Various aspects of fungal succession, and interactions between fungi, are reviewed and the problems encountered highlighted. Suggestions for future research are presented, outlining some of the techniques currently available. Particular attention is focused on the need to understand the chemical interactions involved in succession.

Key words: Colonisation, confocal microscopy, fungal interactions, immunofluorescence, molecular phylogeny, sporulation.

Introduction

Studies of fungal succession on various substrata have established similar patterns in occurrence with time, i.e. early, intermediate or late colonisers (or alternative terms), with the time scale varying with each study and the substratum examined (e.g. Eaton and Jones, 1971a; Sivichai et al., 2000; Tsui et al., 2000). Fungal occurrence may also be characterized as common, frequent, infrequent or rare, with the percentage frequency of occurrence varying with each study, with no common agreement as to what these terms mean in an ecological sense (e.g. Jones, 1963). Sivichai et al. (2000) regarded high frequency of occurrence as over 50% and up to 100%, and a similar approach is adopted by Kane et al. (2002, this volume). In comparison, other researchers have regarded common species in the range of 9-41% (Lake Barrine, Queensland: Hyde and Goh, 1998), 12-70% (Plover Cove, UK: Goh and Hyde, 1999) and 9-15% (Lake Fuxian, China: Cai et al., 2002). Comparison between studies is therefore difficult due to lack of standardization. Other parameters are also variable including sample size, period of exposure or submergence, and subsequent incubation time.

Mycologists studying microfungi, have generally paid more attention to the identification of the fungi present on substrata, rather than to consider the
activity of these organisms, or even their interactions with the substratum. Studies have generally focused on the overall biodiversity of the fungi, rather than trying to elucidate their role, or interactions between the fungi. Studies of fungal diversity are, however, important, as we presently know only an estimated 5% of fungi (Hawksworth, 1991, 2001). There are no studies that compare the levels of propagules in the environment, and try to correlate this with their frequency of occurrence on the substratum. For example: Helicomyces roseus was the most common species on Dipterocarpus alatus test blocks in a stream at Khao Yai National Park, Thailand, with a frequency of occurrence of 60-100% over a 12 month period of submergence. What was the spore loading of this species in the stream water? Why were Chaetopsis penicillata and Dendrosporium lobatum only found once on the wood during the study period? We have few answers to questions of this sort and these are areas that require future investigation.

Problems in studying fungal succession

Fryar (2002, this volume) has discussed the issue of fungal succession versus sequence of fruiting bodies and points out that the nutritional hypothesis, supported for so many years, has proven false (Garrett, 1963; Harper and Webster, 1964). This aspect will not be further treated here. There are however, numerous problems associated with studying fungal succession at the substratum level and these are addressed below.

Many of the papers in this volume view fungal succession by documenting the fungi sporulating on natural substrata, or baits exposed in particular habitats. For practical reasons they have not considered non-sporulating mycelium within the substratum and consequently, cannot conclude that the fungi observed are the only species active in the succession process (Jones, 1963; Eaton and Jones, 1971a,b; Tsui et al., 2000). Development of techniques to assess the presence of microorganisms within substrata that do not rely on direct observation of fruiting bodies, and which do not rely on culturability, are essential to make accurate estimates of biodiversity that can be linked to ecological function within ecosystems.

Microbial communities are complex structures comprising many organisms that cannot be identified because techniques do not exist that can recognize them. These forms include viable, but non-cultur able species, and dormant or senescing spores. Some organisms have never been isolated and therefore remain "invisible". In most papers on fungal succession, the role of other microorganisms are not considered, e.g. bacteria, grazing protozoa.

In studies of fungal communities the situation is complex because, in addition to non-cultur able forms, many fungi occur throughout the substrata in
a vegetative form that is hard to identify. The composition of fungal communities is therefore poorly understood and can only be clarified by the development of techniques that complement existing approaches. Colony boundaries of individual species may be difficult to recognize when more than one species is present (Vrijmoed et al., 1986), so neither the contribution of a single species to the fungal population in the substrate, nor its interaction with other species can be determined. For a complete understanding of succession the changing contribution of each species in the population would have to be assessed over time, from settlement to senescence, and this could only be achieved with the recognition of the vegetative mycelium within the substrate.

Denaturing gradient gel electrophoresis (DGGE) is a technique that can be used to identify specific nucleic acid molecules in complex mixtures that differ by one pair (Muyzer et al., 1993). The target region for this analysis is the rRNA gene cluster. These genes have both highly conserved and variable domains that can be used in phylogenetic analysis to depict relationships amongst eukaryotes. One of these regions present in the 28S rRNA gene, V3 is sufficiently conserved to demonstrate differences between ascomycetous and basidiomycetous fungi (Guadet et al., 1989; Fell and Kurtzman, 1990). Other sections of this cluster include the ITS regions, and these have been used to separate closely related species and strains. Both of these regions are approximately 200-400 base pairs and, as such, are suitable for DGGE analysis. The problem is that this technique is not yet well developed for fungi. Protocol to assess fungi throughout substrata have not been established and there is certainly a need for developmental research in this area.

In many cases in succession studies, substrata have been incubated in moist chambers in the laboratory for periods, ranging from 2-4 weeks to 3-6 months (Vrijmoed, 2000). The supposition is that given time, and the right conditions, those fungi present as mycelium in the substratum, may eventually sporulate. Often the conditions under which the substrata are incubated bear little relationship to those applicable in the environment from which they have been recovered. For example, submerged wood recovered from a temperate river is usually incubated at room temperature, and we do not know the effect this likely to have on the fungi sporulating on the wood. This is the dilemma facing mycologists who wish to record the diversity of fungi colonising a substratum and desiring to make statements about their general ecology.

The issue of non-sporulating fungi on / in a substratum can be overcome to a degree by their isolation onto a range of media. However, many fungi will not sporulate even in culture and then the sterile mycelia must be compared with other non-sporulating mycelium isolated from germinating spores (Rayner and Todd, 1974). An alternative way, as briefly outlined above, is to develop
molecular techniques for the identification of non-sporulating fungi (Guo et al., 2001).

**Problems enumerating fungi on a substratum?**

A number of other problems arise in documenting fungal diversity on substrata. Firstly, there are problems in recording taxa when some are present as single fruiting bodies, while others sporulate over the entire surface of the substratum. Presently most fungi are recorded as being present, but are not quantified. This obviously creates problems in terms of statistical analysis of the data. How relevant are diversity and similarity indices when we cannot enumerate the amount of substratum colonised by the fungi present? Is there any gain in subjecting such data to numerical analysis when we are not comparing like with like? Most similarity indices have been developed for plants and animals that can be counted in a given area, and which can be meaningfully, statistically analysed. For fungi, there is no single unit that can be counted, even if all species did sporulate on the substratum.

A better approach is not only to record the taxa present, but also to estimate the area they occupy on the substratum. Sivichai et al. (2000) attempted to correlate percentage species occurrence with the area covered by the sporulating fungi. Similar evaluations have been undertaken by Hudson (1962) and Eaton and Jones (1971a). Unfortunately, the above procedure suffers from the problem that not all species will sporulate on the substratum under investigation and techniques are not readily available to quantify taxa present only as mycelium. Data on the diversity of fungi on natural substrata are often subjected to statistical analysis, without considering the complexity of the problem, variable samples, size, nature of the substratum, recently exposed or exposed over a period of time. This brings into question the meaning of the statistical figures generated, for example, there are problems in generating occurrence data when comparing the conidium of an anamorphic species with the apothecium of a Discomycete?

**Interference competition**

Much has been written about fungal competition, interference versus exploitation competition and the chemical basis for such interactions (Rayner and Todd, 1979; Glee, 1995; Shearer, 1995; Widden, 1997). This aspect will therefore not be considered in any detail here as it has been adequately addressed elsewhere. Shearer (1995) succinctly summarized the current situation with respect fungal competition “Less well known is the extent to which competitive interactions occur in fungal communities in nature, the importance of competition in determining fungal community structure, and
whether antifungal antibiotics are important in such interactions". She listed five experimental field studies that required investigation but few have followed up these suggestions (Panebianco et al., 2002; this volume; Fryar et al., pers comm.). Studies on interactions between wood decay fungi have advanced at a greater rate than those concerned solely with fungal succession (Dawson et al., 1988a,b; Robinson et al., 1993; Griffith et al., 1994).

How many fungi actually sporulate on any substratum? What are the chances that all species present will sporulate under the conditions of the experimental set up? Some fungi may inhibit the sporulation of other species. Tan et al. (1995) showed that the sporulation of some fungi could be affected by the presence of another species. Therefore, some species may not be detected. Similar observations have been made by Fryar et al. (pers. comm.) for freshwater fungi and alluded to by the field studies of Panebianco et al. (2002, this volume).

**Accounting for fungal succession on substrata**

In all the succession studies published in this volume, it is not clear what determines the occurrence of the fungi on the substrata. In the case of dung fungi and saprobic ammonia fungi it has been shown to depend on the rate of growth and sporulation of species. However, in most studies it is not clear why a succession of fruiting bodies occurs and few researchers have attempted to resolve this aspect in fungal ecology. Two major elements are implicated in the colonisation and dominance of fungi on a substratum. Initially it is the ability of species to settle, germinate and acquire domain. This in turn depends on the propagule loading in the surrounding environment, and their ability to settle on what may be a toxic surface. These aspects have been fully discussed by Jones (1994). Once germination has taken place then the rate of growth is important in the acquiring of domain, when it is vital to out compete other species. The second phenomenon is interference competition, when fungi may secrete compounds that can inhibit the growth of another colonising species. Most examples of this are confined to laboratory experiments. Such studies report on the interactions observed between pairs of species, with little supporting chemical data (Miller et al., 1985; Shearer and Zare-Maivan, 1988; Yuen et al., 1999). Strongman et al. (1987), however, have shown that the marine ascomycete *Leptosphaeria oreaeiris* does produce antifungal compounds that affect the growth of other marine fungi. Under high oxygen tension the sesquiterpene diol culmorin was produced, but at low oxygen tension accumulation of orthoquinone obionene occurred (Miller and Savard, 1989; Miller, 2000).
The importance of antibiosis in shaping the population in natural substrata is difficult to ascertain, when the hyphae of the individual species cannot be recognized. Again, the results of antibiosis investigation performed with pairs of species on artificial media can not be extrapolated directly to the natural situation, as they are usually concerned with a single, uniform substratum, in a stable environment (Wicklow, 1981). These are conditions that do not operate in a like manner in nature.

Similarly, there have been few field experiments to test whether such compounds are produced in nature, and if so at what concentrations are they effective? Such data will be difficult to acquire, as the concentrations may be very low and chemically undetectable. Panebianco et al. (2002) have attempted to follow the effect of pre-inoculating selected marine fungi on to test blocks and determine their subsequent colonisation by native species. The pre-inoculated test blocks with Halosphaeriopsis mediofetigera, did not support the sporulation of native species for up to 9 months submergence, indicating some form of inhibition. Jensen et al. (1998) have shown that the seagrass Thalassia testudinum secreted an antifungal compound (luteolin a new flavone glycoside: 7-O-beta-D-glucopyranosyl 2”-sulfate) that prevented the settlement and attachment of Schizochytrium aggregatum. Further field studies of this nature are required before we begin to understand the complex interactions that operate under field conditions.

Methods for the study fungal succession
Immunofluorescence

Detection and quantification of fungal mass in a particular substratum can be carried out using a variety of techniques. Fluorescence microscopy using conjugated lectins (Morrell et al., 1985) or fluorescent enhancers, such as Calcfluor, can reveal the presence of hyphae within their natural substratum, but the response is not specific as the hyphae of all the fungi present may also be labelled. By conjugating specific antibodies to a fluorescent compound, or by using the indirect method of a specific antibody followed by a commercially produced fluochrome-conjugated-species-specific antibody a means is obtained by which to visualize the homologous antigens, when viewed under UV illumination. The most frequently used fluorochrome is a fluorescein compound FITC (fluorescein isothiocyanate), which fluoresces bright green. Alternatives can also be used. Non-specific labelling can also be a problem, but various techniques are available to reduce or even eliminate this.

Immunological techniques can be used to follow the colonisation of substrata by a fungus, although it has not been widely used in fungal ecology. While much is known about the phenology of ascomata, conidia and
ascospores, the nature of their vegetative hyphae remains obscure, and may not even be mentioned in the species description of a new taxon. However, some authors do include a description of the typical appearance of the colony on a defined medium (Koch and Jones, 1984; Nakagiri and Tokura, 1994).

Specific recognition of the vegetative mycelium will allow both identification of species without the reliance on the production of fruiting structures, and the determination of any one species from a mixed population within its natural substratum. It is possible to achieve such recognition by the application of specific antibodies and standard immunological techniques. This has been achieved with various species of terrestrial fungi, but the procedures have been applied less frequently to marine fungi (Fries and Thorentolling, 1978; Newell et al., 1986; Fallon and Newell, 1989).

Immunological techniques are well-established, but with fungi the choice of antigen is important (hyphae, or spores) and the production of antibodies is expensive. This is a more powerful tool when linked with enzyme-linked immunosorbsorbent assays (ELISA). This uses the inherent binding properties of polystyrene microtitre plates for proteins, polysaccharides and liposaccharides, and the binding capacity of an antibody for the appropriate antigen to form a complex within the wells of the microtitre plate. This can then be visualized by addition of an enzyme (coupled to an antibody) and a chromogenic substrate solution.

Antigens from saprobic or parasitic fungi have been extracted directly from their natural (host) substrata and the extracts employed in ELISA, for example: Newell et al. (1986) and Fallon and Newell (1989) with fungi on Spartina.

To sum up: of the systems available ELISA is the most sensitive of those mentioned above. Newell et al. (1986) and Fallon and Newell (1989) have demonstrated its applicability to quantifying fungal biomass, achieving sensitivity levels of 0.5 μg mycelial dry weight / ml. Such extreme sensitivity suggests that very early colonisation of wood could be detected, thereby eliminating the reliance on the recognition of fruiting structures, and thus greatly reducing the time factor involved in such studies. Immunofluorescence is less discriminating than ELISA but has been used to quantify mycelium in substrate samples (Frankland, 1981). Polyclonal antibodies can also be used in determining the presence or absence of mycelium in a variety of substrata, and with many different species.

Immunogold cytochemistry can also be used to determine fungal activity in substrata as demonstrated by Daniel et al. (1990) in detecting Mn (II)-dependent and lignin peroxidases in wood degraded by white rot fungi. Au et
al. (1996) have also used this technique to chemically characterize fungal adhesives in the attachment of fungal spores to surfaces.

**Confocal laser scanning microscopy**

Another technique that offers scope in following fungal colonisation is confocal laser scanning microscopy and this has been applied to follow the colonisation of wood by bacteria and fungi. Xiao et al. (1999, 2000) discuss the merits of this method to follow the colonisation of bacteria and fungi within thick wood sections. They combined this technique with multi-fluorescent staining for the detection of Ophiostoma piceae in unseasoned radiata pine sapwood and in the examination of two fungi in the same sample.

These methods offer a potentially useful tool for examining the early stages of fungal colonisation when only a few taxa are involved. However, when the number exceeds 5, the method is not practical. This has to be considered when some ecological studies and colonisation pattern runs to over 80 species (Sivichai et al., 2000).

**Molecular techniques to detect fungi in the environment**

Future studies must tackle the issue of molecular identification of fungi in the substrata under study. While some progress has been made, these have largely been in the field of bacteriology (Ward et al., 1990), with only few studies on the role of fungi, and these tend to focus on identifying specific taxa in the environment (Goodwin et al., 1989; Groppe and Boller, 1997; Guo et al., 2001).

The initial step is the extraction of nucleic acids to isolate micro-eukaryotic DNA and RNA from the environment, design PCR primers and probes to amplify and identify 28S rRNA genes and ITS regions for taxa that are present (Liew et al., 1998). Specific techniques may be required for identifying fungi from different environments and/or substrata and this aspect has been addressed by Liew et al. (1998) and Torsvik et al. (1998). For example, from soil and sediments (Atlas, 1993; Holben, 1997; Viaud et al., 2000), aquatic environments (Ma et al., 1997; Fell and Kurtzman, 1990), animal (Bock et al., 1994) and plant hosts (Doss and Welty, 1995; Groppe and Boller, 1997). A more detailed discussion is beyond the scope of this concluding chapter. However, it is meant to underline the importance we place on developing techniques for the identification and enumeration of fungal taxa in various substrata, so as to follow more precisely the sequence of colonisation and the chemical interactions involved.
Conclusions

As indicated earlier, mycologists have been torn between trying to document fungal diversity and at the same time wishing to study the process of succession, which has usually resulted in recording the sporulation of fungi. This type of documentation provides data on diversity of fungi and changes in diversity over time, however, we have a poor understanding of the process of succession. This can only be achieved by focusing on the interactions that take place in the substratum under study, designing experiments that will give data on interactions. Future studies must also examine what is happening under field conditions, as laboratory based experiments may give rise to spurious results.

While molecular techniques may in due course enable us to identify non-sporulating fungi, we must remain vigilant when interpreting such data. Will it discriminate between dormant spores, actively growing mycelium and senescing mycelium? Clearly there is much experimental work to be undertaken before these problems are resolved. Wintzingerode et al. (1997) and Liew et al. (1998) have outlined some of the problems that have to be overcome, but remind us of the great potential of the method, “unrestricted by the limits of pure-culture techniques”. However, much interesting work can be done by using simple models but under field conditions, when only a few species are involved. Shearer (1995) has outlined some ways forward, but there has been little progress to date. Useful systems to try may be lignicolous marine or coprophilous fungi. Not only do we need to know what fungi are present on / in the substratum, but this must be accompanied by chemical data to determine if chemical interactions are taking place as well. Data on the role of chemical defence by marine animals and plants should pave the way for these studies (Paul, 1992; Jensen et al., 1998).

Acknowledgments

The senior author is grateful to S. Quelch for her assistance.

References


Fungal Diversity


Fungal Diversity


(Received 1 May 2002; accepted 1 June 2002)