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Agricultural management affects communities of culturable root-endophytic fungi in temperate grasslands

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Abstract

Three grassland sites of similar physical characteristics but differing management histories were chosen to test the hypothesis that agricultural disturbance has a deleterious effect on the diversity of culturable root-endophytic fungi and favours potentially pathogenic species. Species abundance data were collected for fungi isolated from surface sterilised root samples. Brillouin index of diversity, Twinspan and detrended correspondence analysis were applied to the community data. Quantitative ordination separated the samples by site showing that the communities differed in fields of contrasting management and this was supported by data from a microcosm experiment. Species presence and absence appeared to be affected seasonally; site differences were manifested in relative abundance. Diversity did not appear to vary by site, but a methodological explanation for this is proposed. Sterile dark septate endophytes were shown to be among the most abundant groups at all sites.

Keywords: Fungal ecology; Dark septate endophytes; *Fusarium*; Agricultural disturbance; Diversity index; Temperate grasslands; Root-colonising fungi

1. Introduction

Grassland is now the dominant semi-natural ecosystem in the UK, comprising 34% of rural land use, reaching 54% in Wales (Walford, 1997). Much of this is grazed agricultural grassland, some regularly ploughed and reseeded, frequently with the addition of lime and fertilisers. Such agricultural practices reduce diversity in plant communities, but the effects on fungi are less well documented and understood (Miller and Lodge, 1997). However, the results of Daniell (1999) link low fungal and low floristic diversities and there is an increasing need for the mechanisms linking plant and fungal systems to be understood.

Disturbance of a community can be either anthropogenic or natural in origin, and has the potential to disrupt a habitat's community structure, as well as change the substrate availability and physical environment (White and Pickett, 1985). For fungi living in plant roots, the consequences will be both a result of direct effects on the soil microbiota and also of influences on the plant that they colonise. For example, fertilisation of grassland will alter the relative abundance of a number of soil fungi (Donnison et al., 2000) and may select for arbuscular mycorrhizal fungi that can colonise plants of higher P content (Sylvia and Neal, 1990).

Most studies of the mycota of grassland soils have been descriptive, and focussed on soil or rhizosphere saprotrophs, which are important to decomposition processes and nutrient cycling. Fungi colonising the interior of healthy plant roots will have a less direct role in the decomposition process than soil or rhizosphere fungi, but they interact in a variety of ways with the host plant, and considerable ecological overlap may also occur (Jumpponen and Trappe, 1998). For example, endophytes and necrotrophic pathogens inhabiting healthy roots may still be primary decomposers, becoming active saprotrophs very early in root senescence (Garrett, 1981). By existing in healthy roots prior to senescence, such fungi are increasing their stake in early exploitation of the available resources. Thus the specific roles of fungi in apparently

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healthy roots may be hard to elucidate, but their importance should not be overlooked.

Two contrasting groups of root endophytes are members of the form-genus *Fusarium* and the dark septate endophytes (DSE). *Fusarium* spp. are commonly and easily isolated from grassland and arable soils. They are a cosmopolitan group that exhibit parasitic (Garrett, 1951), saprotrophic (Salerno et al., 2000) and pathogenic (Booth, 1971) behaviour under different circumstances (Samuel and Greaney, 1937). The role of *Fusarium* spp. in natural systems and in the colonisation of healthy roots has been little studied, despite the extensive literature relating to their importance as plant pathogens. DSEs are abundant in many plant genera and many habitats worldwide (Jumpponen and Trappe, 1998) and have been reported to confer a positive effect on plant growth (Read and Haselwandter, 1981). However, most work on DSEs relates to arctic or woodland systems (with few exceptions, e.g. Deacon (1973)), and their abundance as root endophytes in grassland systems has received less attention.

Microcosm plant experiments have shown that a degree of grazing disturbance increases botanical diversity by allowing subordinate species greater opportunity to compete (Grime et al., 1987), whilst studies of prairie grassland plant diversity indicated that the community of species present is dependent on the nature and frequency of disturbances, creating a patchy environment in which a greater number of species find their niche (Loucks et al., 1985). Various authors have attempted to assess the effects of disturbance on fungal communities (Wicklow, 1972; Zak, 1992; Frey et al., 1999). Whether the same theories apply to grassland fungi is unclear, though intermediate disturbance has been shown to maximise diversity in a model microbial system (Buckling et al., 2000) and is believed to maximise fungal diversity by increasing the patchiness of the environment and niche availability without imposing catastrophic species loss (Petraitis et al., 1989). In our study, we hypothesised that the extreme and uniform anthropogenic disturbance imposed on reseeded agricultural grassland would reduce the diversity of root endophytic fungi within the area of that management regime, and that clear differences in the composition of fungal communities would be observed between these and less disturbed sites.

Our aim was to examine the effects of agricultural management on fungal communities colonising healthy roots using statistical methods more common to plant ecology. Sampling root endophytic fungi eliminates the bias of taxa that sporulate heavily in the root surface region but will include the wide range of species that are biologically active in the soil and capable of colonising the outer layers of root tissue, with the roots representing a form of 'bait' for biologically active fungi. Use is made of multivariate techniques to examine quantitative differences between the communities, and to relate the differences to environmental variables to aid the explanation of differences observed.

2. Materials and methods

2.1. Sites

Three sites that have been managed differently over the last 20 y were studied. They were located within and adjacent to the National Botanic Garden of Wales (NBGW), Middleton, Carmarthenshire, UK (Table 1). All three sites

Table 1
Description of three field sites at the National Botanic Garden of Wales

Code	Name	National grid reference	General description	Soil texture	Soil pH (KCl)	Mineralisable N (mg 100 g^{-1} soil ± SD)	Mean soil water potential (p^F) (± SD)	Three most abundant plant species and National Vegetation Classification (Rodwell, 1991)
U (undisturbed)	Waun Las	SN528178	No management other than light sheep grazing for the last 20 y. Diverse with respect to the genus *Hygrocybe* (Rotheroe, 2001)	Clay loam	5.44 (±0.05)	0.705 (±0.51)	3.27 (±0.53)	*Agrostis capillaris*, *Holcus lanatus*, *Anthoxanthum odoratum* (NVC U4b)
I (intermediate)	Pantwgan	SN533184	Overgrazed and probably limed. No ploughing	Sandy clay loam	5.69 (±0.08)	0.580 (±0.25)	2.94 (±0.58)	*Agrostis stolonifera*, *A. capillaris*. *H. lanatus* (NVC MG11a)
D (disturbed)	Plas Uchaf	SN536175	Reseeded ryegrass lay on dairy farm adjacent to botanic gardens	Clay loam	5.74 (±0.08)	0.914 (±0.52)	3.05 (±0.50)	*Lolium perenne*, *H. lanatus*, *Trifolium repens* (NVC MG7a)

All sites are within 1 km of each other and lie on similar soil series.

have similar soil properties, and are located on either the Sannan or Barton soil series, both of which are free-draining brown earths (Rudeforth et al., 1984).

2.2. Sampling

A 30 m transect was established at each site, covering an area considered by eye to be homogeneous, of unchanging topography and aspect. Samples were taken every 2 months during 2000. On each sampling occasion, three 1 m × 1 m quadrats were sampled from the transect according to random numbers between 0 and 1 multiplied to provide an integer between 1 and 30. From each randomly chosen quadrat one $10 \times 10 \times 10\ cm^3$ turf was collected for mycological analyses and one 500 g soil sample from a 10 cm deep core for soil water potential, pH and nutrient analyses.

2.3. Root sample preparation

Care was taken to isolate fungi only from within roots that appeared healthy according to the definition of Hooker et al. (2000), who observed discoloration and darkening of roots as they approached mortality. Roots were surface-sterilised to kill mycelium and spores on the root surface without eliminating mycelium growing in the outer cortex. Paler roots of healthy appearance were washed free from soil in tap water, cut away as close to the shoot base as possible, and surface sterilised for 30 s in 10% domestic bleach followed by four washes in sterile distilled water, shaking vigorously. The roots were then dried in a stream of sterile air (to reduce bacterial growth), and random 5 mm segments plated on a selective medium containing per litre 39 g Lab M potato dextrose agar (PDA), 1 ml 10% chloramphenicol in ethanol, 400 μl 0.5% dichloran, 10 g sucrose, 10 ml 1% Triton X-100 and 990 ml sterile distilled water. Dichloran (a dye) (Bragulat et al., 1991) and Triton (a nonionic detergent) (Beuchat and Dedaza, 1992) reduce the radial growth rate of fungal mycelia and prevent rapidly growing species from over-growing and obscuring the slower growing species. Eight root segments were placed on each plate, and for each quadrat a total of 15 plates were inoculated. Plates were incubated in diffuse daylight at room temperature (20–25 °C). After 3 weeks the number of colonies per plate was recorded, colonies re-isolated where necessary, and each identified to species level where possible.

2.4. Identification of fungal isolates

The identities of some fungi that remained sterile in axenic culture, and of some *Fusarium* isolates, were established by PCR amplification and sequencing of the internal transcribed spacer (ITS) region of the ribosomal RNA locus, followed by BLAST (Altschul et al., 1997) and FASTA (Pearson and Lipman, 1988) searches of the European Molecular Biology Laboratory (EMBL) database (unpublished data).

2.5. Soil analyses

Soil mineralisable N was determined using the methods of Black (1965), extracting N in molar KCl and titrating against HCl. Approximate soil moisture content was determined as percentage of oven dry weight after sieving to 4 mm. Percentage moisture does not give a directly meaningful value for water available to soil biota, so soil water potential was estimated from the moisture data, using the methods of Fawcett and Collis-George (1967), by calibrating the relationship between percentage moisture and water potential values for each site. These data were recorded for every sample taken. pH values (Table 1) were recorded in May 2000, using a 1–2.5 ratio of fresh soil to 1 M KCl (Baize, 1988) and a Denver 'basic' pH meter.

2.6. Microcosm experiment

Seeds of *Holcus lanatus* were surface sterilised using the same procedure described for root samples above, and sown separately in remaining soil from either the disturbed or the undisturbed site. When the roots had grown to a depth of 9 cm they were cut from the plant and their fungal endophytes cultured in a manner identical to the treatment of field samples. Six replicate plants were analysed for each soil type and from each plant a total of 80 × 5 mm sections were plated onto selective medium.

2.7. Statistical analysis

Two-way ANOVA was used to test significance of variation in total colonisation with site and time as factors. The Brillouin diversity index (Magurran, 1988; Harper and Hawksworth, 1994; Begon et al., 1996) and species richness (total number of species isolated) were calculated. For these analyses, number of isolates of each of the taxa shown in Table 2 from each site-time combination were used, an approach analogous to the use of percentage cover (spatial extent) in plant ecology. The Brillouin Index is rarely used because of difficulties in calculating the factorial function when the number of taxa is large. However, it is the most appropriate for these data as it describes a collection, appropriate when the presence of unculturable species means that the sample may not be random or reflect the true nature of the population (Magurran, 1988). Calculation of the index for these data was made possible by use of a program kindly written in house by Dr Leighton Pritchard (available from http://users.aber.ac.uk/gwg/brillouin.htm).

TWINSPAN (two-way indicator species analysis) (Brown and May, 2000) and detrended correspondence

Table 2
Frequency of fungi (percentage of isolates) growing from surface-sterilised roots sampled in the field at bi-monthly intervals

Taxon	January	March	May	July	September	November	Overall abundance rank
MSP6 *Leptodontidium* sp.*(94%)	29.6	8.9	20.9	27.6	18.3	10.3	1
Morphospecies 10	20.0	12.6	12.8	16.5	17.1	13.9	2
MSP9 *Phialophora* sp. *(90%)	6.8	24.6	5.7	14.4	10.8	25.0	3
MSP1 *Lachnum* sp. *(94%)	8.0	6.1	4.9	4.8	16.1	19.6	4
Morphospecies 2	4.5	8.7	8.1	4.5	3.6	3.4	5
Morphospecies 4	5.7	4.5	4.1	5.2	2.6	3.1	6
Acremonium	4.7	3.7	0.4	0.3	5.6	5.1	7
Penicillium	1.8	4.8	5.6	5.3	0.6	0.7	8
Cladosporium	0	9.5	2.5	1.9	0.4	0.6	9
Fusarium	4.8	1.8	3.2	0.8	2.8	0.7	10
MSP3 *Phialocephala* sp. *(91%)	0	0	2.3	3.0	4.7	4.1	11
Cylindrocarpon	5.8	1.6	2.3	0.7	2.6	1.1	12
Trichoderma	1.8	3.9	1.5	0.4	1.7	1.3	13
Coelomycete (unidentified)	0	0	9.9	0	0	0	14
Morphospecies 14	0	1.5	1.7	1.6	2.6	1.1	15
Morphospecies 7	0	0	1.7	4.0	0.7	1.7	16
MSP13 *Hypocrea koningii* *(99%)	0.6	0	2.0	0.5	1.7	2.7	17
Aspergillus sp	0.9	3.1	2.5	0	0	0.1	18
Morphospecies 12	0	0	3.1	1.4	1.1	0.9	19
Basidiomycete (unidentified)	2.6	0.9	0	1.1	0.7	1.1	20
Morphospecies 11	0	0.9	1.0	0.8	1.9	0.5	21
Morphospecies 5	0	0	0.3	0.8	2.6	0.4	22
Morphospecies 8	0	0.8	1.2	1.1	0.6	0.4	23
Zygomycete (unidentified)	0.4	0.8	0.9	0.1	0.1	0.1	24
Verticillium lateritium	0	0	0	1.8	0	0.3	25
Verticillium spp.	0.1	0.4	0.4	0.3	0	0.3	26

Taxa marked with an asterisk were sterile but were identified by means of ribosomal DNA sequencing. The names attributed to these are based on the best hits to sequences on the public database (figures in brackets indicate percentage sequence identity with these hits). Morphospecies (MSP) 3, 6, 10 and 12 were dark in colour; the other morphospecies were hyaline. Ranks 27–41 were rarely recorded: *Paecilomyces*, *Alternaria*, *Scopulariopsis*, *Aspergillus niger*, *Chaetomium*, *Epicoccum*, *Trichophaea*, ascomycete (unidentified), *Dictyosporium*, *Aspergillus glaucus*, *Harposporium*, *Absidia*, *Gliomastix*, *Gonytrichum* and *Moniliella*.

ordination (Heilmann-Clausen, 2001) were performed using in-house programs written by Dr David Causton at the University of Wales, Aberystwyth. The ordination used here is a quantitative technique, unlike the qualitative TWINSPAN, and analyses the community data using abundance values as well as presence or absence for any given taxonomic group. TWINSPAN is a polythetic divisive classification system. One-way ANOVA was performed on the abiotic data to determine significant differences between the values corresponding to groups described by TWINSPAN. TWINSPAN has been used only rarely to describe fungal community data (Termorshuizen, 1991; Derooijvandergoes et al., 1995). Quantitative detrended correspondence ordination was also used to describe the results of the microcosm experiment.

3. Results

3.1. Extent of colonisation

Total extent of root colonisation, expressed as the mean number of colonies isolated per 5 mm root segment, varied both spatially ($F = 5.19^{**}$, $DF = 2$, $P = 0.01$) and temporally ($F = 8.51^{***}$, $DF = 5$, $P = 0.00$) (Fig. 1) although with significant interaction; the temporal colonisation pattern is different for samples from the three sites. However, all three sites showed a similar overall trend to higher colonisation in spring (January) and autumn (September). The undisturbed and intermediate sites were most similar, differing only in the timing of the lowest colonisation value, the undisturbed site reached a minimum value in July ($n = 0.71$) and the intermediate site in May ($n = 0.63$). There was lower colonisation in the disturbed site (minimum $n = 0.53$), and the spring peak occurred later.

3.2. Temporal variation in community composition

Total species richness varied over the year (Fig. 2). For species richness the three replicate samples were pooled to maximise sample size, due to the presence of a large proportion of rare taxa. The largest numbers of taxa were found in July 29 and November 29. The July peak (Fig. 2) corresponded with the period of low overall colonisation (Fig. 1). The two months when the least number of taxa were recorded were March 22 and January 23, periods of high total colonisation.

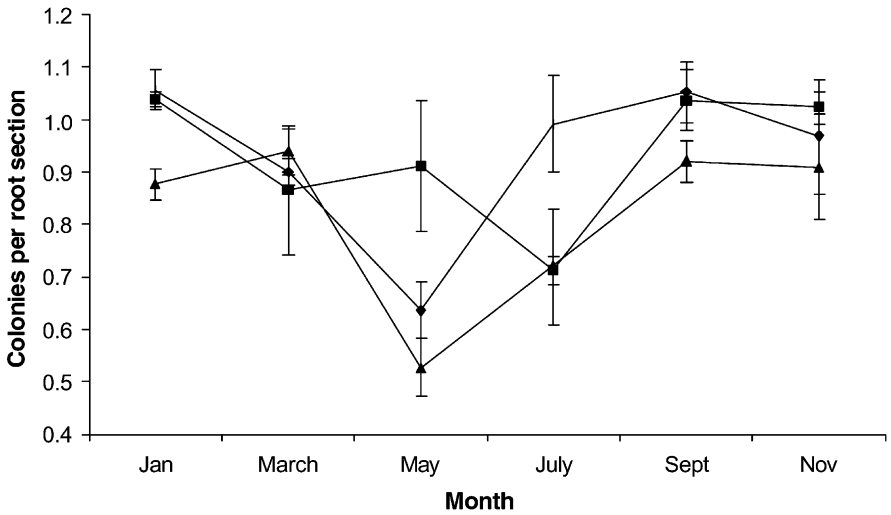

Fig. 1. Bi-monthly variation of mean root colonisation in undisturbed (■), intermediate (◆) and disturbed (▲) sites during 2000. Mean root infection is expressed as the proportion of discrete colonies isolated-to-the number of root segments plated. Error is given as standard deviation.

Over the course of the year, the dominant taxa isolated from roots (comprising the top six in terms of overall rank abundance) were all sterile morphospecies, many exhibiting dark pigmentation. None of these isolates sporulated (even after prolonged culture on woody substrates), and were therefore, classified by colony appearance. This approach has been used in studies of endophytic fungi (Hall, 1987) and has been shown to be reliable by comparison to genetic analysis by Guo et al. (2000). Our own polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses of several of the morphospecies were consistent with this observation (data not shown). Morphospecies 3, 6 and 10 fitted the description of DSEs given by Jumpponen and Trappe (1998). A few of the sporulating taxa were also isolated at every sampling, including fungi in the genera *Acremonium, Cylindrocarpon, Fusarium, Penicillium*, and *Trichoderma* as well as some of the sterile morphospecies (Table 2). Some genera were isolated at one sampling only, including *Absidia*, *Dictyosporium*, *Gliomastix*, *Gonytrichum* and *Moniliella.*

3.3. Effects on the community

Multivariate analysis of the fungal data by site and time yielded data grouping the site-month sets according to similarity of the colonisation patterns. The methods gave contrasting results. TWINSPAN grouped the sets of data according to month, with the site sets for January and March separately classified in their own groups (Fig. 3). The data for the second half of the year were less well grouped in relation to time. Detrended correspondence ordination grouped the data sets more by site, with the disturbed site

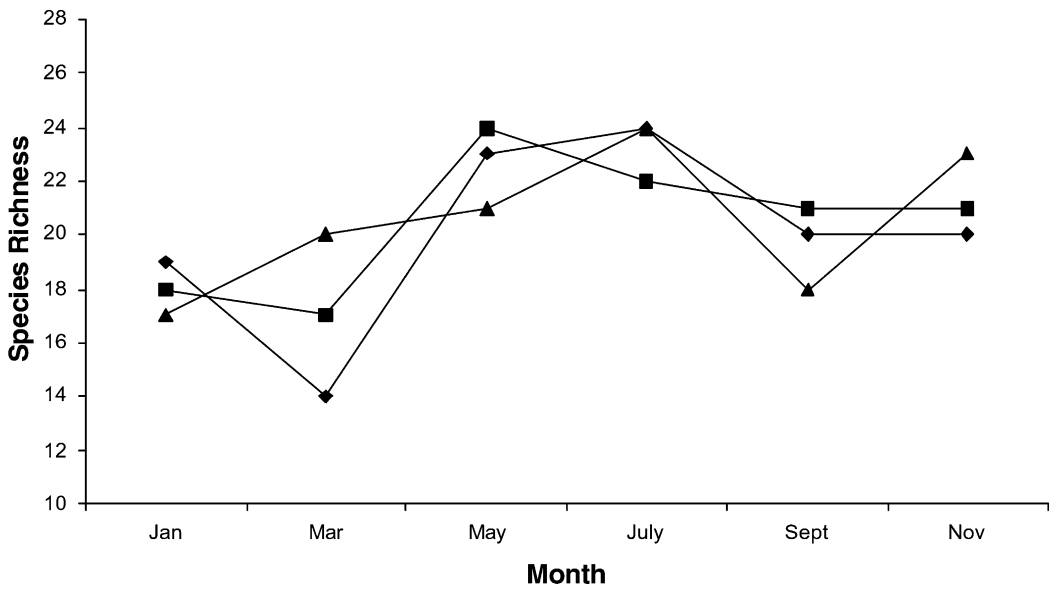

Fig. 2. Bi-monthly variation in total site species richness Values shown are for undisturbed (■), intermediate (◆) and disturbed (▲) sites.

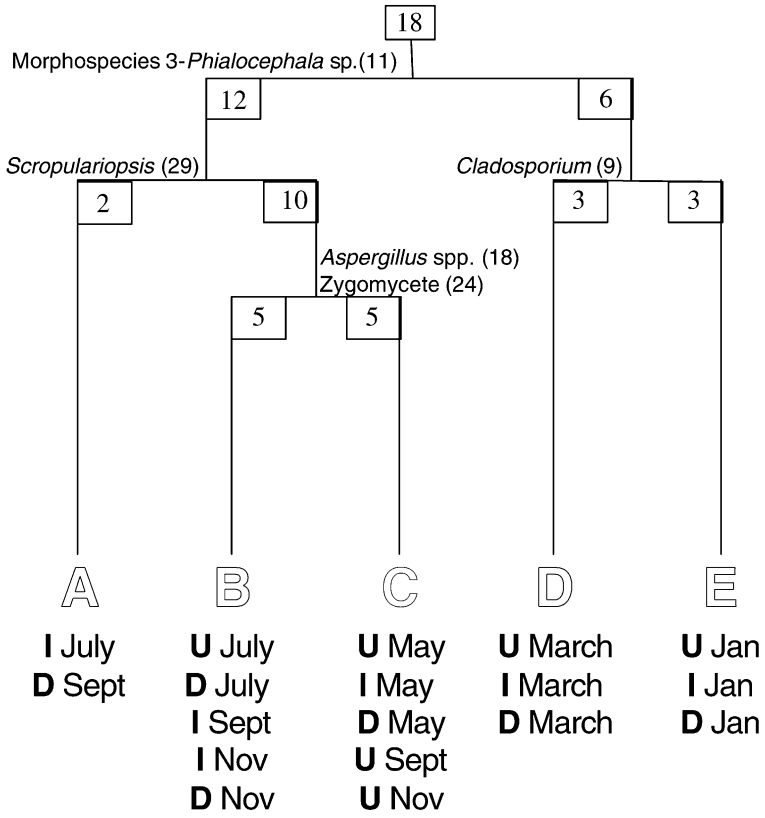

Fig. 3. Qualitative TWINSPAN dendrogram. Numbers in boxes represent total samples (quadrats) in each part of a division. Site codes are U (undisturbed), I (intermediate) and D (disturbed). A to E represent the final groups described by the divisions of the TWINSPAN program. The indicator taxa are listed with rank abundance (Table 2) in brackets.

(D) falling with high values on both axes, the undisturbed site (U) with low values on both axes and the intermediate site (I) between the two (Fig. 4).

3.4. Diversity

The general trends in diversity over time were similar between sites. The Brillouin Index (Fig. 5) peaks in March for the undisturbed and intermediate sites, and May for the disturbed site. There was a trough in recorded diversity over the autumn and winter months in all cases. The maximum diversity recorded (3.53) was in the disturbed site.

Fusarium spp. colonisation varied seasonally in all three sites (Fig. 6). However, colonisation in the disturbed site (D) was several times greater than in the intermediate (I) and undisturbed (U) sites, though this does not reflect any site differences in overall colonisation. This is in contrast to some of the pigmented sterile morphospecies (putative DSEs), for example, morphospecies 10, which occurred in all three sites in large numbers (Fig. 7).

3.5. Effects of soil abiotic factors

One-way ANOVA was performed on water potential and total mineralisable N values according to the groups described by TWINSPAN. This revealed differences between the total mineralisable N values, ($F = 3.22*$, $DF = 4$, $P < 0.05$) and water potential values ($F = 6.22**$, $DF = 4$, $P < 0.01$) of the soils that form these groups (Table 3).

3.6. Microcosm experiment

The results of detrended correspondence ordination of the community data from the *Holcus* microcosms are shown

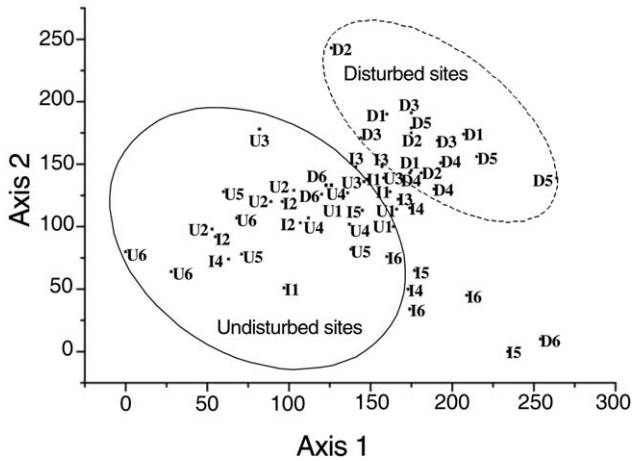

Fig. 4. Detrended correspondence ordination of month-site data. Samples are named first by month (1: January; 2: March; 3: May; 4: July; 5: September; 6: November) and then site (U:undisturbed; I:intermediate; D:disturbed). Three replicate quadrats are plotted per site per time. The principal grouping is indicated for the disturbed and undisturbed sites.

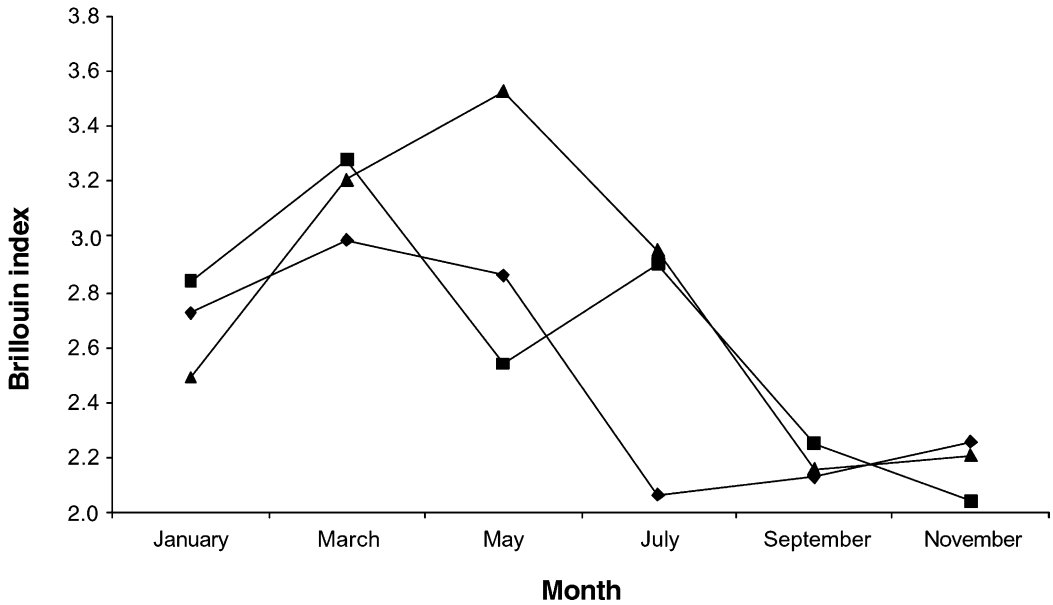

Fig. 5. Brillouin index of diversity. Values shown are for every 2 months for the undisturbed (■), intermediate (◆) and disturbed (▲) sites.

in Fig. 8. The taxonomic data are not shown but are available on request. Samples from roots grown in the two soils were separated distinctly from each other by this quantitative analysis.

4. Discussion

4.1. General pattern of results

Our results demonstrated quantitative differences between fungal root communities in relation to management using multivariate methods, and also showed a significant statistical correlation with environmental variables. Though the occurrence of both dark and hyaline sterile fungi within roots has long been known (Waid, 1974), this study is the first to have examined these fungi in such detail and to have ascribed approximate taxonomic affiliations. The diversity of culturable fungi did not differ greatly between sites, but more interestingly, clear seasonal variation in the species present and spatial variation in species abundance distributions were apparent, as well as temporal variation in overall colonisation and species richness.

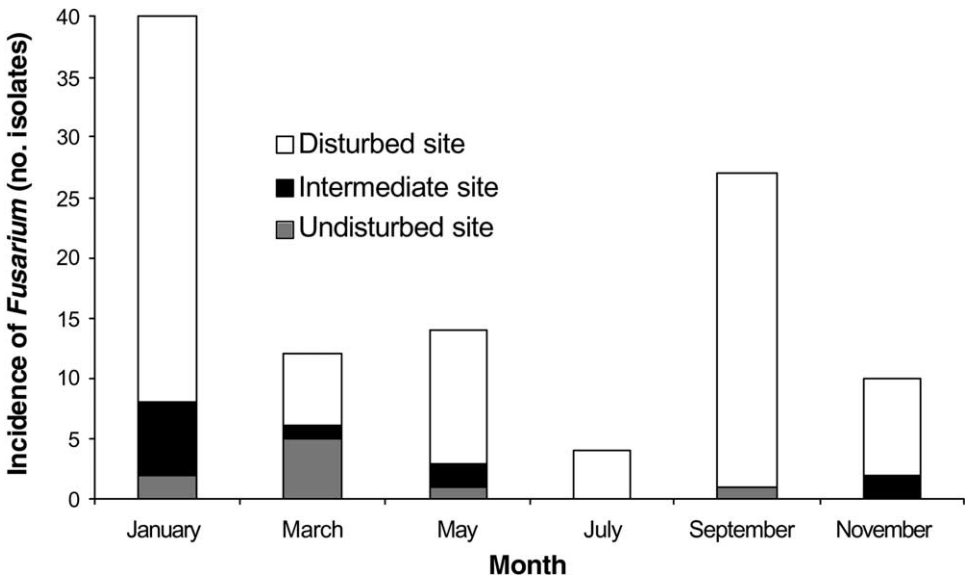

Fig. 6. Number of *Fusarium* isolates recovered from root segments from sites of differing disturbance. Numbers of isolates are given from 360 root sections per site per month.

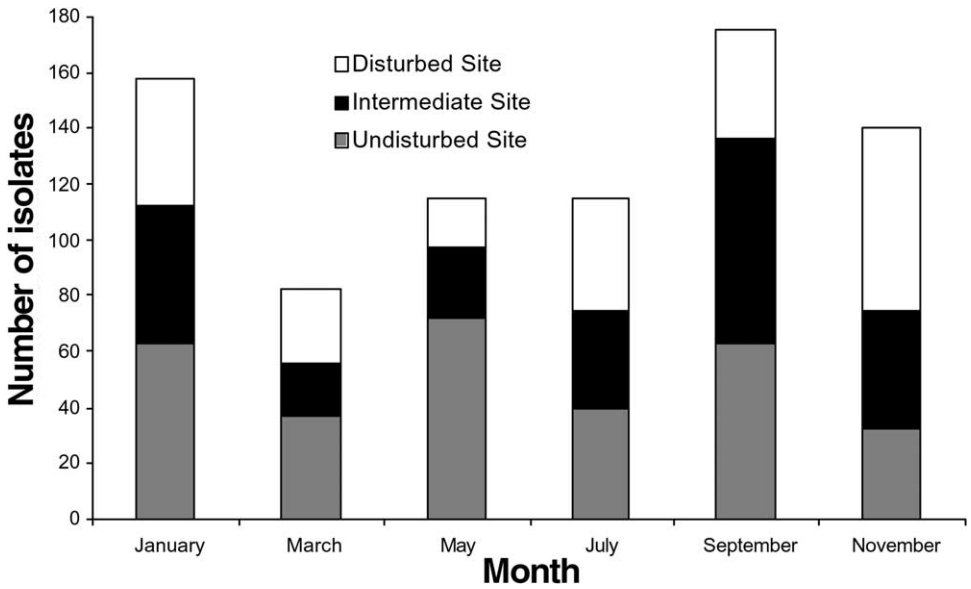

Fig. 7. Number of morphospecies 10 isolates recovered from root segments from sites of differing disturbance. Numbers of isolates are given from 360 root sections per site per month.

4.2. Seasonal variation in colonisation

Seasonal variation in extent of root colonisation, including spring and autumn peaks, has been reported by Griffiths and Siddiqi (1961) and Persiani et al. (1998). The spring peak coincides with the period of peak root growth, which in a *Lolium* meadow (disturbed site) occurs between January and May (Troughton, 1957). The autumn peak corresponds with the saprotrophic behaviour associated with late summer root death, which occurs from May onwards. The low colonisation in the disturbed site during the summer months may be attributable to reduced fungal activity in the soil as both total fungal biomass and the proportion of microbial biomass formed by fungi appear to be reduced by tillage (Frey et al., 1999), which is consistent with the findings of McGonigle and Miller (1995) that tillage reduces parasitic fungal infection of roots. The disturbed site also had visibly lower litter accumulation compared to the undisturbed site, and the soils themselves contained only 10.6 and 12.2% organic matter, respectively. The decomposing litter present in the undisturbed site may act as a pool for the introduction of root colonising species that is absent in the agricultural (disturbed) field. Many of the root inhabiting species isolated would certainly be present in the litter layer, as a large proportion of the genera isolated, such as *Cladosporium*, *Trichoderma*, and *Penicillium* (even some of the less frequently isolated genera such as *Chaetomium*) have the ability to exist saprotrophically and competitively in senesced material.

4.3. Diversity

Understanding the individual ecological roles of the component fungi may also help to explain phenomena such as the late spring/early summer peak in diversity. The increased resources created by root growth would create environmental conditions in which a greater number of species could be supported. High diversity is generally attributed either to high competition, causing niche restriction, or low competition due to predation (Huston, 1979), for example, the grazing by soil animals of extra-radical mycelium. In early spring, new grown root material may contain a low diversity of fungi, as only species capable of rapidly reaching and colonising the new resources will be present. Mostly these will be generalist parasitic fungi, infecting roots through the areas of exudation, particularly just behind young root tips (Garrett, 1981; Turlier et al., 1994) where dividing cells are undifferentiated and defence provided by adjacent cells. By early summer the abundance of resources will provide capacity to support a higher diversity of competing species and sufficient time has elapsed for more species to colonise; there is a reduced probability of any one group becoming dominant. In the autumn, saprotrophic species would be prevalent in senescing root material; it has been shown that in the late

Table 3
Results of ANOVA testing the variance of soil abiotic factors with biotic characteristics grouped by TWINSPAN

TWINSPAN group	Mean water potential (p^F)	Mean net mineralisable N (mg 100 g^{-1} soil)
A	3.375[a]	1.071[a]
B	3.377[a]	1.209[ab]
C	2.996[ab]	0.883[ab]
D	2.492[b]	0.651[ab]
E	3.150[a]	0.571[b]

Breakdown analysis is shown with letters where the same letter denotes no significant difference between given levels of that treatment.

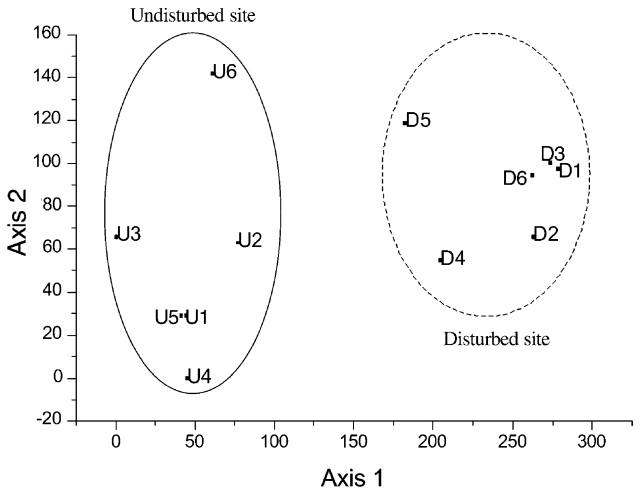

Fig. 8. Detrended correspondence ordination of community data from *Holcus* roots grown is soil from disturbed (D1–D6) and undisturbed (U1–U6) sites. Strong grouping is shown in relation to axis 1.

stages of root decomposition the fungal spectrum may be reduced due to nutrient depletion and extreme competition (Upadhyay and Bharat, 1982), thereby reducing diversity.

4.4. Multivariate analysis

The use of multivariate statistics widely applied to plant community data analysis has permitted the classification of communities of root endophytic fungi without consideration of the autecology of individual species. There are very few examples of this kind of analysis in fungal ecology and those that have been published involve analysis of the distribution of macroscopic fruit bodies (Termorshuizen, 1991; Heilmann-Clausen, 2001). TWINSPAN grouped the communities entirely by time for the first two samples, suggesting that there is greater qualitative similarity between sites at this time of year. The presence or absence of a given species in the winter and early spring may be determined to a greater extent by seasonal factors, which would be uniform across the sites; these might include temperature and soil moisture. Temperature has already been shown to affect the distribution of some species of *Fusarium* (Saremi et al., 1998). The classification of the data sets from the second half of the year is less well grouped by time, suggesting management or biotic factors that are site dependent are interacting with the putative seasonal effect.

Since both soil N and moisture show significant differences between the means for the TWINSPAN groups, they may be examples of site-dependent environmental variables affecting the fungal communities. Soil N was highest in TWINSPAN groups 1 and 2 (July sites U, I, D, September site I, D, November sites I, D), i.e. the summer and autumn months, with a slight bias to the disturbed site. Seasonal variations in N could be a determinant of the pattern of variation in these root endiphytic fungi, mediated either directly through nutrient availability in soil or via effects on the plants. Nutrient availability has been demonstrated to affect soil fungi (Guillemat and Montègut, 1960; Popova, 1993) but to date this has not been shown for culturable root endophyte communities.

In our study, the patterns in relation to soil moisture are complex. Some differences observed are likely to be related to soil topography, for example, site I is better drained than the other two on account of a steeper gradient and slightly sandier soil. With so many interacting factors it is not possible to infer the causes and effects of different water potential values. Griffiths and Siddiqi (1961) found no influence of soil moisture or temperature considered independently on fungi in grassland soil, however, the significant differences reported here suggest that further and more rigorous testing could be informative.

Site-community differences were clearly revealed using the quantitative method of ordination (Fig. 4). These differences must arise not only from contrasts in the species present but also from their relative abundance, as the use of this quantitative technique has grouped the data sets by site more clearly than the qualitative TWINSPAN. Potential confounding variables, such as the differences in the plant communities between the three sites, should not be overlooked, but the strong experimental evidence from the *Holcus* microcosm demonstrates that the site effect is real.

4.5. Distribution of Fusarium and morphospecies 10

Clear site related differences in abundance were also revealed for *Fusarium* spp. This group exemplifies why site differences were seen more clearly using ordination. *Fusarium* isolates are present in almost every site-time sample so qualitative techniques would be uninformative, yet there is a clear site related difference in abundance with a greater degree of colonisation in the disturbed site. Understanding the reasons for site abundance differences in a genus like *Fusarium* is crucial to understanding the consequences of disturbance for the mycological communities. Having the ruderal characteristic of producing large numbers of asexual spores (species such as *F. oxysporum* have no known teleomorph), and not relying on extensive mycelial networks, it is likely to be favoured by agricultural perturbation such as ploughing and reseeding (Pugh and Boddy, 1988). If the species is pathogenic, this could have profound consequences for the health of the grassland. A spring peak in *Fusarium* colonisation has also been seen in other studies (Clarke and Christensen, 1981), and is likely to relate to new root growth, although in one study a higher autumn than spring peak of *Fusarium culmorum* was found (Griffiths and Siddiqi, 1961). In addition, it is noteworthy that it is in cosmopolitan species, such as *Fusarium* (Fig. 7), which are capable of non-pathogenic colonisation of many hosts (Alabouvette et al., 1993) that site differences in abundance appear to be manifested, rather than in taxa generally considered more specific to root tissues such as morphospecies 10.

The dominance in all sites of sterile morphospecies matching the description of DSEs was unexpected,

especially as they are mostly reported to occur in less fertile arctic and sub-alpine ecosystems (Ahlich and Sieber, 1996; Schadt et al., 2001), possibly due to a reduced rate of decomposition. They may be of particular importance as various reports have suggested that they may have a beneficial effect on the host plant either via N uptake, or antagonistic effects on colonisation by root-infecting pathogens (Read and Haselwandter, 1981; Jumpponen, 2001). They appear not to be host-specific, a trait supported by their widespread distribution in the present study. The contradictory reports on the ecological roles of some genera identified as belonging to the DSE group such as *Phialocephala fortinii* are highlighted by Ahlich and Sieber (1996); this fungus and other DSEs have been cited as having both pathogenic and mycorrhizal roles.

4.6. Methodological considerations

The fact that our original hypothesis that diversity would be highest in the undisturbed site was rejected may be partly attributable to methodological bias. Plating onto selective media only isolates a subset of the microorganisms present and favours fast growing non-basidiomycetous fungi. PDA has been shown to yield fewer isolates than synthetic nutrient-poor agar (Bateman and Kwasna, 1999). We did not use low nutrient agar because the high radial growth rate of fungi on such 'weak' media necessitates a reduction in sample plating density, whereas PDA permitted greater sample throughput and also enhanced colony pigmentation, an aid in taxonomic identification of isolates. It is significant for plant pathologists that whilst intensively managed grassland is known to have low arbuscular mycorrhizal diversity (Helgason et al., 1998) the same is not true for the fast-growing ascomycetes.

Another methodological consideration relates to the choice of root. For logistical reasons, plant roots from each site were pooled from all plant species present as the thick litter thatch inhibited the separation of individual plants and identification of species. However, on the basis of previous findings that both root colonising fungi such as dark septate endophytes (Jumpponen and Trappe, 1998; Schadt et al., 2001), arbuscular mycorrhizas (Eriksson, 2001) and some of those more typically isolated from soil, such as *Fusarium* (Jenkinson and Parry, 1994) lack host specificity, it was not anticipated that this would confound site effects. Saar et al. (2001) concluded that plant host specificity in endophytes is 'fairly rare'. Any species showing obligate host specificity were less likely to be isolated and included by the cultural techniques employed in our study. Many of the DSE and *Fusarium* isolates from both sites were subsequently inoculated onto grass species present at the field sites (in monoxenic culture) and all were capable of colonising healthy roots. Furthermore, the results of the microcosm experiment using *Holcus lanatus* (which occurred at all three sites) sown in soil from the field sites confirm that there is a real and strong site effect even when plant species is eliminated as a confounding factor.

The aim of surface sterilising root tissue was to reduce the bias inherent in soil fungal profiles due to the growth of colonies from spores. By bleach treatment and serially washing the material, rhizosphere spores should be eliminated, and isolates should mainly originate from mycelia (Waid, 1974; Hall, 1987). Ordination of the endophyte data with community data from rhizosphere isolations that were performed simultaneously results in tight, separate clustering of the two groups (data not shown) due to a far greater dominance of the heavily sporulating taxa in the rhizosphere, demonstrating the benefits of surface sterilisation. It should also be noted that although roots were selected on the basis of healthy appearance, it is known in cereals and to a lesser extent in grasses that the earliest indications of root cortex senescence (disappearance of nuclei) occur long before there is any change in macroscopic appearance (Kirk and Deacon, 1986). A greater knowledge of the progress of root senescence will be required for the complete elimination of such samples from similar studies based on 'healthy' root communities.

Disturbance is known to affect soil fungal communities (Wicklow, 1972; Zak, 1992; Frey et al., 1999). Our study demonstrates that this also occurs with culturable root endophytes. Some consequences can be inferred, in particular with regard to the relative dominance of *Fusarium* in the disturbed meadow. An increase in dominance of potentially pathogenic taxa such as *Fusarium*, or reduced endophyte diversity, may have significant consequences for the susceptibility of a plant community to fungal diseases. There is some debate whether community diversity necessarily confers stability (Givnish et al., 1994). Given the mechanisms of biological control and interactions in plant-fungal relations as determinants of plant fecundity (Griffiths and Siddiqi, 1961; Cook, 1993; Newsham et al., 1994) the issue of diversity of fungi in the roots is clearly an important one. It seems likely that a high diversity of root fungi would reduce the deleterious effects of root pathogens, since biocontrol of pathogens is exerted by the natural soil flora, by mechanisms including competition for infection site and substrates (Steinberg et al., 1999).

4.7. Conclusions

Differences between the management treatment effects on endophytes were observed, though these were not manifest in diversity indices, as originally hypothesised. Site communities were separable based on the identity and abundance of taxa living in grassland root systems, especially during the late summer and early autumn. Ideally several fields for each management type should be sampled. The difficulties identifying causality in the relationships between land management and fungal communities are recognised (Eriksson, 2001). Localised variations in soil texture, drainage, and plant composition all have a confounding effect. Whilst

causality may be more difficult to prove, it has been demonstrated conclusively that differences between these sites do exist and soil N content may be one mechanism by which management affects root colonising fungi. The analytical methods employed here were successful in proving a statistically significant link between community and environment and merit further use in fungal ecology.

Because of the problems identifying some taxa and the bias of studying only culturable endophytes, the next logical step would be to investigate molecular diversity of fungi in the roots, as recommended by Smit et al. (1999). Further microcosm based investigations into the different colonisation patterns in the sites' soils would increase understanding of the relationship between physical site disturbance and the development of the endophytic communities in roots. Little is known about the life cycles of plant and fungal material underground compared to studies on the aerial parts of plants. It remains a challenge to clarify the effects of the numerous influences on terrestrial fungal communities and gain a better understanding of the dynamic interactions between plant roots and their endophytes.

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