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Mycorrhizal and non-mycorrhizal Douglas fir grown in hydroculture. The effect of nutrient concentration on the formation and functioning of mycorrhiza

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SUMMARY

A series of experiments using the Douglas fir as the subject of research were performed in hydroculture. Different relative nutrient addition rates were used prior to and after plants had been inoculated with *Laccaria bicolor*. The effect of the resulting nutrient conditions on mycorrhiza formation was studied together with the effects on growth and internal nutrient levels.

The level of external nutrient concentration influenced mycorrhiza formation and functioning. In general, very low and limiting external nutrient conditions resulted in rapid and good mycorrhiza formation, with the mycorrhiza plants having the same or a lower fresh weight than those of the non-mycorrhizal plants. While under higher nutrient conditions mycorrhiza formation was slower and less abundant, and the mycorrhizal plants had a larger total fresh weight than non-mycorrhizal plants.

Key-words: growth, internal nutrient content, *Laccaria bicolor*, nutrient concentration, *Pseudotsuga menziesii*.

INTRODUCTION

Ectomycorrhizae may have several distinct effects on trees: they may increase the uptake of nutrients and the internal nutrient concentration and growth may subsequently increase (Bowen 1973; Bowen & Smith 1981; Harley & Smith 1983).

Several factors affect the formation and functioning of mycorrhizae. One such factor is the external nutrient concentration (Marx *et al.* 1977; Crowley *et al.* 1981; Shaw *et al.* 1982; Ruehle & Wells 1984). Several studies have been performed on nitrogen supply. These studies showed that a high level of nitrogen supply reduces mycorrhizal infection (Menge *et al.* 1977; Ekwebelam & Reid 1983; Alexander & Fairly 1985). However, it also became apparent that high nitrogen levels enable the growth of other mycorrhizae (Alexander & Fairley 1983).

In the experiments presented here the effect of different levels of external nutrient concentrations on mycorrhiza formation and functioning of the Douglas fir (*Pseudotsuga*

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menziesii) was studied. The nutrients were added to the nutrient solution at concentrations which increased exponentially (Ingestad 1982). The nutrient conditions were varied, either by varying the initial amount of addition, by varying the nutrient addition rate, or by giving the plants a high nutrient pretreatment before inoculation.

Hydroculture was chosen, as it allows a more accurate analysis of the formation and functioning of mycorrhizae as dependent on a nutrient concentration. *Laccaria bicolor* was chosen as the fungal partner. In general, ectomycorrhizae are studied using a solid substrate. Only a few experiments have been reported in which ectomycorrhizae are grown in hydroculture, and none of these have involved the Douglas fir. Kähr & Arveby (1986) developed a method by which ectomycorrhizal *Pinus sylvestris* seedlings can be grown in hydroculture. This method was also used in the present experiments.

Although we used a variety of experimental designs, we were able to describe the general effects of nutrient status on mycorrhizal development and functioning. Moreover, the effect of mycorrhiza on growth of the host plant was studied under these conditions.

MATERIALS AND METHODS

Germination

Seeds of Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco), provenance Arlington-05.stII, were supplied by the Institute for Forestry and Urban Ecology, 'De Dorschkamp', Wageningen, The Netherlands.

The seeds were collected from a single tree in 1985 and stored at 4°C. Before germination the seeds were superficially sterilized for 75 min in a 30% solution of H_2O_2. They were then stirred in distilled water for 24 h after which they were put in moist vermiculite in a climate room. Growth conditions were: a 12-h light period at an irradiance of 200 μmol m^{-2} s^{-1} (PAR), a temperature regime of 22/18°C and a relative humidity of 60/70% (day/night).

Hydroculture

Mycorrhiza formation cannot proceed on submerged roots. The roots need to be very moist but in contact with air. Therefore the special hydroculture set-up developed by Kähr and Arveby (1986) was used.

In this set-up, the roots grew on slopes with the nutrient solution flowing along the roots. Plastic containers (30 l) were used, with four slopes in each container. Ten seedlings were placed on each slope. The seedlings were mounted in pieces of black styrofoam, placed in holes in a lid that covered the tank, with the roots resting on the slopes underneath. Nutrient solution (10 l) was continually circulated through the tanks and a cotton filter using a centrifugal pump. Nutrients were supplied to the water in the containers from a concentrated stock solution at 1-h intervals by means of a peristaltic pump and valves operated by a small computer. The amount of nutrients added in this way increased exponentially with time (Ingestad 1982), controlled by a computer program. The aim of the experiment was to ensure that the added nutrients would be taken up in a very short time; this depends upon the relative addition rate and the initial nutrient amount (Table 1). The end result is that different amounts of nutrients are left in the containers (see Results). The stock solution was composed of $(NH_4)_2SO_4$, KH_2PO_4, NH_4NO_3, $Ca(NO_3)_2$, $Mg(NO_3)_2$ and KNO_3 in such a way that a solution with the following molar ratios was obtained: N:P:K:Ca:Mg:S = 100:13·6:18·1:1·4:2·9:3·9. This molar ratio closely resembled that described by Van den Burg (1971) (also cited in Ingestad 1979) as being optimal for Douglas fir. The nitrogen source was a mixture of NH_4^+ and NO_3^- in the ratio 47:53.

Table 1. Initial conditions (RAR [% day^{-1}]), initial N amount (μmol N $plant^{-1}$) on Day 1, mean total fresh weight (mg) on Day 1 and conditions at the time of inoculation (day, total fresh weight) with *Laccaria bicolor*, the type of pump used and time of harvest for the different experiments using Douglas fir seedlings (SEM in brackets). Before inoculation $n=80$, after inoculation $n=40$ for inoculated plants and non-inoculated plants

	Start				Inoculation		
	RAR	Initial amount	Total fresh weight	Pump	Day	Total fresh weight	Experimental period
Experiment 1a	1	5	80 (1)	10	31	184 (8)	195
Experiment 1b	4	20	130 (2)	10	14	169 (6)	
From day 32	2						
From day 43	1	10					155
Experiment 2a	2	4·2	223 (5)	10	21	617 (7)	128
From day 14	2	2·6					
Experiment 2b	2	2	200 (6)	10	24	461 (12)	107
Experiment 2c	2	2	200 (5)	12	16	400 (10)	83

Micronutrients were given at levels proportional to N as follows: N:Fe:Mn:B:Cu:Zn:Mo = 100:0·6:0·11:0·34:0·068:0·062:0·001. This solution was composed of $MnSO_4$, H_3BO_3, $CuSO_4$, $ZnSO_4$ and Na_2MoO_4. The culture solution was renewed once every 2–3 weeks to avoid accumulation of one or more ions. Furthermore, the solution was analysed for pH, NH_4^+, NO_3^- $H_2PO_4^-$, K^+, Ca^{2+} and Mg^{2+} at regular intervals.

Culture of Laccaria bicolor *mycelia and mycorrhiza formation*

Cultures of *Laccaria bicolor* (Maire) P. D. Orton originally isolated from *Pseudotsuga menziesii*, were obtained from Dr A. E. Jansen, Department of Phytopathology, Agricultural University, Wageningen, The Netherlands. *Laccaria bicolor* was grown in Petri dishes on MMN agar (Marx 1969). In order to produce the inoculum, pieces of the mycelia were transferred to Erlenmeyer flasks containing 200 ml MMN culture medium. The flasks were put on a shaker for 4 weeks. The suspension was filtered and washed twice with demineralized water. The residue was resuspended in water. The suspension obtained in this way was used as an inoculum for the roots.

Inoculation

The length of the period between the start of the experiment and the moment the seedlings were inoculated, varied according to the experiment (Table 1). The roots were dipped in the inoculum suspension and mycelial fragments adhered to the roots (Kähr & Arveby 1986). Thereafter the seedlings were replaced in the hydroculture.

Pretreatment

In Experiment 2, the seedlings were treated prior to the actual experiment in order to manipulate the internal nutrient conditions and thus the initial growth rate. During this pretreatment, the seedlings were placed in a normal hydroculture (without slopes) for 2–3 weeks in a complete nutrient solution with a nitrogen concentration of 1 mM and the same

molar ratios as described above, which was refreshed once a week. The pH was adjusted to a value of 4·0.

In Experiment 1, plants were not pretreated in a normal hydroculture, but put directly on the slopes (Experiment 1a). In Experiment 1b, the relative addition rate (RAR) was rather high, 4%, and started at a high level, 20 μmol plant^{-1} day^{-1}.

Harvest

At harvest, total fresh weight was determined. In Experiment 2, the external mycelia of the mycorrhizal plants were carefully removed from the roots in order to allow separate weight measurements and mineral analysis of the mycorrhizal roots and mycelia. The data obtained were used to calculate total fresh weight of the host of the mycorrhizal plants in Experiment 1 where total fresh weight of the host plant including mycelium was measured.

Mineral analysis of plant material

The harvested material was separated into shoots and roots and dried at 80°C for 24 h. Roots and mycelia, separated (Experiment 1) or together (Experiment 2) were thoroughly rinsed with distilled water and dried before analysis. The nutrient concentration of the dried material was analysed. Nutrient concentrations were analysed per plant or on pooled plant material. Samples (250 mg) were dissolved in 5 ml of a mixture containing H_2SO_4, HNO_3 and $HClO_4$ (4:6:0·25, v/v). For Ca and Mg determinations $La(NO_3)_2$ was added. The K, Ca and Mg concentration of the samples was measured by atomic absorption spectrophotometry. P was determined colorimetrically as $H_2PO_4^-$ at 680 nm after adding ammonium dissolved in H_2SO_4 and a solution containing ascorbic acid and stannous chloride in H_2SO_4 (Sommarin *et al.* 1985). Total nitrogen was determined using a slightly modified Kjehldahl method (Bradstreet 1965). Samples (50 mg) were dissolved in 2 ml of Na-salicylate containing H_2SO_4. A mixture of K_2SO_4, $CaSO_4$ and Na_2SeO_3 (15:5:0·085, w/w) was used as a catalyst (Eastin 1978). The NH_4^+ produced after digestion was determined colorimetrically at 410 nm by use of Nessler's reagent.

Analysis of the culture solution and mycorrhizal growth

K^+, Ca^{2+}, Mg^{2+}, NH_4^+ and $H_2PO_4^-$ concentrations were determined as described above. NO_3^- was determined by its absorption at 210 nm (Doddema & Telkamp 1979). Mycorrhizal plants were observed under the binocular microscope at regular time intervals. The mycorrhizal phases were divided into three stages. During stage I, mycorrhizal root tips developed on the places where the inoculum adhered to the roots. Other root tips remained non-mycorrhizal. In stage II, all root tips became infected and thin strands of mycelia could be seen along the roots. During stage III, all root tips were infected and the roots were completely covered with a thick layer of mycelia (Fig. 1).

Experimental procedure

Time of transfer of the seedlings to the slopes was called Day 1. In Table 1, total fresh weight on Day 1, RAR and the amount of nutrients added on Day 1, time of inoculation and total fresh weight at inoculation are given.

Ten-litre pumps were used to circulate the nutrient solution, except for Experiment 2c, where a 12-l pump was used from Day 1. The 10-l pumps circulated 10 l nutrient solution and had a maximal flow rate of 270 l h^{-1}, and the 12-l pump circulated 12 l nutrient solution and had a maximal flow rate of 390 l h^{-1}. The actual flow rate along the roots was in the order of 3 and 5 l h^{-1}, for the 10-l and 12-l pumps respectively.

Determination of optimal growth

To determine maximal relative growth rate (RGR_{max}), 34 plants were grown on a complete nutrient solution of 2 mM total nitrogen in a normal hydroculture, without the hourly nutrient addition and without using slopes. The nutrient solution was refreshed once a week and the pH was adjusted to 4·0. The plants were weighed at regular intervals. On Day 83 50% of the plants were harvested and on Day 111, the final harvest was carried out. The resulting maximal growth curve is given in Fig. 2. The dried material was analysed for nutrient concentration. The so-called maximal addition (M.A.) curve was calculated from these data and this is also shown in Fig. 2. This curve shows the amount of nitrogen (μmol $plant^{-1}$ day^{-1}) required to be taken up in order to allow maximal growth and to maintain an unchanged internal nitrogen concentration of 330 μmol (g total fresh weight.)$^{-1}$. Data on the nitrogen concentration of the seedlings will be discussed in the Results.

Statistical analysis

The results were statistically evaluated using Student's *t*-test at the $P = 0{\cdot}05$ level.

RESULTS

Besides an experiment on maximal growth in hydroculture, six experiments were performed to establish the effect of nutrient concentration on mycorrhiza formation and the effects on growth and internal nutrient concentration (Kamminga-van Wijk 1991). We selected two of these six experiments for use in reporting the above topic.

Maximal growth

The maximal growth curve with the corresponding calculated M.A. curve, is shown in Fig. 2. From Day 60 onwards, maximal growth decreased. Table 2 shows N concentration of very young, just germinated or pre-grown seedlings and the N concentration of plants which were grown at a maximal growth rate. In the very young seedlings, N concentration was very high, due to the high N reserves of the seed, but this level quickly decreased. A pretreatment concentration of 2 mM N instead of 1 mM N resulted in a higher N content in the plants although growth was not stimulated. Plants of the growth experiment which had a total fresh weight of 3407 mg had an N concentration of 283 μmol N (g total fresh weight.)$^{-1}$. However, the N concentration of older plants with a total fresh weight of 6360 mg was higher again. With regard to dry weight alone, there was no difference between the N concentration of plants of the growth experiment with time. Based on these data it was decided to choose an N concentration of 330 μmol (g total fresh weight)$^{-1}$ for the calculations concerning the M.A. curve. This value was the mean N concentration of the young seedlings with a total fresh weight of at least 200 mg that had been grown on a pretreatment solution and the plants of the maximal growth experiment.

Cations

The K^+, Ca^{2+} and Mg^{2+} concentrations of the nutrient solution during the experiment were measured. The resulting concentrations of cations not taken up were very low: the K^+ concentration always remained below 25 μM and the Ca^{2+} and Mg^{2+} concentrations always remained below 10 μM.

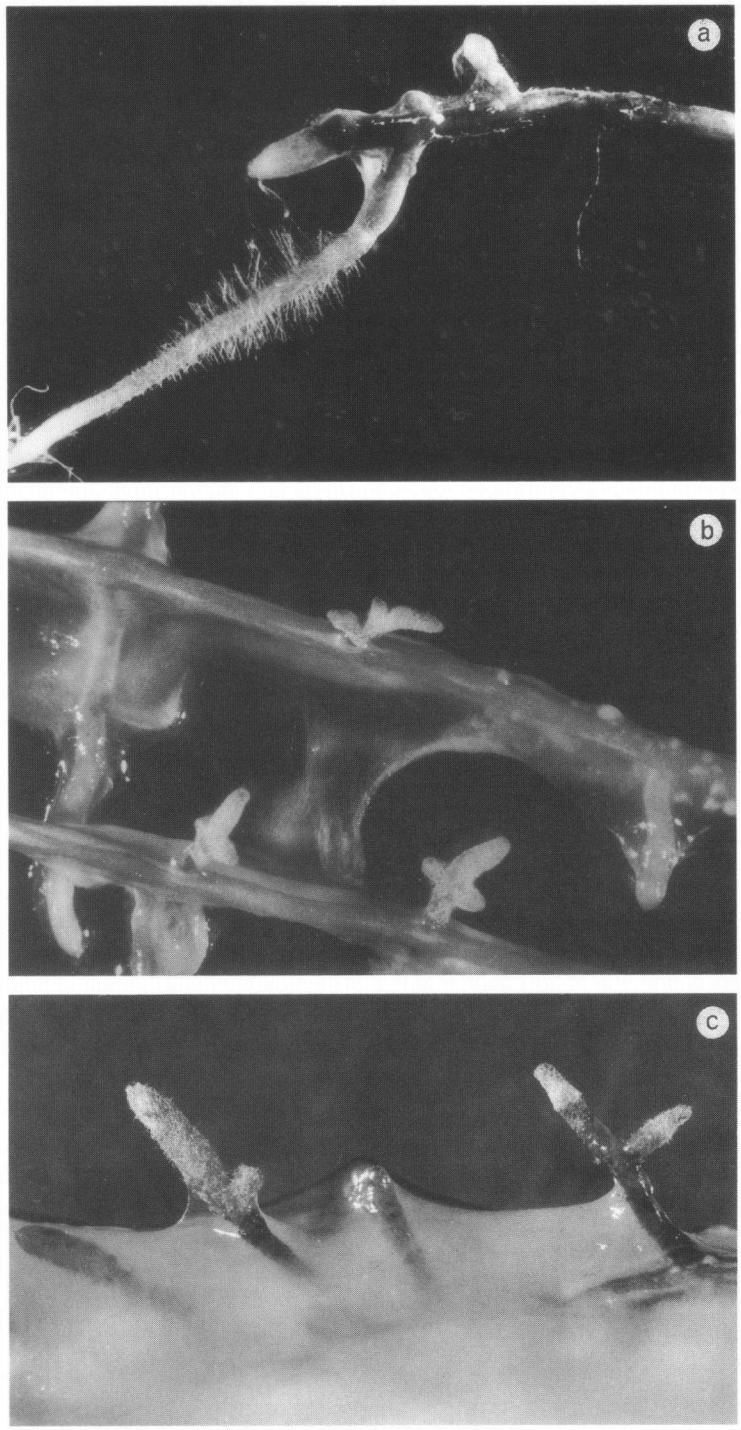

Fig. 1.

Experiment 1

Time course of nutrient addition and solution concentration. In this experiment the different nutrient conditions prevailing at the time of inoculation were compared. In Experiment 1a, nutrient addition increased at a rate of 1% day^{-1}. The initial amount of nutrients added on Day 1 was somewhat higher than could be absorbed by the young seedlings (Fig. 3a). At the time of inoculation RAR was below M.A.; this was in contrast to Experiment 1b. At the start of Experiment 1b, both the increase in addition rate (4%) and the initial level of nutrients (20 μmol) were very high (Table 1). Twenty-nine days after inoculation the amount of nutrients was drastically lowered and the increase in addition rate remained at 1%. As a result, the difference in rates of addition was reflected in the concentrations actually measured, which were much higher at the beginning of Experiment 1b (mean NH_4^+ and $H_2PO_4^-$ concentrations were below 350 μM), compared with Experiment 1a (mean NH_4^+ and $H_2PO_4^-$ concentrations were below 100 μM). After the switch to low nutrient addition, the mean NH_4^+, $H_2PO_4^-$ and NO_3^- concentrations remained below 25, 40 and 200 μM respectively. The mean pH value between Day 64 and harvesting was 3·9 (SEM = 0·06) in Experiment 1a, and the mean pH value between Day 42 and harvesting was 3·8 (SEM = 0·10) in Experiment 1b.

Mycorrhiza formation and effects on growth and nutrient concentration. It took 80 days once inoculation had taken place for the first root tips to become infected. Mycorrhization in Experiment 1a was better than that in Experiment 1b. Mycorrhiza formation proceeded slowly 80 days after inoculation. Approximately 100 days after inoculation, mycorrhization reached stage I while in Experiment 1a, mycorrhiza formation had proceeded beyond stage I (Fig. 1).

The mean fresh weight of the mycorrhizal plants (Table 3) was higher than that of non-mycorrhizal plants. The mycorrhizal plants tended to have a lower % dry weight, indicating the increased water content of mycorrhizal plants. Nutrient concentrations (Table 4) at the end of the experiment were almost the same for both mycorrhizal and non-mycorrhizal plants.

Experiment 2

Time course of nutrient addition and solution concentration. In this experiment a low RAR (2% day^{-1}) and a low initial nutrient amount (2 μmol $plant^{-1}$ day^{-1}) were given from the start of the experiment. In Experiment 2a, the initial nutrient amount was slightly higher (4·2 μmol N $plant^{-1}$ day^{-1}) and this was gradually decreased during the first 2 weeks to 2·6 μmol on Day 14 (Fig. 3b). This procedure was used to compensate for the difference between the pretreatment and the nutrient addition rate. Thereafter the amount of nutrients was again actively increased by 2% day^{-1}. The different ions measured in the nutrient solution were very low. Only at the start of the experiment were nutrient concentrations slightly higher, occasionally as high as 35, 100 and 20 μM for NH_4^+, NO_3^- and $H_2PO_4^-$ respectively, and this was probably because of the adjustment of the seedlings to the slopes after having been transferred from the pretreatment solution which contained 1 mM total N.

Fig. 1. Examples of the different mycorrhizal stages. (a) An example of mycorrhizal stage I is shown; two mycorrhizal root tips and a non-mycorrhizal root (with root hairs). (b) Represents an example of mycorrhizal stage II; all the root tips are mycorrhizal and thin strands of mycelia can be seen along the root. (c) An example of mycorrhizal stage III is shown; mycorrhizal root tips stick out of the thick layer of mycelia that covers the root.

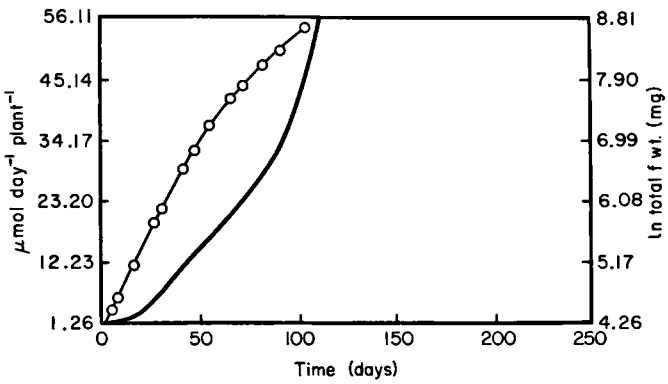

Fig. 2. Maximal growth curve of Douglas fir seedlings (ln (total f wt.), —O—, right axis) and maximal addition curve (μmol day^{-1} plant^{-1}, ——, left axis).

Table 2. Total fresh weight (n = 15–40), % dry weight and N concentration in Douglas fir seedlings (n = 3), that have just germinated, been pretreated or grown under optimum nutrient conditions. SEM in brackets

	Total fresh weight (mg)	% dry weight	μmol N (g total f wt.)$^{-1}$
Seedlings	60 (1)	17	633 (3)
Pretreated (mM N)			
1	223 (5)	20	307 (5)
2	217 (4)	22	389 (7)
Maximal growth experiment			
After 83 days	3407 (215)	22	283 (12)
After 111 days	6360 (336)	29	351 (17)

While growing on the slopes, the mean NO_3^- concentration was below 50 μM, while the concentrations of the other nutrients were even lower: less than 10 μM for NH_4^+ and $H_2PO_4^-$. Thus, during almost the entire experiment all the added nutrients were absorbed; this indicates that external nutrient conditions did limit growth. Mean pH in this experiment was 4·5 (SEM = 0·03).

Mycorrhiza formation and effects on growth and nutrient concentration. Mycorrhiza formation was very rapid. In Experiment 2a, the first infected root tips appeared 14 days after inoculation. Between Day 14 and Day 50, more and more root tips became infected, and by Day 50 mycorrhization reached stage III. Thereafter, mycorrhization remained at this stage. All newly formed root tips immediately became infected. Ten days later the first signs of the decline of the fungal material became visible as indicated by the mycorrhiza changing colour from purple–white to brownish.

In Experiment 2b, the first mycorrhizal root tips were spotted 2 weeks after inoculation and mycelia appeared to be growing over the entire root. However, the layer of mycelia covering the roots remained thinner than that of plants from Experiment 2a. Nevertheless, all root tips were infected by Day 45 and were, or had been, intense purple in colour. After

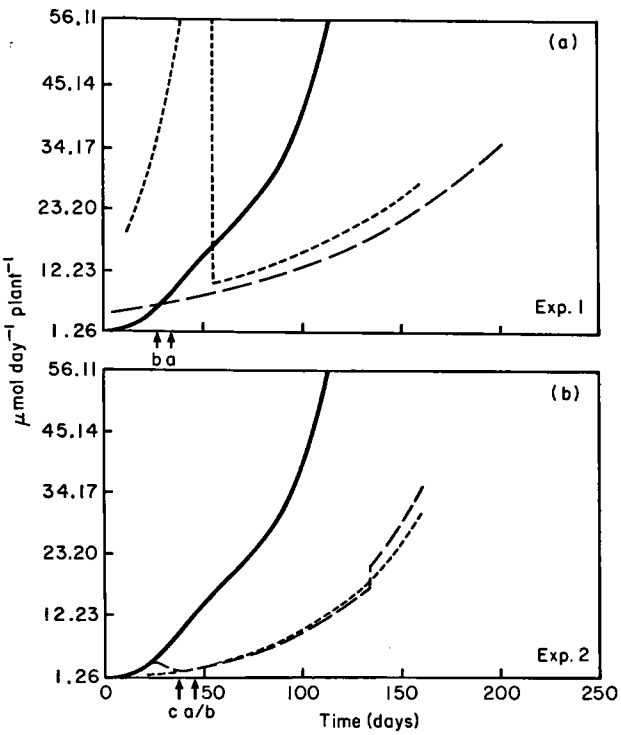

Fig. 3. The addition curve (μmol plant^{-1} day^{-1}) for Experiment 1a (— — —) and Experiment 1b (- - -) (Fig. 3a) and for Experiment 2a (— — —) and Experiments 2b and 2c (- - -) (Fig. 3b). In all the figures, the maximal addition curve is also given (——) for the purpose of reference. The arrow(s) indicate moment of inoculation, the letters refer to the experiment, and give moment of inoculation.

Day 45 these processes did not seem to change much and mycorrhization typically remained at stage II. Three weeks later the mycelia gradually changed to a brownish colour. In Experiment 2c, mycorrhiza formation was completely different from that in Experiments 2a and 2b: initially mycorrhiza formation was restricted to those places where the inoculum had adhered to the roots and very little further growth of mycelia occurred on the roots. Mycorrhization could typically be classified as being at stage I up to 56 days after inoculation.

The differences in mycorrhization between the separate experiments are schematically presented in Table 5. Data on growth of the mycorrhizal and non-mycorrhizal plants are shown in Table 3. Differences in growth parameters between mycorrhizal and non-mycorrhizal plants depended on the experimental conditions: in Experiment 2a the final weight of mycorrhizal plants was 10% less than the final weight of non-mycorrhizal plants. In Experiment 2b, there was no difference between the mycorrhizal and non-mycorrhizal plants, but in Experiment 2c, the mycorrhizal plants produced significantly higher fresh and dry weights (50%) than the non-mycorrhizal plants. In this experiment, the shoot/root ratio of the mycorrhizal plants was also significantly higher than that of the non-mycorrhizal plants at the moment of final harvest.

The N, P, K and Mg concentration of the mycorrhizal shoots in Experiment 2a was higher than that of the non-mycorrhizal shoots, however, the opposite was the case for the roots. Taking the whole plant into account, there was not much difference between the N,

Table 3. Growth parameters (g f wt.) of Douglas fir seedlings either inoculated (myc) or not inoculated (non) with *Laccaria bicolor*. The values for the mycorrhizal plants include those of the mycelia or are corrected for the mass of mycelia (Experiment 1); in Experiment 2 the values for the mycorrhizal plants do not include the mycelia. The % dry weight figures do not include the mycelium. The value of *n* and the day of harvest are given. SEM in brackets

	Mycorrhizal state	*n*	Total fresh weight	Corrected fresh weight	Shoot/root ratio (f wt.)	% dry weight
Experiment 1a						
Day 195	myc	16	6·07 (0·86)	5·83	0·63 (0·05)	0·26
	non	16	4·84 (0·81)		0·87 (0·28)	0·30
Experiment 1b						
Day 155	myc	10	*5·05 (0·60)	*4·88	0·37 (0·04)	0·24
	non	10	1·87 (0·25)		0·51 (0·05)	0·30
Experiment 2a						
Day 128	myc	34	*2·45 (0·13)		0·28 (0·01)	0·22
	non	34	2·70 (0·27)		0·23 (0·02)	0·27
Experiment 2b‡						
Day 104	myc	23	3·25 (0·16)		0·45 (0·04)	0·21
	non	22	3·23 (0·43)		0·47 (0·04)	0·23
Experiment 2c§						
Day 86	myc	18	*3·15 (0·24)		*0·58 (0·04)	0·20
	non	22	2·18 (0·17)		0·25 (0·01)	0·22

*Indicates that the mycorrhizal values are significantly different from the non-mycorrhizal value at $P<0·05$ (Student's *t*-test).
‡Combined data from Day 100 (myc $n=14$; non $n=13$) and Day 107 (myc $n=9$; non $n=9$).
§Combined data from Day 83 (myc $n=4$; non $n=8$) and Day 89 (myc $n=14$; non $n=14$).

P and K content of the mycorrhizal and non-mycorrhizal plants. In Experiment 2b, there was only a slight difference between the fresh weights of mycorrhizal and non-mycorrhizal plants.

In Experiment 2c, however, N and P concentrations were highest in the non-mycorrhizal plants both with regard to fresh and dry weight measurements.

DISCUSSION

It should be realized that for research concerned with mycorrhiza and mineral nutrition, the experimental growth phase (seedling stage at the first flush of growth) as well as the growth phases of older trees should be studied.

According to Ingestad (1982) plants with a constant RGR (exponential growth) have a constant internal N concentration. Moreover, there is a linear relationship between RGR and internal N concentration. However, it should be noted that secondary N-compounds are not taken into consideration. Most of the experiments with trees undertaken by Ingestad *et al.* dealt with growth in the exponential phase (Ingestad *et al.* 1986; Kähr & Arveby 1986). In their experiments, seedlings were given a treatment prior to the actual experiment to bring them into steady-state growth conditions. In general, plants were harvested when total fresh weight of the plants was around 1500 mg. The present growth experiments were started with or without a pretreatment and the final harvest was carried

Table 4. Concentration of macronutrients (μmol (g total f wt.)$^{-1}$) in Douglas fir plants either inoculated (myc) or not inoculated (non) with *Laccaria bicolor*. The data of the mycorrhizal roots include fungal material for Experiment 1 and do not include fungal material for Experiment 2. $n = 13$–20 in Experiment 1a, 1 in Experiment 2b, and 2 or 3 from pooled samples in Experiments 1b, 2a and 2c. SEM in brackets

Experiment	N	P	K	Ca	Mg
Experiment 1a					
Myc					
shoot	368 (21)		82 (3)	11 (0·9)	12 (0·8)
root	337 (15)		64 (2)	6 (0·5)	7 (0·3)
Non					
shoot	368 (29)		85 (4)	9 (0·8)	12 (0·8)
root	343 (15)		79 (4)	6 (0·5)	7 (0·5)
Experiment 1b					
Myc					
shoot	425 (27)		56 (2)	5 (0·3)	12 (0·8)
root	209 (13)		*28 (0·2)	2 (0·2)	3 (0·2)
Non					
shoot	473 (40)		66 (14)	5 (0·3)	12 (1)
root	263 (10)		44 (3)	3 (0·2)	5 (0·1)
Experiment 2a					
Myc					
shoot	*715 (19)	*93 (2)	*91 (0·7)	22 (0·3)	*28 (0·3)
root	*223	*21 (0·1)	*41 (3)	*5 (0·1)	*6 (0·1)
fungus	278 (3)	13 (0·2)	17 (0·4)	1 (0·1)	3 (0·1)
Non					
shoot	543 (26)	61 (1)	83 (0·2)	23 (0·2)	25 (0·4)
root	296 (9)	28 (0·5)	57 (1)	7 (0·1)	8 (0·2)
Experiment 2b					
Myc					
shoot	310	49	73	7	14
root	168	19	33	2	4
fungus	331	17	31	1	4
Non					
shoot	325	43	73	7	14
root	132	18	33	2	4
Experiment 2c					
Myc					
shoot	*310 (19)	40 (0·7)	77	10	19
root	*88 (6)	*12	26	1	3
fungus	243				
Non					
shoot	564	54 (3·3)	87	9	26
root	167 (18)	22 (0·7)	43	3	5

*Indicates that the mycorrhizal value is significantly different from the non-mycorrhizal value at $P < 0.05$ (Student's t-test).

out when plants were much older than in the studies of Kähr and Arveby (1986); in general more time was needed to study mycorrhization in Douglas fir.

In order to compare the separate experiments we used the so-called maximal addition curve. The calculation of this curve was based on an N concentration of 330 μmol

Table 5. Ectomycorrhiza development of Douglas fir seedlings inoculated with *Laccaria bicolor*, time of harvest and weight of the mycelia as percentage of the total weight. The day on which the final mycorrhization stage was reached is given in brackets. All data indicate days after inoculation

	Experiment				
	1a	1b	2a	2b	2c
First root tip infected	80	80	14	18	25
Mycelium covered all the roots			21	25	32
Mycorrhiza stage	I (100)	I (100)	III (50)	II (45)	I (56)
First signs of decline of mycelium			110	67	not seen
Harvest time	164	141	128	83	67
Mycelium as % of total fresh weight			33%	16%	3·3%
Mycelium as % of total dry weight			15%	6%	1·8%

(g total fresh weight.)$^{-1}$. This value was based on figures obtained from young pretreated seedlings and from concentrations of plants with a RGR_{max} that had already passed the exponential period (Table 2). This value correlated well with the N concentration of young *Pinus sylvestris* seedlings that had had a similar pretreatment in a nutrient-rich solution (Kähr & Arveby 1986). Although RGR decreased in time (Fig. 2) the exponential addition, which was maintained at the same percentage per day, still remained below M.A. As a result of this, an increase in the internal concentrations should be expected during the later growth period. This was indeed observed.

Formation and functioning of mycorrhiza as affected by mineral supply

The general conclusion is that the level of external nutrient concentration did affect mycorrhiza formation and functioning. Very low or limiting external nutrient concentrations rapidly resulted in the abundant mycorrhization stage (III) as observed in Experiment 2a. Mycorrhiza formation was poorer (stage I) when nutrient concentrations were higher (Experiment 1a and b).

Mycorrhization was found to occur in plants growing with a low RGR (Experiments 1 and 2). Good mycorrhization was also found in plants growing at a high RGR (Kamminga-van Wijk 1991), and this was also observed in *Pinus sylvestris* seedlings (Kähr & Arveby 1986).

Another important observation is that the nutrient concentration before inoculation has no effect on mycorrhization. In Experiment 1a, nutrient concentration at the time of inoculation was close to M.A., while in Experiment 1b it was much higher than M.A. and was lowered 29 days after inoculation without significantly affecting mycorrhization. There are indications that, under very low nutrient conditions, the time of inoculation is a sensitive period. There was a slight difference between Experiments 2a and 2b in the time of inoculation. The slightly higher initial amount of nutrients added at the beginning (during the first 2 weeks) of Experiment 2a is the only difference between Experiments 2a and 2b (Fig. 3b), but different mycorrhizal stages were nevertheless reached (II and III).

At very low external nutrient concentrations, abundant mycorrhiza formation occurred (Experiment 2a) which was accompanied by a negative effect on the growth of the host. However, under natural conditions mycorrhization probably improves plant growth as

compared to non-mycorrhizal plants. This growth will be achieved by enlarging the absorbing surface. This effect could not be observed in this particular experiment as nutrient concentrations remained limiting. At relatively low nutrient concentrations (NH_4^+, $H_2PO_4^-$ and NO_3^- lower than 25, 40 and 200 μM, respectively), mycorrhizal plants were larger than non-mycorrhizal plants (Experiments 1a and b). At limiting nutrient concentrations (NH_4^+, $H_2PO_4^-$ and NO_3^- lower than 10, 10 and 50 μM, respectively) non-mycorrhizal plants were larger or the same as mycorrhizal plants. At high nutrient concentrations, mycorrhizal plants were again smaller than non-mycorrhizal plants (Kamminga-van Wijk 1991).

In this series of experiments, mycorrhiza formation was described by classifying the observed symptoms in different stages. The plants in Experiment 2c behaved differently to those in both Experiments 2a and 2b, the only difference in experimental set-up being the 12-l pumps used right from the start. Stage I mycorrhization could be due to the increased flooding brought about by these pumps. The experiment lasted only 86 days and it seems possible that mycorrhization may have reached a different stage at a later date. In this experiment, mycorrhizal plants were larger; this is possible only when plant nutrient concentration is low because of the limiting effect of external nutrient concentration. As this was indeed observed in these experiments, it is possible that mycorrhizal growth and not tree growth was retarded by the higher flooding of the pump. Such changes in growth could be mediated by changed hormonal balances between mycorrhizal fungus and tree, however, this remains to be proved.

A number of studies show that mycorrhizal infection is reduced at high N and/or P levels (Ekwebelam & Reid 1983; Danielson *et al.* 1984; Castellano *et al.* 1985; Väre 1989). Gagnon *et al.* (1988) very clearly demonstrated that there is an optimum N concentration for mycorrhizal infection. *Picea mariana* seedlings, infected with *Laccaria bicolor* and grown in a greenhouse had significantly more mycorrhizal roots when fertilized at an N level of 8·5 mg compared with a level of 15 mg per seedling during a 20-week growth period. The number of mycorrhizal roots was not significantly greater than those of plants fertilized with 6 mg N. It was only at 6 mg N that the mycorrhizal seedlings absorbed significantly more N than the non-mycorrhizal seedlings.

In contrast, the nutrient level in other studies did not affect mycorrhizal development using *Pseudotsuga menziesii–Laccaria laccata* (Molina & Chamard 1983) or using *Larix laricina-Laccaria laccata* (Chakravarty & Chatarpaul 1990).

After 67–100 days the first signs of mycelia decline could be observed, but this does not mean that the whole mycorrhiza was declining. This could be seen in hydroculture experiments while in experiments using solid substrates these processes could not be observed.

It is difficult to make a comparison between the present results obtained in hydroculture experiments and the results described above, which were obtained in fertilization experiments on solid substrate. Meyer (1974) pointed out that fertilization will affect the rooting of the tree and will thus influence mycorrhization in that way.

CONCLUSIONS

It is clear that the concentration of the nutrient solution is a decisive factor in mycorrhiza formation. On the one hand, the nutrient addition determines the RGR and the level of the internal nutrient status of the plant. On the other hand, the amount of absorbed nutrients determines the concentration of the remaining nutrient solution. All these factors were important for the formation and functioning of mycorrhizae in hydroculture.

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