates recovered from the OWW rotation were clearly related to the take-all pathogen, *G. graminis* var. *tritici* (Fig. 2 and Table 2). The colony morphology, avenacin sensitivity, and ability to cause substantial disease on wheat but not on oats are consistent with the identification of these isolates as *G. graminis* var. *tritici*. It has previously been reported that *G. graminis* var. *tritici* may be isolated from wheat grown after oats but that it is more common and causes more severe disease on wheat grown after wheat (33, 36). The oat-attacking variety of *G. graminis*, *G. graminis* var. *avenae* (which is pathogenic to both wheat and oats), was not recovered from any of the three crop successions.

Since the fungi isolated in these experiments were obtained from surface-sterilized roots, they are likely to be able to grow within the root tissue of their host of origin, although some may be saprophytes which have either escaped the surface sterilization procedure or which have colonized dead or dying root material. Consequently those fungi which originated from oat roots may be expected to be resistant to avenacin A-1, and this is indeed the case (Table 2). The proportion of avenacin A-1-resistant fungi isolated from the first wheat after oats was lower, and it is clear that many of the fungi isolated from this crop succession were not represented in the OOO isolate collection. In general, fungi which were pathogenic under the assay conditions employed here caused greater disease symptoms on wheat than on oats (Tables 2 and 3). Those fungi which caused extensive discoloration or lesions on oat roots were all resistant to avenacin A-1, while avenacin-sensitive fungi which were able to produce substantial disease symptoms on wheat roots failed to produce similar symptoms on oats. Of the two isolates with 5.8S rDNA sequence 9, which were similar to *M. nivale*, OOO19 was highly resistant to avenacin A-1 and showed substantial pathogenicity to oats, while OOW3 was sensitive to avenacin A-1 and did not cause disease on oats, despite being a successful pathogen of wheat (Table 2). OOO19 was able to degrade avenacin A-1 in these experiments, while OOW3 was apparently unable to do so. Collectively these observations suggest that resistance to avenacin A-1 is required for pathogenicity to oats, as has been shown to be the case for *G. graminis* var. *avenae* (3). Clearly, avenacin resistance is not the sole determinant of pathogenicity to oats, since a number of avenacin-resistant fungi were only weakly pathogenic to oats despite being highly pathogenic to wheat. There is evidence for other plant-fungus interactions that tolerance of plant antibiotics may be a prerequisite for infection. Successful pathogens of cyanogenic plants are all able to tolerate hydrocyanic acid (17), while the ability of tomato-infecting isolates of *Fusarium oxysporum* to infect tomato roots has been associated with resistance to the steroidal glycoalkaloid α-tomatine (11, 12, 37).

While there are likely to be a number of different mechanisms by which oat-infecting fungi may tolerate avenacin A-1, many of the avenacin A-1-resistant fungi in these experiments were able to degrade the saponin, apparently by the removal of sugars in a manner similar to that seen with the avenacinase of *G. graminis* var. *avenae*. It remains to be seen whether the avenacinase activities of these other fungi are encoded by genes which are related to the avenacinase gene (*AVN1*) of *G. graminis* var. *avenae* (3). Only four of the 52 avenacin A-1-resistant isolates which were included in the assays for avenacinase activity failed to degrade avenacin A-1 under the conditions tested. These isolates may have some other mechanism of avenacin A-1 resistance, for instance, one that is mediated by membrane sterol content (19), or alternatively they may have an avenacinase activity which is not expressed under the conditions used in these experiments.

By using plants essentially as bait we have explored the plasticity of the reservoir of fungi present in soil which are able to infect oat or wheat roots. The composition of the collection of fungi which we have isolated is likely to have been influenced by many factors, including location, soil type, climatic conditions, agronomic practices, cereal cultivars sampled, growth stage of the plants, time of sampling, the root surface sterilization procedure, and the media used for fungal isolation. Further studies are required before general conclusions can be drawn about the nature of the fungi which colonize the roots of different cereals and the factors which determine colonization. However, these experiments suggest that avenacin A-1 can have a major influence on the development of fungal communities within oat roots. This saponin has also been extracted from the soil around roots at concentrations at which it would be expected to be inhibitory to many fungi (data not presented), and so it is likely to influence the development of microbial communities not only within the root but also in a zone around it.

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