

At the frontier between Basidiomycotes and plants: reciprocal interactions between mycorrhiza formation and root development in an *in vitro* system with oaks and Hymenomycetes

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The relation between mycorrhization dynamics and elongation of the lateral root system was compared in an *in vitro* system with oak microcuttings between five ectomycorrhizal basidiomycetes. The experiment allowed the recognition of two schemes of mycorrhization depending on whether the endogene rhythmic growth, characteristic for oaks, is involved. Additional experiments with one representative mycobiont of each scheme showed a diversity of mycorrhization patterns. This might be explained by competition between the fungus and the roots for plant resources and that their variations are related to rhythmic growth cycles of the oak. Based on this interpretation, we propose to integrate the classical carbon and hormone theories in a common hypothesis that considers the formation of ectomycorrhizas by a wide diversity of Ascomycota and especially of Hymenomycetes (Basidiomycota) as a result of a fine tuning between a few mechanisms, i.e., the individual carbon demand of each fungus, the production levels of growth promoting factors by the fungus and by the plant, and the rhythmic growth. Rhythmic plant growth has never been considered in mycorrhizal research so far. The application of molecular tools should allow assessment of the hypothesis in future.

Most higher plants do not have roots, they have mycorrhizas.” This quote from a popular text book of ecology (BEAGON, HARPER & TOWNSEND 1996), a scientific field with conceptual difficulties regarding mutualism (BOUCHER 1985), reflects the place that mycorrhizal symbioses occupy in biology approximately 120 years after their first description by FRANK (1885). In the meantime, fossil evidence has shown that the first land plants emerging from the aquatic environment were associated with fungi related to the actual mycobionts of arbuscular mycorrhizas (AM) (REDECKER, KODNER & GRAHAM 2000, REDECKER 2002). This fungal group was long regarded as an order (Glomales) within the Zygomycota. Recently, molecular considerations have led to placing it into a new phylum, the Glomeromycota (SCHÜBLER, SCHWARZOTT & WALKER 2001).

From the bryophytes to the spermatophytes, 80 % of modern terrestrial plants form mycorrhizas of the AM type (see for example the list of HARLEY & HARLEY 1987). The main ecological function of AM fungi that dominate in mineral soils and soil horizons is the mobilization of phosphorus (see

review by BUSCOT et al. 2000), an essential biological element with low concentration and mobility (PAUL & CLARK 1996). Another important type of mycorrhizas, the ectomycorrhizas (EM), is formed on forest trees of the boreal, temperate and sometimes also tropical regions by fungi in the Ascomycota and especially in the Hymenomycetes (Basidiomycota). Although not demonstrated formally, it seems likely that genesis of the first soils – characterised by high inputs of plant debris and their transformation into more or less stable soil organic matter that accumulates in specific horizons (WILD 1993) – was the key evolutionary process leading to the emergence of the EM symbiosis, besides the occurrence of trees. Soil organic matter constitutes a reservoir of nondirectly available nutrients for plants. In such soil compartments, the limiting factor apart from phosphorus is nitrogen, which besides its sparse concentration is distributed heterogeneously within a wide range of minerals and complex organic forms (READ 1993, READ & PEREZ-MORENO 2003). It makes sense that to mobilize resources from such a heterogeneous medium, a high biodiversity of fungi with different specific metabolic capabilities is necessary. This consideration may explain how more than 5000 fungal taxa are estimated to form EM versus approximately 200 for AM (SMITH & READ 1997).

From their parasitic biotrophic associations with plant leaves and stems, basidiomycota are known to be often extremely specific, and this trait led to a fascinating coevolution between host plants and parasitic mycobionts (see OBERWINKLER 1993). However, such specificity is not observed in basidiomycetous EM fungi, most of which are known to have a wide range of potential plant partners. Nevertheless, the morphological and anatomical features of EM are species specific and can be used to identify EM mycobionts (AGERER 1987-1998). Of the traits used in this morphotyping, fungal features like the organisation of the hyphal mantle and the branching pattern of the EM rootlets are considered to be quite constant within associations between two given partners.

In contrast to these species-specific morphological traits, EM mycobionts are known to display a wide functional diversity at both inter- and intraspecific levels. Experimental studies showed that their capability to form EM and their effects on plant growth or nutrient acquisition is extremely variable between species and even strains of the same species (see review in SMITH & READ 1997). Differences in the carbon demand for optimal growth or in the production of growth promoting factors have also been reported between and within species (HUTCHINSON & PICHE 1995). This variability is of importance as both factors have long been considered to be involved in the EM formation itself (NYLUND 1988).

The duality between a quite constant species-specific morpho-anatomical organisation of EM formation, on the one hand and their wide functional diversity, inclusive of traits involved in EM formation, on the other hand reword the mechanisms that control the interaction. The convergent occurrence of EM in many different families of the Ascomycota and Hymenomycetes (Basidiomycota) to which saprotrophs and parasites also belong strongly suggests that EM development results rather from a special pattern of regulation of the genome than from the acquisition of specific EM symbiosis-related genes at a defined point of the fungal evolution. In this article, we try to identify such regulative processes by making detailed morphological observations on a model system that involves oak microcuttings for synthesis of EM under strictly controlled conditions.

Different culture systems for synthesis of EM have been developed (PETERSON & CHAKRAVARTY 1991). In particular Petri dish systems in which the roots grow two-dimensionally allow nondestructive observations of the plant development in relation to the EM formation under strictly controlled conditions (KOTTKE et al. 1987). Compared to *in vitro* systems that handle young seedlings, the model system we use here has the advantage of involving microcuttings revealing morphological and physiological characters of adult trees (HERRMANN, MUNCH & BUSCOT 1998, HERRMANN et al. 2003,

HERRMANN, OELMÜLLER & BUSCOT 2004). We compared the patterns of EM formation in relation to plant development with several homobasidiomycetous mycobionts (*Hebeloma crustuliniforme*, *Laccaria amethystea*, *Laccaria laccata*, *Paxillus involutus*, *Pisolithus tinctorius*). The diversity of patterns and interactions is discussed in the perspective of competition between fungus and roots for resources and their variations, related to the growth rhythmicity characteristic for many ectomycorrhizal plant species.

Material and methods

EM fungi *Paxillus involutus* (Batsch) Fr. strain F 1667, *Laccaria amethystea* (Bolt.: Hocker) Murr. strain F 1629, *Hebeloma crustuliniforme* (Bull.: St. Amans) Quél. strain F 1584, *Pisolithus tinctorius* (Pers.) Coher & Couch strain F 1626, and *Laccaria laccata* (Scop.: Fr.) Berk. & Br. strain F 1647 were provided from the TUMY type collection by the *Lehrstuhl für Spezielle Botanik und Mykologie* at the University of Tübingen Germany. Cultivation was performed on MMNC agar medium, where C is given for additional casein hydrolysate (KH₂PO₄ 0.5 g; (NH₄)₂HPO₄ 0.25 g; MgSO₄·7 H₂O 0.15 g; CaCl₂·2H₂O 0.066 g; NaCl 0.025 g; glucose monohydrate 10 g; malt extract 5 g; casein hydrolysate 1 g; thiamine hydrochloride 100 µg (see MARX 1969, KOTTKE et al. 1987) supplemented with Murashige and Skoog minor elements H₃BO₃ 6.2 mg; MnSO₄·H₂O 16.9 mg; ZnSO₄·H₂O 10.59 mg; KJ 0.83 mg; Na₂MoO₄·2H₂O 0.25 mg; CaCl₂·6H₂O 0.025 mg; CuSO₄·5H₂O 0.025 mg and Fe-EDTA from Murashige and Skoog; Na₂·EDTA 37.25 mg, FeSO₄·7H₂O 27.85 mg (see MURASHIGE & SKOOG 1962) and 20 g agar (Merck), pH 5.6. Incubation was performed at 20 °C in darkness.

Quercus robur L. was micropropagated and rooted as described in HERRMANN, MUNCH & BUSCOT (1998). For mycorrhizal synthesis, rooted microcuttings were transferred into Petri dishes (80 or 140 mm diameter) on a MMN1/10 medium, which is a MMN medium with following modifications: KH₂PO₄ 0.05 g, (NH₄)₂HPO₄ 0.025 g, no carbohydrate source (HERRMANN, MUNCH & BUSCOT 1998) containing alternatively 2 g activated charcoal (AC). Roots grew two-dimensionally in the Petri dish between two nylon discs (70 µm mesh size). Inoculation with EM fungi precultivated on MMNC was performed 14 days later on the same Petri dish. Incubation during mycorrhizal synthesis was performed in growth chambers at 25 °C with 16 h illumination. After a short acclimatization period at a high relative humidity of 95 %, the humidity was reduced to 70 %.

As previously described (HERRMANN, MUNCH & BUSCOT 1998), the root system of the micropropagated oaks consists of an orthotrope main root (corresponding to the taproot of the seedlings) and plagiotrope ramified roots. This plagiotrope root system is subdivided into first- and second-order lateral roots, termed mother roots (MR) after they ramify, and in first- and second-order short roots. Lateral roots (and therefore mother roots) and short roots differ on the basis of their size.

Elongation and formation of the mother and short roots and mycorrhiza formation were monitored during the whole experiments. For the first-order mother roots, the elongation is reported in a non-cumulative manner for each 10 day period in the first experiment and in a cumulative manner for the 7 day periods in the second and third experiments. Measurements were performed by a digitized image-analysis system as presented by HERRMANN, MUNCH & BUSCOT (1998). For each of the three independent experiments presented, six replicates per treatment were performed.

Results

In a first assay, we compared the dynamic of EM formation between five mycobionts in relation to the rhythmic growth of the roots. In the two following assays, conducted with *L. amethystea* and *P.*

tinctorius, we identified different mycorrhization patterns and related their occurrence to root morphogenesis during the growth flushes.

Preliminary comparative study of mycorrhization by five different mycobionts

After 14 days preculture in small Petri dishes, oak microcuttings were inoculated with *P. involutus*, *L. amethystea*, *H. crustuliniforme*, *P. tinctorius* or *L. laccata*. The co-cultures were performed on media with or without addition of activated charcoal (AC). Table 1 summarises the development and mycorrhization of one representative plant per treatment. Variations in the length of the first-order mother root (MR) segments produced each 10-day period reflect the rhythmic growth of the root system. Short root formation occurred within a few days after the development of each MR segment.

Paxillus involutus and *Laccaria amethystea* were able to form high percentages of mycorrhizas independently of the presence or absence of AC. With *P. involutus*, the general root development was distinctly stimulated and the highest number of mycorrhizal short roots (over 200) obtained. This intensive formation of mycorrhizas was continuous over 40 days and not influenced by the root growth flushing. With *L. amethystea*, mycorrhizas formed rapidly after inoculation, and were therefore the most abundant on the oldest segments of the MR. In the absence of AC, the growth of MR slowed down immediately after early mycorrhization. In this case the limiting factor for further mycorrhizal formation was the weak formation of short roots itself.

In the presence of *Hebeloma crustuliniforme*, mycorrhization was low, and AC had no enhancing effect. Both the number of mycorrhizas and the percentage of mycorrhizal short roots remained the lowest among the different inoculated assays. Mycorrhizal formation was strictly limited in time and apparently related to the maximum elongation period within the rhythmic growth flush of the MR.

In co-cultures with *Pisolithus tinctorius* or *Laccaria laccata*, AC was able to enhance mycorrhiza formation. With both fungi, the number of mycorrhizas was doubled by addition of AC. Mycorrhization onset was observed over a prolonged period of the co-culture. In presence of *P. tinctorius* and AC, the high mycorrhization rate indicated that short root formation was the factor limiting mycorrhization.

For two EM fungi, *L. amethystea* and *P. tinctorius*, we observed that mycorrhiza formation was related to several different root ramification patterns. In order to find out under which conditions these patterns occurred, we studied their appearance in relation to the lateral root morphogenesis. The results are presented in the following two sections.

Ramification patterns during EM formation with *Laccaria amethystea*

The observation of the final root architecture of an oak microcutting inoculated in a small Petri dish with *L. amethystea*, a mycobiont with a violet mycelium, revealed two different patterns of root ramification and distribution of mycorrhizas (Fig. 1). In the proximal upper part of the root system, the mycorrhizas were formed at the distal end of the lateral roots, which displayed limited growth and did not produce short roots (Figs 1A, 1B). In the distal part of the main root, lateral roots displayed prolonged growth. Their violet colour indicate colonisation by *L. amethystea*, but their terminal tip did not form mycorrhizas. These were only produced when several short roots were developed at the basis of the lateral root (Figs 1C, 1D) that were consequently transformed into mother roots.

These two mycorrhization patterns can be enlightened by analysing root development before and during the co-culture. Two weeks before inoculation (see I in Fig. 1), the root system was limited to the main root. One week after inoculation, several lateral roots but no short roots had developed (see II in Fig. 1). Two weeks after inoculation (see III in Fig. 1), most lateral root tips at the upper part of

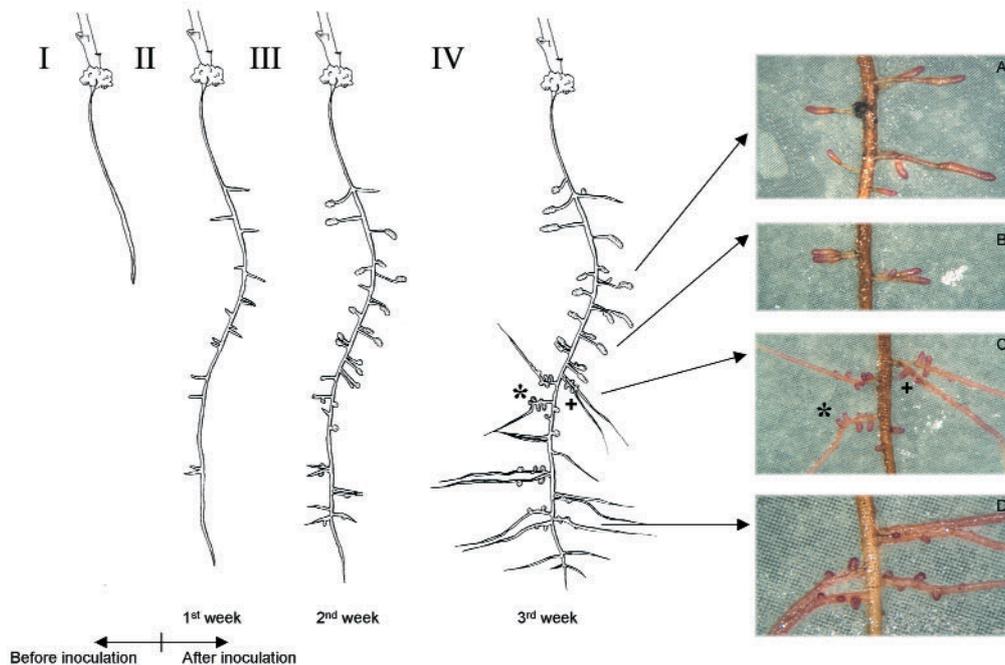


Fig. 1: The root architecture of oak microcuttings at different developmental steps is given before and after inoculation with *Laccaria amethystea* (I, II, III, IV). Two types of mycorrhization patterns (A-B) and (C-D) are developed along the root system. Intermediary ramification patterns are indicated by * and + (see text)

the root system were mycorrhizal and had stopped their elongation, which corresponds to pattern **A-B** (Figs 1A, 1B). At the lower part of the root system, the lateral roots themselves displayed no mycorrhization but continuous growth, and began to produce mycorrhizal short roots as seen in pattern **C-D** (see III in Fig. 1 and Figs 1C, 1D). A week later, root flushing was maximal but essentially expressed in the lateral roots of the lower root system, and pattern **C-D** became evident (see IV in Fig. 1 and Figs 1C, 1D). Relating the course of mycorrhization to the dynamics of root development provides an explanation for the establishment of both patterns. In phases of moderate root growth, as between the first and second week after inoculation, EM were able to form around the apical meristem of lateral roots, which apparently blocked further development of these roots and resulted in pattern **A-B**. In phases of high root growth, as between the second and third week after inoculation, EM could not form on the meristem of the dynamically growing lateral roots, but they could on the short roots that they produced, which resulted in the formation of pattern **C-D**.

A closer inspection of Figure 1C allows detection of an intermediate situation in the middle part of the root system. Two weeks after inoculation, the tips of lateral roots at this middle part were mycorrhizal (see III in Fig. 1). But in the third week (see IV in Fig. 1), these mycorrhizal roots displayed considerable increase in length either after perforation of the mycorrhizal mantle by the root meristem itself (indicated by + in Fig. 1) or through the development of an apical collateral meristem (indicated by * in Fig. 1). This intermediate situation, i.e., the formation of a terminal mycorrhiza on

lateral roots that did not impeach their further growth probably, corresponds to a stage at which root growth was high but not maximum. This is also reflected by the fact that the final length of the lateral roots in question was not as high as that of the most distal part of the root system.

Ramification pattern during EM formation with *Pisolithus tinctorius*

In plants already having lateral roots, that had been pre-cultivated in large Petri dishes over 14 days before inoculation with *P. tinctorius*, approximately one half to two thirds of the MR displayed a “normal” mycorrhization pattern, consisting of regular production of mycorrhizal short roots. However, four further “particular” patterns of mycorrhization were observed on the rest of the root system (Figs 2a-d). In pattern **a** (Fig. 2a), second-order lateral roots that were mycorrhizal at their tip and that had limited growth were regularly distributed along 2- to 4-cm portions of first-order MR, resulting in a herringbone ramification pattern. In pattern **b** (Fig. 2b), a high density of mycorrhizal short roots formed toward the end of first-order lateral roots. Pattern **c** (Fig. 2c) represented one 3- to 6-mm long portion with high density of mycorrhizal short roots at a non-terminal position along first-order mother root. Pattern **d** (Fig. 2d) consisted of a second-order ramification system with mycorrhizal second-order lateral and short roots.

The distribution of these four particular mycorrhization patterns on the root system and their formation was examined on 5 plants over 8 weeks after inoculation. As previously mentioned, these plants already had lateral roots at the beginning of the pre-culture. In the second week after inoculation they all began to produce additional lateral roots along the main root. In total, three categories of first-order lateral roots were distinguished: (i) lateral roots already present at the pre-culture stage characterised by prolonged growth over > 4 weeks after the inoculation; (ii) lateral roots also characterised by a prolonged growth, but that formed after inoculation, simultaneously within each single plant and often during a period of maximal elongation of main or lateral roots of category (i); (iii) lateral roots initiated at different times of the co-culture and characterised by a limited growth period (≤ 4 weeks). The roots of all plants presented at least one rhythmic growth flush in which the maximal elongation of the main and lateral roots took place between weeks 3 to 5 after inoculation (Fig. 3). This developmental stage of the roots occurred concomitantly with swelling of the apical shoot bud. As the experiment was performed in large Petri dishes and over a period of 10 weeks, some plants also displayed a second root growth flush. This began in weeks 6, 7 and 8 after inoculation for plants C, D and A, respectively (Fig. 3).

Figure 3 shows the growth dynamics of each category of lateral roots (i, ii, iii) for the five individual plants of the assay. It also gives the number of mycorrhizas formed weekly during the co-culture. Additionally, the formation time of those root segments on which each particular mycorrhiza pattern was observed at the end of the assay is marked on the respective graphs of the three lateral root categories. The different graphs of Figure 3 show that pattern **a** occurred on segments of the three categories of lateral roots (i, ii, iii) produced during fast growth periods. This pattern was abundantly represented on plant A, which was characterized by the highest root development during the first rhythmic root growth flush. Pattern **b**, which was formed in distal position, also occurred on all three categories of lateral roots, but mostly after a prolonged development stagnation and shortly before elongation began again. This is particularly evident for plants C and D on which a second growth flush was observed in lateral roots, but also for plant A on which pattern **b** occurred in week 8 after inoculation, i.e., at the end of a maximum elongation period of the main root indicating that the root system is developing a second growth flush. When pattern **b** occurred, the growth reinitiation of the lateral roots stopped, which might have been caused by a depletion of resources by the fungus.

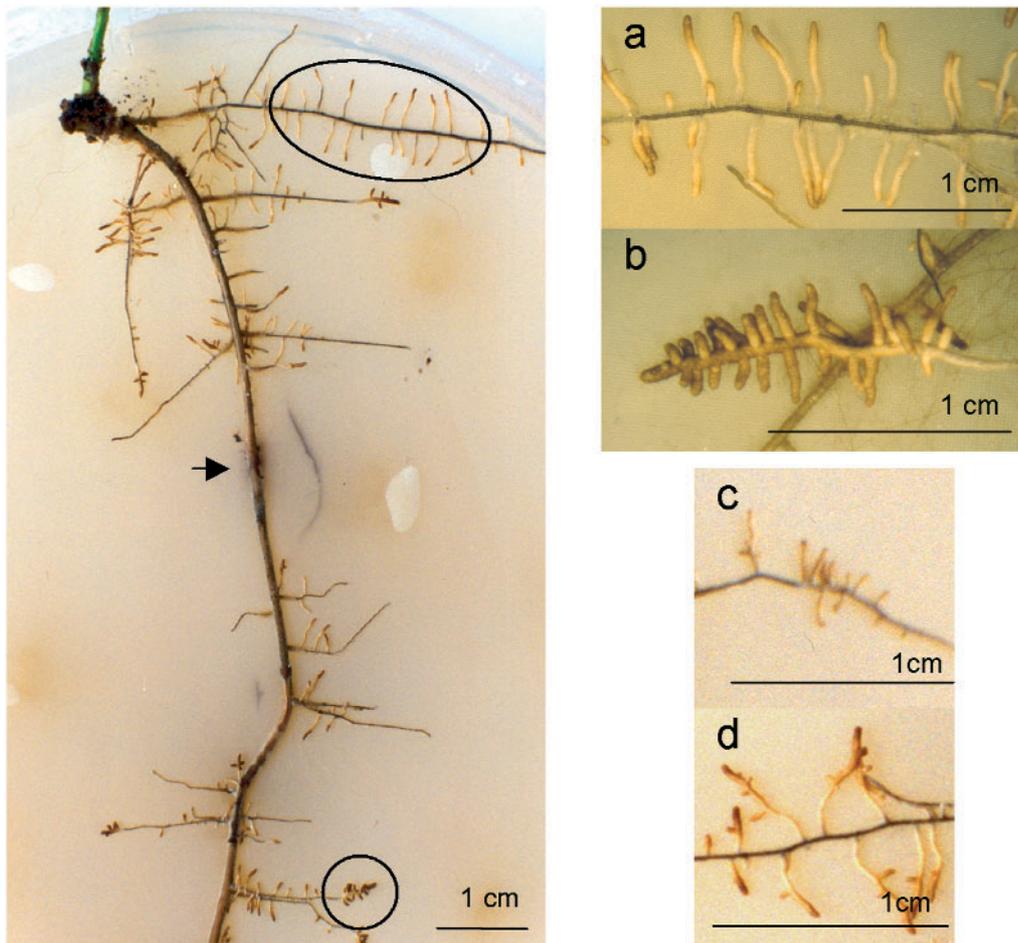
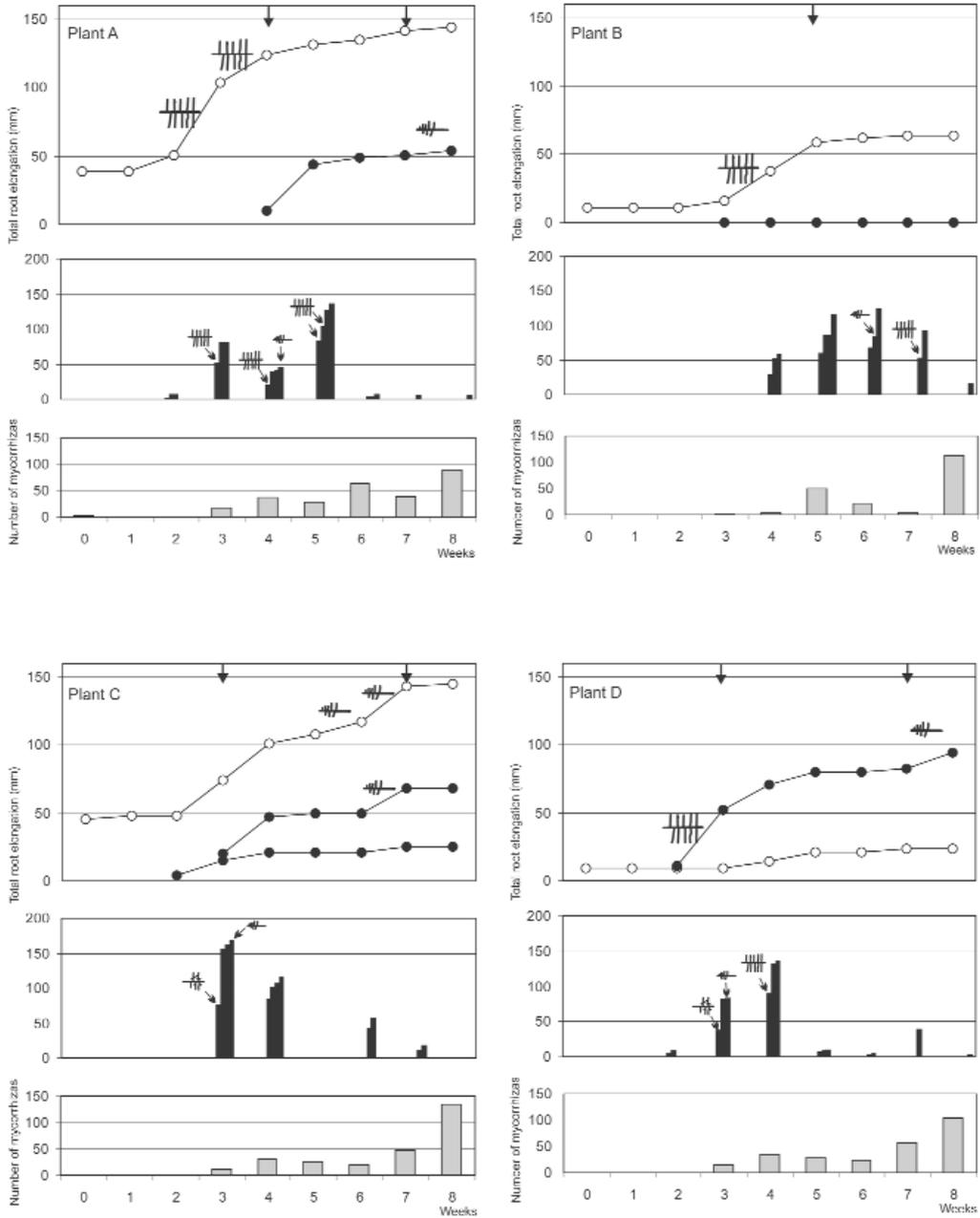


Fig. 2: Root system of a oak microcutting eight weeks after inoculation with *Pisolithus tinctorius*. Elongation start of the main root after transfer in the Petri-dish system in is indicated by an arrow. **a**, **b**, **c** and **d** correspond to four different particular patterns of root ramification during mycorrhization (see text)

According to this interpretation, the resources could be used to promote either reinitiation of root elongation or the formation of densely packed mycorrhizas of pattern **b**, representing a case of localized competition for resources between the symbionts.

Allocation repartition of localized resources between the symbionts probably also governed the establishment of pattern **c** (Fig. 2c) that occurred in plant E on segments of root categories ii and iii (Fig. 3), which formed between week 5 and 6. In this plant, lateral roots of category i only displayed a reduced elongation and the major contribution to lateral root growth was the one of roots of category ii and, to a lesser extent, of category iii. Category ii lateral roots displayed two growth flushes, and after the stagnation preceding the second flush, some of them produced densely mycorrhizal short roots of the pattern **c**. The fact that the elongation of these lateral roots was not stopped by the mycor-



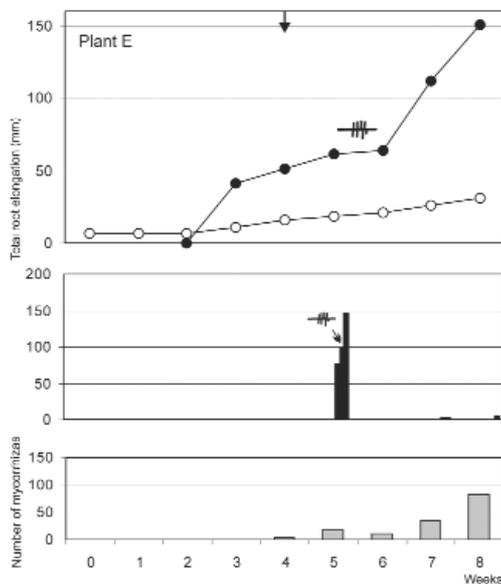


Fig. 3: Elongation of three categories of lateral roots after inoculation with *Pisolithus tinctorius* by 5 oak microcuttings A, B, C, D and E.

○ roots initiated before inoculation with growth prolonged over four weeks (cumulative elongation).
 ● roots initiated after inoculation with growth prolonged over four weeks (cumulative elongation).
 ■ roots initiated after inoculation with growth limited to a maximum of 4 weeks: all roots initiated in the same week are represented together and the bars represent their cumulative weekly elongation during growth time.

↓ Maximal elongation of the main root.
 □ Number of mycorrhizal tips developed weekly on all 3 categories of lateral roots (non-cumulative)

Distribution of the four particular mycorrhizal ramification patterns on elongation segments of the 3 categories

|||| pattern a -#- pattern c
 -#- pattern b -#- pattern d

rhiza formation could reflect a higher amount of resources compared to that resulting in pattern **b** on plants A, C, or D (Fig. 3). This high amount of resources is reflected by the steep slope that represents the elongation rate in the second growth flush of category ii lateral roots and, in addition for lateral roots of category iii, by the height of the bar that represents their elongation in the third week after their first occurrence (Fig. 3). A high amount of resources must have been locally available to induce this important elongation and their partial depletion to support the dense formation of mycorrhizal short roots was not enough to prevent further root elongation, resulting in pattern **c**.

The last mycorrhization pattern (**d**) formed in plants C and D (Fig. 3) and exclusively on certain lateral roots of category iii (characterized by a limited growth period). The lateral roots were all initiated concomitantly to a root growth flush in week 3 after inoculation and pattern **d** only occurred on the segment formed in the week of their initiation. As a second root growth flush occurred in week 7 after inoculation, the category iii lateral roots displayed almost no elongation any more. In contrast, their mycorrhizas elongated and ramified. Because the category iii lateral roots have a limited elongation time window, the resources potentially available for a second root growth flush were probably invested in elongation and ramification of the mycorrhizas.

Discussion

Choice of an experimental approach

Mechanisms involved in EM formation can be approached by considering morphological markers such as the root-shoot ratio or the root ramification pattern (RUPP & MUDGE 1985, REGVAR & GOGALA 1996, SCAGEL & LINDERMAN 1998, NIEMI HAGGMAN & SARJALA 2002, NIEMI et al. 2002)) or by measuring physiological criteria (RUDAWSKA & KIELISZEWSKA-ROKICKA 1997, BIDARTONDO et al. 2001, NIEMI HAGGMAN & SARJALA 2002, NIEMI et al. 2002, LERAT et al. 2003). In both cases however, analyses are based on experiments with statistical assessment of a number of replicates, and their resolution

is limited by the fact that due to the heterogeneity of the plant material, the impact of high-intensity processes is intensified and *vice versa*. On our oak microcuttings model system that handles genetically identical plants, statistically based analyses can be done either by comparing precise developmental stages between non-synchronously growing individuals (HERRMANN, MUNCH & BUSCOT 1998), or by analysing samples of synchronously growing individuals (HERRMANN, OELMÜLLER & BUSCOT 2004). The focus of the present work was different. It consisted in detailed morphological observations of single plants inoculated with different mycobionts in order to identify patterns of mycorrhization and to relate their occurrence to root development. Replicates were performed to avoid the description of single atypical events, but not for performing statistical analyses.

Dependence of mycorrhization on resource allocation in plants and influence of the rhythmic growth

In the first assay, the five mycobionts compared displayed different mycorrhization dynamics, which reflects the high functional diversity of EM fungi. However, some common traits in the dynamics of mycorrhization and root development allow two main schemes to be distinguished. In the first one, observed in *P. involutus*, *P. tinctorius* and *L. laccata*, mycorrhiza formation occurred during a wide period of time (30–40 days), and root length increased by a high factor (3–18 x). In this case, formation of mycorrhiza occurred independently of the rhythmic variations in the elongation rate of the lateral roots. In the second scheme, observed in *L. amethystea* and *H. crustuliniforme*, the mycorrhization window was narrower (10–20 days), and the root length increased moderately (1.3–5 x). A coincidence between mycorrhization and the period of maximum elongation of the lateral roots was noted in this last scheme.

Two additional and more detailed experiments to describe precise mycorrhization patterns were performed with fungi representing one of each of the schemes described above. For *L. amethystea*, two mycorrhization patterns were described. Their occurrence appeared to be essentially determined by the endogenous rhythmic root growth. In phases of low elongation, the lateral roots themselves formed mycorrhizas, while in phases of high elongation they produced mycorrhizal short roots. For *P. tinctorius*, four particular mycorrhization patterns were detected in addition to the current one (regular distribution of mycorrhizas along MR), and their occurrence could always be related to the rhythmic growth variations of the lateral roots. In addition, considering that root growth flushes should correspond to an enhanced resource allocation in the root compartment, the four particular mycorrhization patterns (Fig. 2) could be explained in terms of competition for these resources on one hand between mycorrhiza formation (patterns **a**, **b**, **c** & **d**), eventually their elongation and/or ramification (patterns **a** & **d**), and the elongation of the lateral root system on the other hand (Fig. 3).

This interpretation regarding competition for resources between mycorrhiza formation and lateral root elongation and the influence of rhythmic growth on resource shifts is also compatible with the observations of the first two experiments. In the first scheme of the first experiment (Table 1), the continuous EM formation, apparently independent of the rhythmic growth, would correspond to the fact that, as the general growth of the plants was high, the resource level in the root system permanently allowed the support of both the root elongation and the mycorrhiza formation. In the second scheme (Table 1), where plant development in general was weaker, the resources would have allowed support of both elongation of lateral roots and their EM formation only during periods of root growth flushes. In the second experiment with *L. amethystea*, the formation of mycorrhizas on lateral roots in phases of decreased root development might correspond to a shift of the limited resources into the EM formation at the expense of root elongation. In contrast, in phases of root flushing, the rein-

Tab. 1: Elongation of 1st order mother roots (MR) and mycorrhizas formed in 10 day periods during co-culture of oak microcuttings with *Paxillus involutus*, *Laccaria amethystea*, *Hebeloma crustuliniforme*, *Pisolithus tinctorius*, *Laccaria laccata* in presence or absence of activated charcoal (AC). For each treatment, one single plant is represented. The period of maximal lateral root elongation during the root flush is given in bold. The abundance of mycorrhizas on each successive growth segment (Myc/MRSeg) produced every 10 days by 1st order mother roots is given by 0, -, +/+, + or ++. Nd is given for not determined

| | Before inoculation | | After inoculation | | | | | Total length new MR | Mycorrhizal status at the end of the co-culture | | |
|---|--------------------|-------|-------------------|---------|---------|---------|---------|---------------------|---|------------------|-------|
| | ←→ | | | | | | | | | | |
| | 10 days | 0 day | 10 days | 20 days | 30 days | 40 days | 50 days | | | | |
| <i>Paxillus involutus</i> Strain F1667 +AC | MR (mm) | 56 | 0 | 31 | 61 | 174 | 129 | 18.7 | 414 | Nb. mycorrhizas | 245 |
| | Myc/MRSeg | 0 | | ++ | ++ | ++ | ++ | 0 | | % mycorrhization | 97.2% |
| <i>Paxillus involutus</i> Strain F1667 -AC | MR (mm) | 120 | 44 | 22 | 86 | 98 | 151 | 85 | 486 | Nb. mycorrhizas | 220 |
| | Myc/MRSeg | + | ++ | ++ | ++ | ++ | 0 | 0 | | % mycorrhization | 85.6% |
| <i>Laccaria amethystea</i> Strain F1629 +AC | MR (mm) | 240 | 0 | 343 | 81 | 10 | 4.6 | nd | 439 | Nb. mycorrhizas | 173 |
| | Myc/MRSeg | 0 | | ++ | ++ | ++ | ++ | nd | | % mycorrhization | 83.2% |
| <i>Laccaria amethystea</i> Strain F1629 -AC | MR (mm) | 76 | 21 | 8.3 | 4.5 | 19 | 46 | nd | 99 | Nb. mycorrhizas | 43 |
| | Myc/MRSeg | ++ | + | 0 | 0 | 0 | 0 | nd | | % mycorrhization | 84.3% |
| <i>Hebeloma crustuliniforme</i> Strain F1584 +AC | MR (mm) | 102 | 0 | 153 | 135 | 72 | 133 | nd | 493 | Nb. mycorrhizas | 58 |
| | Myc/MRSeg | + | | +/- | 0 | 0 | 0 | nd | | % mycorrhization | 34.9% |
| <i>Hebeloma crustuliniforme</i> Strain F1584 -AC | MR (mm) | 185 | 65 | 26 | 112 | 106 | 181 | nd | 490 | Nb. mycorrhizas | 45 |
| | Myc/MRSeg | 0 | - | - | + | - | 0 | nd | | % mycorrhization | 41.3% |
| <i>Pisolithus tinctorius</i> Strain F1626 +AC | MR (mm) | 89 | 0 | 60 | 10 | 50 | 121 | 0 | 241 | Nb. mycorrhizas | 95 |
| | Myc/MRSeg | + | | + | + | + | 0 | | | % mycorrhization | 96.9% |
| <i>Pisolithus tinctorius</i> Strain F1626 -AC | MR (mm) | 25 | 86 | 82 | 0 | 68 | 172 | 54 | 462 | Nb. mycorrhizas | 40 |
| | Myc/MRSeg | 0 | - | + | + | + | 0 | 0 | | % mycorrhization | 33.3% |
| <i>Laccaria laccata</i> Strain F1647 +AC | MR (mm) | 77 | 0 | 15.9 | 110 | 142 | 199 | 81 | 548 | Nb. mycorrhizas | 98 |
| | Myc/MRSeg | 0 | | + | + | + | + | 0 | | % mycorrhization | 51.0% |
| <i>Laccaria laccata</i> Strain F1647 -AC | MR (mm) | 48 | 14.3 | 25 | 0 | 46 | 424 | 160 | 669 | Nb. mycorrhizas | 49 |
| | Myc/MRSeg | 0 | + | + | + | + | 0 | 0 | | % mycorrhization | 49.1% |

forced resource allocation in the root compartment would allow support of both lateral root elongation and their production of mycorrhizal short roots. This hypothesis is based on three assumptions: first, formation of mycorrhiza is a process of competition for the resources available in roots; second, roots display a rhythmic growth; third, root growth flushes correspond to increased resource allocation into the root compartment of the plants.

Concerning the first assumption, the idea of “competition” of EM fungi for resources available in roots has not been mentioned directly until now. However, there is molecular evidence that fungi generate a strong monosaccharide sink in colonized roots (see the review by NEHLS et al. 2001). In experiments with AM plants where leaves were labelled with $^{14}\text{CO}_2$, the C-sink strength in mycorrhizal roots was linearly correlated with the degree of mycorrhization (LERAT et al. 2003). In EM roots, WRIGHT et al. (2000) found that the expression of two hexose and one sucrose transporter genes of birch were reduced to less than one-third of the expression level compared to in non-mycorrhizal roots, indicating that competition for resources between the fungus and the roots is actively ruled at the gene level.

Concerning the second assumption, it has to be emphasized that the rhythmic root growth, evident in our system, remained unclear for a long time. Rhythmic plant growth and its endogenous origin have been largely demonstrated in trees of tropical and temperate regions (HALLE & OLDEMANN 1970, HALLE & MARTIN 1968, LAVARENNE 1966), whereby these observations only related to the shoot. Rhythmic growth in roots has not been referred to so long as the studies were performed on young seedlings (LAVARENNE 1968, BELGRAND et al. 1987, ALAOUI-SOSSÉ et al. 1994, HALLÉ & MARTIN 1968). But in works with cuttings or microcuttings that behave much more like adult plants, alternate episodes of shoot and root growth with maximal root growth at the beginning of a shoot flush were demonstrated (REICH et al. 1980, KUEHNY & HALBROOKS 1993, HERRMANN, MUNCH & BUSCOT 1998, HERRMANN, OELMÜLLER & BUSCOT 2004).

Concerning the third assumption, KUEHNY, MILLET & DECOTEAU (1997) found that changes in endogenous C and N concentrations and allocation patterns in *Ligustrum* were linked to the control of episodic shoot and root growth. A link between C allocation and rhythmic growth has also been shown in *Quercus robur* (ALAOUI-SOSSÉ et al. 1994, 1996).

Carbon versus hormone theory

Our results and their interpretation in the light of the available literature support the thesis that mycorrhiza formation is dependant on plant resources. They therefore primarily appear to reinforce the carbon theory first formulated by BJÖRKMAN (1942), according to which carbohydrate concentration is determinant for the formation of EM. However, some aspects of the results also support, at least indirectly, the second main theory on EM formation, the hormone theory. According to this theory, auxins of fungal origin are the regulatory factor of EM formation (SLANKIS 1973). In our first experiment (Table 1), a crucial factor for mycorrhization dynamics was the intensity of root development. *P. involutus*, *P. tinctorius* and *L. laccata* displayed a better root growth than *L. amethystea* and *H. crustuliniforme*. These differences, which influenced the mycorrhization dynamics and intensity, might be related to differential root growth promoting effects of the mycobionts, as EM fungi are known to display variable levels of auxins production (ULRICH 1960, HO 1986, 1987, NIEMI et al. 2002). In addition, we show that resource allocation governed by rhythmic plant growth is crucial for mycorrhiza formation. For *Hevea* trees, it has been shown that IAA content in the apical bud varied and was maximal just after out-bursting of apical buds (HALLE & MARTIN 1968), the stage that is concomitant with the beginning of root growth flushes in our system. Additionally, auxin is known to be translo-

cated basipetally from the apex to the root part (see MARKS 1996). Thus, our observations suggest that, if IAA plays a role on EM formation, a combined influence of external (fungal) and endogenous (plant) IAA has to be considered in studies on hormonal regulation during EM formation. WALLANDER, NYLUND & SUNDBERG (1994) and RUDAWSKA & KIELISZEWSKA-ROKICKA (1997) agree that the relationship between IAA concentration and EM colonisation is not as clear-cut as SLANKIS (1973) suggested. Available works on hormones and mycorrhiza showed that IAA cannot be considered independently from other hormones, like ethylene, jasmonic acid or cytokinins (RUPP & MUDGE 1985, RUPP, MUDGE & NEGM 1989, GOGOLA 1991, REGVAR & GOGALA 1996, REGVAR, GOGALA & ZNIDAR-SIC 1997, SCAGEL & LINDERMAN 1996, BARKER & TAGU 2000). Hormones in the context of mycorrhizas are currently being investigated at the molecular level (CHARVET-CANDELA et al. 2002, REDDY et al. 2003).

Synthesis

Our results also suggest that the carbon and hormone theories might be compatible. This point of view is supported by previous works with this oak microcutting model applying the EM basidiomycete *Piloderma croceum*. We were able to demonstrate that EM formation by *P. croceum* is directly resource limited, because it occurred only when plants have acquired a minimal photoassimilation capacity (HERRMANN, MUNCH & BUSCOT 1998, HERRMANN, OELMÜLLER & BUSCOT 2004). On the other hand, we showed that *P. croceum* during its long pre-mycorrhizal stage stimulates shoot and root growth and protects the photosynthetic apparatus, helping the plants to acquire the capacity to support fully developed EM (HERRMANN, OELMÜLLER & BUSCOT 2004). A comparable root-growth stimulating effect was described by NIEMI et al. (2002) for the EM fungus *P. involutus* without any formation of mycorrhizas. They attributed the root stimulation not directly to IAA production but rather to exogenous diamines (NIEMI, HÄGGMAN & SARJALA 2002). In our system, EM formation and root elongation could result from a permanent tuning between resource allocation by the plant and the production of growth-promoting substances by the fungus. This novel integrative hypothesis results from our handling with a system that allows the expression of plant rhythmic growth both in shoot and roots. This is a crucial trait that has not been considered so far in investigations on mycorrhizas. The objection is possible that culture models are far from natural field situations, in which the adsorbing potential of soils might buffer fine shifts in resources or hormone allocation. Our experiences with activated charcoal – a substance with strong absorbing capacity like that of soil particles –, however, shows that, if the single mycorrhization rate and dynamics of the compared mycobionts may be modified in some cases, an identical level of diversity in the dynamics of mycorrhization was noted.

Conclusion and perspectives

The variety of mycorrhizal behaviour and patterns found in our study express functional diversity of EM and might be explained by a balance between few simple basic mechanisms in plants and fungi including rhythmic plant growth, production levels of growth promoting factors by the plants and the fungi, and individual carbon demand of the fungi. As mentioned in the introduction, the capacity to form EM has occurred many times convergently during the evolution of higher fungi in the Asco- and Basidiomycota (see OBERWINKLER 1993), and it makes sense that this symbiotic behaviour results from a few mechanisms essential for regulation in contrast to a trait that would have been acquired once in evolution by one monophyletic group and which requires a specific genetic adaptation. In our model, we have begun to reveal plant genes regulated during the recognition and early formation stages of EM (HERRMANN et al. 2003, Krüger et al. (subm.)). Once we have identified a minimum amount

of such genes, we can elaborate on the present study by analysing the fine regulation of such genes at different stages or in different patterns of mycorrhization, with array techniques or *in situ* expression profiling.

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