The effect of pre-inoculation of balsa wood by selected marine fungi and their effect on subsequent colonisation in the sea

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A field study was undertaken in order to examine the effect of pre-incubation of test blocks of balsa with marine fungi and their subsequent effect on colonisation when submerged in the sea. Four marine ascomycetes, *Ceriosporopsis halima, Corollospora maritima, Halosphaeriopsis mediosetigera* and *Marinospora calyptrata,* were pre-inoculated onto balsa test blocks before submergence in the sea. Control and pre-inoculated test blocks were submerged in the sea at Langstone Harbour, Portsmouth, England, and recovered at 2, 6, 9 and 15 months and the fungi colonising them were recorded. The fungi pre-inoculated on the test blocks were the only species sporulating; there was no sporulation of native marine fungi. The control test blocks were heavily colonised by numerous sporulating marine fungi, and these were similar to those reported in previous studies in Langstone Harbour. These results are discussed in relation to inhibition of sporulation and colonisation and interference competition.

Key words: field observations, fungal ecology, marine fungi.

Introduction

Jones (2000) reviewed a number of factors known to affect the colonisation of wood by marine fungi in the sea, for example: availability of substrata, abundance of propagules in the water, degree of exposure, physical and chemical factors, timber species and a combination of environmental parameters e.g. temperature, salinity, pH, oxygen, light, and depth. Although a significant volume of data is available on the colonisation of wood by fungi in marine environments, little is available on the interactions between fungi in this process (Jones, 1963; Sanders and Anderson, 1979; Vrijmoed *et al.*, 1986; Shearer and Zare-Maivan, 1988; Fryar *et al.*, 2002). Several interpretations of the phenomenon of competition have been expressed by different authors, involving both laboratory and field studies, e.g. production of inhibitory compounds (Wicklow, 1981; Fisher and Anson, 1983; Miller *et al.*, 1985;

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Strongmann *et al.*, 1987), or hyphal interference (Skidmore and Dickinson, 1976; Wicklow, 1981).

There has however, been little correlation between laboratory studies and those in the field (Fryar *et al.*, 2002). Competition is also regarded as an event controlled partly by physical factors (size of the resource), and partly by biological factors. Both events occur after the fungus has settled on the wood (e.g. antibiosis: Sanders and Anderson, 1979); or as a phenomenon related to the rate of growth on the resource, so that nutrient depletion may also be an important factor (Hulme and Shields, 1970, 1972). With respect to marine fungi, most interference studies have been conducted under laboratory conditions (Miller *et al.*, 1985; Strongmann *et al.*, 1987).

Shearer (1995) commented, and succinctly summaries the current situation in fungal succession studies, "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 antibiotics are important in such interactions". She went on to outline five experimental field studies that needed to be addressed, but few have taken up the challenge (Fryar *et al.*, 2002). This paper is an attempt to take up one of these challenges. This paper examines the effect of pre-inoculating balsa test blocks with selected fungi to determine their effect on colonisation and sporulation in the field by native marine fungi.

Materials and methods

The fungi selected are all temperate fungi isolated from collections made in the Portsmouth area and include: *Corollospora maritima*, *Ceriosporopsis halima*, *Halosphaeriopsis mediosetigera* and *Marinospora calyptrata* (marine Ascomycota). All are lignicolous species that occur commonly on wood and have been shown in laboratory experiments to affect the growth of other marine fungi (Panebianco, unpubl. data). They were grown on seawater corn meal agar at 20 C in the dark. Sterile test blocks of balsa (1 × 1 × 2 cm) were placed on top of the growing mycelium in the Petri dishes for 11 weeks, and left undisturbed to ensure good penetration of fungal hyphae within the wood tissue. Test blocks were then placed in nylon litter bags together with sterilized balsa test blocks as a control. The bags were fixed inside a metal cage (60 × 40 × 40 cm) in a vertical position, so that there was good circulation of seawater around each block. Cages were submerged in the sea suspended from a raft in Langstone Harbour, South Coast, England, from October 1990 until January 1992. Replicate test blocks (ranging from 3 to 8) for each fungus and controls were exposed for 2, 6, 9 and 15 months. As some test blocks became contaminated during incubation, the number of test blocks available for

Table 1. Fungi colonising un-inoculated control test blocks of balsa wood.

Months	Test blocks recovered	Taxa observed	Percentage surface colonised	Number of ascomata	Percentage surfaces not colonised
2	7	*Corollospora maritima*	21	41	36
		Halosphaeriopsis mediosetigera	29	62	
		Ceriosporopsis halima	14	31	
6	7	*Ceriosporopsis halima*	29	2080	44
		Lulworthia sp.	4	25	
		Marinospora calyptrata	7	77	
		Lul. sp. / *M. calypt.*	4	14	
		Lul. sp. / *C. halima*	4	12	
		M.calypt. / C. halima	4	185	
		H. medio. /C. halima / M. calytrata	4	130	
9	8	*Ceriosporopsis halima*	9	375	1
		Halosphaeria appendiculata	28	329	
		Marinospora calyptrata	31	153	
		H. app. / M. calypt.	25	240	
		C. hal. / H. med.	3	60	
		H. app. / Lul. sp.	3	79	
15	9	*Marinospora calyptrata*	97	282	-
		Halosphaeria appendiculata	3	15	

exposure was lower than planned. Test blocks were removed from the raft and any fouling organisms present gently removed, washed with sterile seawater and incubated in damp Petri dishes at 4 C. Biofouling was present on the outside of the litter bags and was removed periodically through out the experimental period. Wood was heavily degraded by month 15, but this was the same for the controls and the pre-inoculated test blocks.

Test blocks were examined under a binocular microscope for the presence of sporulating structures, and fungi identified under a compound microscope. Each set of blocks was examined immediately on return to the laboratory, in order to record the occurrence of fungi already fruiting on the wood, and after 6-8 weeks of incubation. For each species identified, the percentage of surface cover by each fungus was calculated and the total number of ascomata recorded. For the former, the test blocks were gridded into 5mm squares and the presence / absence of the fungus noted, and the number of ascomata per grid counted.

Results

The total number of ascomata present and the area colonised (as a percentage) for each treatment are presented in Tables 1-5.

Control test blocks

During this study six ascomycetes *Ceriosporopsis halima*, *Corollospora maritima*, *Halosphaeria appendiculata*, *Halosphaeriopsis mediosetigera*, *Lulworthia* sp., and *Marinospora calyptrata* were recorded on the control un-inoculated test blocks (Table 1). Initial colonisation was by three species: *Halosphaeriopsis mediosetigera*, *Corollospora maritima* (recorded only on the first set of test blocks) and *Ceriosporopsis halima*. At 6 months the number sporulating on the wood was 4 with *C. halima* the dominant species. Species diversity was greatest at 9 months with *Marinospora calyptrata* and *Halosphaeria appendiculata* being co-dominant. *Marinospora calyptrata* was the dominant species at 15 months with a few ascomata of *Halosphaeria appendiculata*. The wood blocks were highly degraded with ascomata of *M. calyptrata* located deep in the wood. Sporulation occurred on 64% (2 months), 56% (6 months), 99% (9 months) of the surfaces and 100% coverage at 15 months.

Test blocks pre-inoculated with *Halosphaeriopsis mediosetigera*

Halosphaeriopsis mediosetigera was the only fungus that sporulated on the wood at 6 months, but at 9 months *Halosphaeria appendiculata* was also present (16% colonisation of the blocks) (Table 2). By 15 months few ascomata were found on the test blocks with *Halosphaeriopsis mediosetigera* and *Marinospora calyptrata* co-dominant. All surfaces supported heavy sporulation by *H. mediosetigera* over the first 9 months, but there was a marked drop in sporulation of this species at 15 months.

Test blocks pre-inoculated with *Corollospora maritima*

Corollospora maritima was the only fungus sporulating on the wood at 6 months (Table 3). At 9 months, *C. maritima* remained dominant with only a few ascomata of *Halosphaeria appendiculata*. At 15 months *Corollospora maritima* was no longer sporulating on the wood and *Halosphaeria appendiculata* and *Marinospora calyptrata* were co-dominant species with respect to percentage cover, but the numbers of ascomata of both fungi were low. Initially, *Corollospora maritima* only colonised 29% of the surface with increasing areas colonised at 6 (50%) and 9 (93%) months.

Table 2. Fungi colonising balsa test blocks pre-inoculated with *Halosphaeriopsis mediosetigera*.

Months	Test blocks recovered	Taxa observed	Percentage surface colonised	Number of ascomata	Percentage surfaces not colonised
2	4	*Halosphaeriopsis mediosetigera*	100	2760	-
6	7	*H. mediosetigera*	100	3391	-
9	8	*H. mediosetigera*	84	1917	-
		H. med. / H. append.	16	407	
15	2	*H. mediosetigera*	13	3	25
		Marinospora calyptrata	63	32	

Table 3. Fungi colonising balsa test blocks pre-inoculated with *Corollospora maritima*.

Months	Test blocks recovered	Taxa observed	Percentage surface colonised	Number of ascomata	Percentage surfaces not colonised
2	7	*Corollospora maritima*	29	106	71
6	7	*C. maritima*	50	80	50
9	7	*C. maritima*	93	598	-
		Halosphaeria appendiculata	4	18	
		C. mar. / H. append.	3	75	
15	5	*Marinospora calyptrata*	50	55	10
		Halosphaeria appendiculata	40	70	

Test blocks pre-inoculated with *Ceriosporopsis halima*

Ceriosporopsis halima was the sole sporulating species on the test blocks at the 2 month stage with 100% colonisation and abundant sporulation (Table 4). However, many species were sporulating on the wood at 6 months: *Lulworthia* sp., *Marinospora calyptrata* and *Halosphaeriopsis mediosetigera*. *Marinospora calyptrata,* with time, became the most abundant fungus on the test blocks (9 and 15 months) and was the dominant species at 15 months with only a few ascomata of *Halosphaeria appendiculata*. Once other fungi sporulated on the wood, the number of *C. halima* ascomata declined rapidly, from 1881 at 6 months to 496 at 9 months and none at 15 months.

Test blocks pre-inoculated with *Marinospora calyptrata*

At 2 months, *M. calyptrata* and *H. mediosetigera* were co-dominant species sporulating on the test blocks with a few ascomata of *C. halima* (Table 5). Five fungi were recorded at 6 months, all with low percentage cover and number of ascomata on the test blocks. *Marinospora calyptrata* was the only species on the wood at 9 and 15 months with the exception of a few ascomata

Table 4. Fungi colonising balsa test blocks pre-inoculated with *Ceriosporopsis halima*.

Months	Test blocks recovered	Taxa observed	Percentage surface colonised	Number of ascomata	Percentage surfaces not colonised
2	7	*Ceriosporopsis halima*	100	1881	-
6	7	*Ceriosporopsis halima*	39	496	18
		Lulworthia sp.	4	4	
		Marinospora calyptrata	11	36	
		Halosphaeriopsis mediosetigera	14	153	
		C. halima / M. calypt.	7	142	
		H. med. / M. calypt.	7	22	
9	8	*Ceriosporopsis halima*	25	162	-
		Halosphaeria appendiculata	13	184	
		Marinospora calyptrata	9	57	
		H. app. / C. halima	9	126	
		C. halima / M. calypt.	25	291	
		M. calypt. / H. app.	13	223	
		C. halima / M. calypt. / H. app.	6	170	
15	4	*Marinospora calyptrata*	94	307	-
		Halosphaeria appendiculata	6	8	

Table 5. Fungi colonising balsa test blocks pre-inoculated with *Marinospora calyptrata*.

Months	Test blocks recovered	Taxa observed	Percentage surface colonised	Number of ascomata	Percentage surfaces not colonised
2	4	*Marinospora calyptrata*	25	31	44
		Halosphaeriopsis mediosetigera	25	49	
		H. med. /C. halima	6	22	
6	7	*Marinospora calyptrata*	14	42	38
		Lulworthia sp.	4	10	
		Halosphaeriopsis mediosetigera	4	6	
		Halosphaeria appendiculata	7	62	
		Ceriosporopsis halima	14	52	
		C. halima / H. med.	4	72	
		Lulw. sp. / *M. calypt.*	7	54	
		H. append. / H. med.	4	50	
		C. halima / H. appen.	4	40	
9	7	*Marinospora calyptrata*	89	825	3
		Halospaheria appendiculata	4	10	
		Ceriosporopsis halima	4	2	
15	5	*Marinospora calyptrata*	100	291	-

of *Halosphaeria appendiculata* at 9 months. The most notable feature was the low sporulation of all the ascomycetes, with the exception of *M. calyptrata* at 9 and 15 months.

Discussion

There was a distinct difference in the sporulation and colonisation of the un-inoculated control balsa test blocks in comparison to those pre-inoculated with selected fungi. These differences may be accounted for in many ways.

Rate of colonisation and sporulation

The colonisation sequence of the control test blocks corresponds to that documented previously for temperate marine fungi (Jones, 1963, 1968; Byrne and Jones, 1974; Grasso *et al.*, 1985; Miller *et al.*, 1985). Early colonisers include *Ceriosporopsis halima*, *Lulworthia* spp. (*L. floridana*, *L. purpurea*, *L. rufa*), while others were less frequent as later colonisers: *Corollospora maritima*, *Halosphaeria appendiculata* and *Remispora maritima* (Jones, 1976). No data is available to resolve whether this is the result of sequential colonisation, rapid growth of particular species or the expression of differential sporulation times. However, the sequence of colonisation of the pre-inoculated blocks differs from the controls. These observations cannot be wholly explained by differences in sporulation time. Obviously pre-inoculated fungi were at a greater advantage in capturing a resource and in their ability to sporulate.

One aspect worthy of consideration is whether the incubation technique employed had any inherent effect on the ability of certain species to sporulate. This procedure has been widely used in marine and freshwater fungal ecology, but data is not available to ascertain whether incubation favours sporulation in some species (Prasannari and Sridhar, 1997; Vrijmoed, 2000).

Factors affecting ascomata formation

In a laboratory experiment that mimicked conditions in nature, Tan *et al.* (1995) demonstrated that certain marine fungi had a profound effect on the sporulation of other species. Three mangrove ascomycetes (*Aigialus parvus*, *Lignincola laevis* and *Verruculina enalia*) were able to grow well and sporulate on three mangrove timbers (*Avicennia alba*, *Bruguiera cylindrica* and *Rhizophora apiculata*). However, inoculation of mixed pairs or triplicate fungi resulted in a delayed onset of sporulation and affected the abundance of ascomata formed. For example, sporulation of *Aigialus parvus* was markedly reduced when paired with *Lignincola laevis*, or in combination with *Lignincola laevis* and *Verruculina enalia*. In contrast, when paired with *Verruculina*

enalia, Lignincola laevis enhanced ascomatal formation by *Verruculina enalia*. Tan *et al.* (1995) suggested these observations were the result of interference competition among the test fungi.

Interference competition

Much has been written on interference competition, especially of fungi in terrestrial habitats (Porter, 1924; Hulme and Shields, 1970, 1972; Skidmore and Dickinson, 1976; Rayner and Todd, 1979; Wicklow *et al.*, 1980; Lockwood, 1981). Indeed it is well documented that a variety of organisms sequestere metabolites to serve as chemical defences (Jensen *et al.*, 1998). Interference competition, mainly under laboratory conditions, have been reported for coprophilous, carbonicolous, lignincolous, phylloplane, and marine and freshwater fungi (Gloer, 1995). Gloer suggested "that fungal antagonism results from the production of biologically active metabolites by a fungus that can exert an effect on potential competition on predators".

Wicklow (1981) has also demonstrated that antibiosis not only exists in laboratory culture, but can be "a major determinant of population structure in many terrestrial fungi" (Miller, 2000). However, research on marine fungi is virgin territory, especially when considering field experiments.

Data from the present study indicates that *Halosphaeriopsis mediosetigera, Corollospora maritima,* and to lesser extent *C. halima,* sporulate early on the wood. On control blocks, *C. halima* was present at 2 months and became the dominant species at 6 months. However, this species appeared to be unable to seriously challenge any of the fungi on the pre-inoculated test blocks (*C. maritima, H. mediosetigera, M. calyptrata*). Earlier studies indicated that *C. halima, Lulworthia* sp. and *Zalerion maritimum* were early and dominant colonisers of beech and Scots pine test blocks submerged in Langstone Harbour (Jones, 1963, 1968; Miller *et al.*, 1985). The absence of *Zalerion maritimum* and low colonisation of blocks by *Lulworthia* sp., is surprising and warrants further investigation. Miller *et al.* (1985) found that the occurrence of *Ceriosporopsis halima* on test blocks dramatically affected the number of ascomata produced by *Lulworthia* spp. when both were present.

Marinospora calyptrata was late to colonise and / or sporulate on the wood in the un-inoculated controls, and wood pre-inoculated with *Ceriosporopsis halima* and *Marinospora calyptrata*. *Marinospora calyptrata* penetrates deep into the wood, requires a longer period to sporulate, and causes significant wood decay (Jones, unpubl. data). Thus in these experiments we are possibly dealing with competition between species and the nature of this interaction requires further investigation. The pre-inoculated fungi may produce bioactive compounds that prevent native species from colonising or

sporulating on the wood. These are interesting aspects to consider in future studies on the colonisation of substrata. The problem here is whether the bioactive compounds produced are in sufficient quantity to be chemically detected. Jensen *et al.* (1998) have shown that such compounds can be detected in the marine seagrass *Thalassia testudinum.*

This study has shown that *Halosphaeriopsis mediosetigera* and *Corollospora maritima* are the most successful test species in the inhibition of colonisation or sporulation by native fungi, followed by *Ceriosporopsis halima*. Initially, *Marinospora calyptrata* test blocks were colonised by a number of fungi, but after 9 months it became the dominant species.

Laboratory studies indicate that *Halosphaeriopsis mediosetigera* was the most active taxon in repelling the growth of 13 marine fungi; followed by *Corollospora maritima, Ceriosporopsis halima* and *Marinospora calyptrata* (Panebianco unpubl. data). The *in vitro* and *in vivo* results are therefore in agreement, although the *M. calyptrata* field data is complicated by the late sporulation of this species. In this respect the results from this study differ from those reported by Fryar *et al.* (pers. comm.) for peat swamp fungi pre-inoculated into wood and exposed under field conditions.

So far, no investigations of the effect of fungi pre-inoculated onto wood on subsequent colonisation by other fungi in the sea have been published. Fisher and Anson (1983) observed that the freshwater ascomycete *Massarina aquatica*, growing on oak (one of its natural substrata), produces compounds inhibitory for a range of microorganisms from its own habitat and also terrestrial species. A number of marine fungi have been shown to produce anti-microbial activity (Kupka *et al.*, 1981; Abbanat *et al.*, 1998; Albangh *et al.*, 1998; Biabani and Laatsch, 1998; Jones, 1998; Schlingmann *et al.*, 1998). Strongmann *et al.* (1987) have shown that *Leptosphaeria oraemaris* produces the sesquiterpene culmorin, with anti-fungal activity to the marine fungi tested. They concluded that this is clear evidence for interference competition in a lignicolous marine fungus.

This study set out to explore parameters operating in the colonisation of wood submerged in the sea. As stated earlier (Jones, 2000) a large number of factors determine which fungi eventually settle and colonise submerged substrata. We cannot conclusively state that the succession pattern observed in this study was due to interference competition and this is in common with most studies of this type (Jones, 1963; Alias and Jones, 2000; Sivichai *et al.*, 2000; Tsui *et al.*, 2000; Ho *et al.*, 2001).

A number of techniques could have been used to better understand the nature of the interactions observed. Firstly, the isolation of sub-samples of the test blocks to determine if all the fungi in the wood had been accounted for, but

few marine ascomycetes readily sporulate once isolated. Secondly, to test if the fungi under study affected sporulation of each other, since this might affect the sporulation of these fungi on the substratum. The same problem is encountered as most marine ascomycetes do not sporulate in culture, although Tan *et al.* (1995) have explored this with selected tropical marine fungi. They were able to show that when species were paired one often affected the sporulation of the other. Thirdly, the use of molecular techniques to identify none sporulating fungi in wood have not yet been sufficiently well-developed, but is the way forward in fungal ecology. Some of these problems are considered in greater detail in a paper elsewhere in this volume (Jones and Hyde, 2002).

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