Plant and Soil 71, 247–256 (1983). A-24

The concept of succession in relation to the spread of sheathing mycorrhizal fungi on inoculated tree seedlings growing in unsterile soils

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Key words Birch Mycorrhizal fruitbodies Mycorrhizal succession Sheathing mycorrhizas Sitka spruce

Summary Repeated annual assessments of the toadstools (fruitbodies) of mycorrhizal fungi associated with a mixed stand of *Betula* spp. indicated that they were produced in a pattern ordered in time and space, suggesting a succession with identifiable early- and late-stage fungi. This concept is supported by below-ground observations of mycorrhizas which, however, need to be augmented.

Both early- and late-stage mycorrhizal fungi form mycorrhizas on seedlings growing in axenic ('aseptic') conditions. In contrast, only early-stage fungi seem able to trigger mycorrhizal formation on seedlings growing in unsterile soils.

During axenic propagation, the early-stage *Hebeloma sacchariolens* and the late-stage *Amanita muscaria* formed similar numbers of mycorrhizas per root system. After being transplanted to a range of unsterile field soils, *A. muscaria* failed to keep pace with the spread of the developing root system: no more *A. muscaria* mycorrhizas were formed. On the other hand the continued development of *H. sacchariolens* mycorrhizas precluded, during the first season after transplanting, the development of mycorrhizas by fungi naturally occurring in field soils. In the second season, however, the development of *H. sacchariolens* mycorrhizas was restricted in acid peat but not in three other types of soil.

The development of *Laccaria* mycorrhizas after inoculating Sitka spruce with this fungus was associated, irrespective of soil type, with accelerated tree growth; with heights at the end of the first season being doubled.

Introduction

The benefits conferred on trees by sheathing mycorrhizas are not in dispute although the substantive field evidence leaves much to be desired. To ensure the early development of effective mycorrhizas, and hence successful plant establishment, forest nursery seedbeds and/or seedlings have been inoculated with naturally infested (i) soils, (ii) raw humus or (iii) leaf litter[17]. But the use of these usually bulky and unwieldy inocula has often incurred risks of introducing root pathogens and weeds. A possibly less hazardous approach, which is attracting increasing interest is the use of pure cultures. When testing pure cultures of mycorrhizal fungi, it was seen that different fungi possessed different *in vitro* cultural characteristics, growth rates and temperature optima as well as

different host preferences[22]. This, and further evidence, now suggests that the (i) development and (ii) effectiveness of tree mycorrhizas depend, as in the legume root nodule complex, upon a range of genetically controlled host and fungal factors. But which fungus, or isolate of a fungus, should be used when attempting to forge beneficial associations which will persist after planting out?

Microbiologists are very much aware that the assemblages of bacteria and fungi colonising the surfaces of leaves and roots change in a systematic manner as their substrates get older. This being so, it should not be surprising if the fungi forming mycorrhizas with a tree, 40 years-old, differ from those associated with saplings – an age related succession. Although the concept of 'mycorrhizal succession' is only now being enunciated there are already several pieces of circumstantial evidence. How significant are they and how will the impact of mycorrhizal succession affect the choice of fungi for the controlled inoculation of tree seedlings?

Concept of succession

With some notable exceptions most of the lists associating fruitbodies of sheathing mycorrhizal fungi with trees have paid little or no attention to the age of the host[21]. But when the occurrence of fruitbodies was recorded during the first ten years after planting a mixed stand of birches (*Betula pendula* Roth and *B. pubescens* Ehrh.), clear evidence was obtained of a sequence in both time and space[15]. Fruitbodies of *Hebeloma crustuliniforme* (Bull. ex St. Amans) Quélet and a *Laccaria* sp. were observed within two years after planting. These fungi were joined by *Thelephora terrestris* Ehrenb. ex Fr. (in year 3), *Inocybe lanuginella* (Schroet.) Konrad and Maublanc and *Lactarius pubescens* (Fr. ex Krombh.) Fr. (year 4), *Hebeloma* spp. (year 5), species of *Cortinarius* and *Leccinum* in year 6 and of *Russula* in year 10.

In year 2, fruitbodies of *H. crustuliniforme* were concentrated in a 'ring', encircling stem bases, with a mean radius of 22 cm which by year 6 had increased to 73 cm. The fruitbodies of *Lactarius*, like those of *H. crustuliniforme*, tended to occur in zones of maximal density which first showed in year 4 with a radius of 40 cm and then progressively increased to 69 cm in year 6. In the same year, fruitbodies of the newly appearing *Leccinum* spp. were nearest the trees (27 cm from stem bases). Thus it was possible to discern the elements of a number of concentric rings with each new species initially appearing close to the bases of trees and then moving progressively outwards in successive years[6]. Interestingly, the fruitbodies of *Laccaria* sp., instead of being arranged in rings, seem to follow the lines of secondarily thickened roots.

In addition to being associated with birch, fruitbodies of *Hebeloma crustuliniforme*, *Laccaria* species, particularly *Laccaria laccata* (Scop. ex Fr.) Cooke, *Thelephora terrestris* and species of *Inocybe* have also been found in association with young conifers. *Hebeloma crustuliniforme* has been recorded in

abundance with seedlings of *Pseudotsuga menziesii* (Mirb.) Franco[23] and *Pinus radiata* D. Don before, but not after, transplanting[2]. In contrast, fruitbodies of species of *Suillus* and *Inocybe* were associated with stands of *P. radiata* 5 or more years old but seldom with younger trees. Thus the sequences of mycorrhizal fruitbodies associated with birches and pines have many features in common. Furthermore, toadstools of *Amanita muscaria* (L. ex Fr.) Pers. ex Hooker were found in plantations of *P. radiata*[2] and *Pseudotsuga menziesii*[3] when more than 10 and 12 years old respectively. As yet they have not appeared in our experimental birch plot which is now 11 years old. This clear age dependence is supported by observations made in southern India where the annual production of *Amanita muscaria* toadstools increased from 32 to 3,720 per thousand trees in plantations of *Pinus patula* Schl. and Cham. 5 and 16 years-old respectively[10].

Together these fruitbody observations suggest that some mycorrhizal fungi (*e.g. Hebeloma*, *Laccaria* and *Inocybe*) are characteristic of young stands of trees whereas others *e.g.* species of *Leccinum*, *Russula* and particularly *Amanita* are characteristically associated with older trees. Although the occurrence of fruitbodies does not give a comprehensive insight of events below ground-level, the idea of mycorrhizal succession based on fruitbody data is given credence by the apparently close relation between the presence and position of (i) mycorrhizas and (ii) the fruitbodies of their associated fungi[8,20]. Furthermore, Warcup (pers. comm.) when working in the stand of *Betula* spp., already mentioned, was able to relate the fruitbodies to their own distinctive types of mycorrhizas (Table 1).

Root isolations by Chu-Chou and Grace[3] confirmed that *Hebeloma crustuliniforme* was only associated with young seedlings of Douglas fir and that *Amanita muscaria* could only be isolated from mycorrhizas when trees were 13 or more years old. Mycorrhizal root observations[9,13,19] on nursery and outplanted saplings have also shown that the range of mycorrhizas associated with forest trees can alter considerably as trees get older. Thus the evidence derived from root isolates and the appearance of mycorrhizas supports the concept of mycorrhizal succession.

We know that fungi found both early and late in the mycorrhizal sequence readily form mycorrhizas in axenic culture[14]. However, the functional importance of succession was recognised when birch seedlings were planted into soil cores sampled immediately beneath fruitbodies of species of *Hebeloma*, *Inocybe*, *Laccaria*, *Lactarius* or *Leccinum*; the seedlings developed numerous mycorrhizas of *Laccaria*, *Inocybe* and *Hebeloma*, a few with *Lactarius* and none with *Leccinum*[4]. Fox[7], similarly, found that birch seedlings planted into basidiospore amended soils readily developed mycorrhizas with *Hebeloma* and *Inocybe* species but not with *Lactarius* or *Leccinum*. Thus, it seems that early-stage fungi, *e.g. Hebeloma* and *Laccaria*, are able to form mycorrhizas on young seedlings growing in unsterile as well as sterile soils, whereas late-stage fungi will only form mycorrhizas on seedlings growing in axenic conditions. But is the

Table 1. The relation between (i) fungi isolated from mycorrhizas and (ii) the occurrence of fruitbodies when observations were made on 5 birch saplings of *Betula pendula* during their fourth season after being planted into a brown earth (Warcup, pers. comm., 1975)

Tree	Fungi isolated from different mycorrhizas	Fruitbodies recorded in association in the autumn
1.	*Thelephora terrestris*	None
2.	*Hebeloma* sp.	*Hebeloma* sp.
	An unidentified basidiomycete	
	An unidentified ascomycete	
3.	*Laccaria 'laccata'*	*Laccaria 'laccata'*
	An unidentified basidiomycete	
	A white ascomycete differing from the unidentified ascomycete ex tree 2.	
4.	*Hebeloma* sp.	*Hebeloma* sp.
	Lactarius pubescens	*Lactarius pubescens*
	Thelephora terrestris	
	An unidentified ascomycete	
5.	*Hebeloma* sp.	*Hebeloma* sp.
	Lactarius pubescens	*Lactarius pubescens*
	Laccaria 'laccata'	*Laccaria 'laccata'*
	An unidentified ascomycete	

development of mycorrhizas solely host/fungus dependent? Can the association be altered by other soil microbes or by different soil types?

The colonisation of birch seedlings by early-and late-stage fungi

To examine rates of spread and abilities of different mycorrhizal fungi to compete with naturally occurring microbes, birch seedlings were planted into a range of unsterile soils after being inoculated, during axenic propagation[16], with *Hebeloma sacchariolens* Quélet (an early-stage fungus), *Amanita muscaria* (a late-stage fungus) or *Paxillus involutus* (Batsch) Fr. (a fungus often associated with young birch trees growing on slag heaps and other sites of dereliction.)

At the end of the 8 week period of axenic propagation at least 65% of the root tips of inoculated seedlings were mycorrhizal. When a second sample was inspected 121 days after being transplanted into one of 4 different soils (2 peaty and 2 mineral) the distribution of mycorrhizas differed greatly. At this time, the end of the first growing season, mycorrhizas, derived from naturally occurring soil inocula and attributed to *Laccaria proxima* (Boud.) Pat., *Inocybe lanuginella* and *Thelephora terrestris*, had developed on the uninoculated controls. Mycorrhizas formed by a similar group of mycorrhizal fungi had developed on the seedlings inoculated with *Amanita muscaria*, this fungus appearing to be totally incapable of forming mycorrhizas on seedlings in unsterile conditions;

Table 2. Percentages of sheathing mycorrhizas, after growing seedlings of *Betula pendula* in a range of 4 unsterile soils, attributable to the fungi introduced two years earlier while propagating in axenic conditions

		Fungi introduced during propagation		
		Hebeloma sacchariolens	*Paxillus involutus*	*Amanita muscaria*
Peaty soils	A	0	44	0
	B	100	29	0
Mineral soils	C	93	14	0
	D	99	43	0

even the mycorrhizas formed during axenic propagation could not be traced. In contrast, *H. sacchariolens* and *P. involutus* both spread to form numerous mycorrhizas to the total exclusion of mycorrhizas formed by naturally occurring microbes. The latter were absent even though many roots were non-mycorrhizal.

Although the different soil types didn't significantly affect the development of *H. sacchariolens* and *P. involutus* during the first year, there were appreciable interactions in the second year (Table 2).

H. sacchariolens continued to spread and colonise new roots on seedlings in 3 of the 4 soils, with at least 90% of root fragments becoming mycorrhizal but in the fourth soil (A), an acid peat, it failed totally. Instead the mycorrhizas formed in the second year in this combination of treatments, were attributable to naturally occurring soil-borne inocula. Mycorrhizas of *Paxillus involutus* developed less prolifically than those of *H. sacchariolens*. They accounted for 40–50% of the mycorrhizas on seedlings in two of the four soils and less than 30% on seedlings in the other soils. At the end of the second year a detailed

Table 3. Percentages of mycorrhizas, two years after planting inoculated birch seedlings into a range of four unsterile soils, attributable to naturally occurring mycorrhizal fungi. (See Table 2)

		Naturally occurring mycorrhizal fungi*				
		*Hebeloma*** sp.	*Inocybe* sp.	*Thelephora terrestris*	*Cenococcum geophilum*	*Leccinum* sp.
Peaty soils	A	0	0	17	0	51
	B	0	5	66	<1	<1
Mineral soils	C	51	0	9	8	0
	D	49	0	31	0	0

* These figures show the percentages of mycorrhizas of naturally occurring mycorrhizal fungi on seedlings irrespective of their original treatment.

** These figures do not include mycorrhizas of *Hebeloma sacchariolens* (see Table 2).

assessment was made of the mycorrhizas formed by naturally occurring fungi irrespective of the inoculation treatment (Table 3). Interestingly, mycorrhizas were only formed by naturally occurring *Hebeloma* spp. when seedlings were growing in mineral soils. This suggests that the occurrence of this genus may be strongly controlled by soil type.

The inability of naturally occurring fungi, during the first year, to colonise seedlings inoculated with *H. sacchariolens* and *P. involutus* suggests that they can be kept in check by the use of judiciously selected fungi. At the end of the second year, it appears that the early advantage afforded by inoculation during axenic propagation may eventually be overtaken, albeit by isolates of other early-stage fungi.

Effects of *Laccaria* sp. and *Paxillus involutus* on the growth of Sitka spruce seedlings

The results of experiments done with birch encouraged the inoculation of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) with *Laccaria* sp. or one of two isolates, 0086 and 16, of *Paxillus involutus*. As before seedlings were inoculated during axenic propagation and then transferred eight weeks later to pots containing two peat and two mineral soils.

Unlike the experiment done with birch there were significant differences in mycorrhiza formation during propagation (Table 4). These differences were maintained so that by the end of the first growing season, mycorrhizas attributable to *Laccaria* sp. were most numerous (67%) and those attributable to *P. involutus* 0086 the least numerous (5%) (Table 4). Moreover, growth appeared to be directly related to the abundance of mycorrhizas. Isolate 0086 of *P.*

Table 4. Percentages of Sitka spruce root fragments with mycorrhizas at the end of the first growing season after inoculating with three different fungi

	Inoculated during propagation with				
Samples	Uninoculated	*Paxillus involutus*		*Laccaria*	L.S.D.
taken at	controls	isolate 0086	isolate 16	sp.	($p = 0.05$)
End of propagation in axenic conditions (days 0–63)	0* (*0.0*)	2 (*7.0*)	12 (*20.0*)	76 (*60.7*)	(*27.72*)
End of first growing season in unsterile soils (days 63–221)	5 (*12.3*)	5 (*12.9*)	22 (*27.9*)	67 (*55.2*)	(*2.35*)

* For statistical analysis, percentages were transformed to angles which are italicised.

Table 5. Effects of inoculating with three mycorrhizal fungi on the heights of Sitka spruce seedlings.

Samples taken at	Inoculated during propagation with: Uninoculated controls	*Paxillus involutus* Isolate 0086	*Paxillus involutus* Isolate 16	*Laccaria* sp.	LSD ($p = 0.05$)
End of propagation in axenic conditions (days 0–63)	2.8 cm	2.9 cm	2.9 cm	3.0 cm	1.02
End of first growing season in unsterile soils (days 63–221)	3.1 cm	3.6 cm	4.8 cm	6.5 cm	0.17

involutus was associated with a 58% height increase whereas *Laccaria* sp. stimulated a greater than 100% increase in shoot height (Table 5). Similarly inoculation with *Laccaria* trebled numbers of root fragments whereas *P. involutus* isolate 0086 increased them by merely 71%.

These results emphasise the importance of inoculating tree seedlings with 'effective' isolates of selected mycorrhizal fungi. Nevertheless, although not too

Table 6. Effects of inoculating Sitka spruce seedlings with three mycorrhizal fungi on subsequent root growth expressed as numbers (n) of root fragments*

Samples taken at	Inoculated during propagation with: Uninoculated controls	*Paxillus involutus* Isolate 0086	*Paxillus involutus* Isolate 16	*Laccaria* sp.	LSD ($p = 0.05$)
End of propagation in axenic conditions (days 0–63)	7** *(0.91)*	14 *(1.17)*	16 *(1.23)*	13 *(1.15)*	*(0.43)*
End of first growing season in unsterile soils (days 63–221)	52 *(1.72)*	89 *(1.96)*	130 *(2.12)*	150 *(2.18)*	*(0.04)*

* For statistical analysis numbers (n) of root fragments per plant were transformed to log (n + 1). Transformed data are in italics.

** For the sample taken at the end of propagation, (n) represents the total number of roots on each seedling, whereas for the end of season sample, (n) represents the total number of roots counted in zones 1 cm wide and spaced 2 cm apart.

Table 7. The characteristics of early- and late-stage fungi associated with birch saplings planted in a brown earth at Bush Estate, Edinburgh in 1971

	Laccaria spp. (early -stage)	*Lactarius pubescens*	*Amanita muscaria* (late -stage)
Years after planting in which fruitbodies of mycorrhizal fungi were first observed	2	4	>10
Years after planting in which fungi were first isolated from mycorrhizas	2	4	>10
Ability (+ or −) to form mycorrhizas when a) basidiospores were added to unsterile soil[7]	+	−	N.t.
b) mycelial cultures were added to unsterile soil[4]	+	+/−*	−
Ability (+ or −) of mycorrhizal fungus to spread after transferring inoculated seedlings from axenic propagation to unsterile soil	+	+	−

N.t. = Not tested.
* +/− = Mycorrhizas very seldom formed.

much should be drawn from the use of one isolate, it seems that the use of *Laccaria* species might prove to be rewarding[18] – this fungus seems easy to establish and appears to be a strong competitior, conferring, at the same time measurable benefits on its hosts.

Critique

The results in this other papers[4,5,7,11,12] suggest that the concept of 'succession' has an important bearing on the selection of fungi for inoculating young tree seedlings. Although fruitbody observations by themselves do not tell the whole story, they have highlighted the occurrence of a sequence of early-, followed by late-stage fungi (Table 7).

Early-stage fungi, such as *Laccaria* spp., are associated with young tree seedlings; they have the ability to colonize roots and develop mycorrhizas in unsterile soil, irrespective of naturally occurring inocula. In contrast, late-stage fungi, such as *Amanita muscaria* are unable to spread and form mycorrhizas on

young seedlings growing in unsterile soils. No doubt, a range of intermediates exist and will be identified in due course. *Lactarius pubescens*, for example, possesses little or no ability to infect seedlings from inoculum added to unsterile soils yet can spread from mycorrhizas, pre-formed in axenic conditions (Table 7).

The concept of mycorrhizal succession seems to provide a rational basis for the selection of fungi for inoculating tree seedlings. However much more needs to be known about the sequence of fungi associated with tree saplings growing in a range of soils, *e.g.* the peats which occupy a central position in British forestry. Knowledge of this sort may help explain the recently reported failure of *Laccaria laccata* and *Hebeloma crustuliniforme* to infect new roots of Douglas fir[1]. It may also enable us to understand the role of *Paxillus involutus* which appears to dominate from the outset on sites of dereliction yet can be replaced by a range of fungi in mineral and peaty soils within 1–2 years.

On balance, the evidence to date suggests, when considering controlled inoculations, that the choice of fungus should, in the first instance, be controlled by its position within the mycorrhizal succession; secondly by its ability to stimulate the growth of its host, some isolates being more beneficial than others; and thirdly it is clear that the persistence of the association will depend not only on the host × mycorrhizal fungus interplay but on a three membered system host × mycorrhizal fungus × other soil microbes, with each component being subject to environmental influences.

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