



# The impact of artificial mycorrhizal inoculation on the growth of common oak seedlings and development of mycorrhiza: Inoculation may not positively affect growth of seedlings

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## Abstract

**Background and Purpose:** This study evaluated the effect of the VAMBAC<sup>®</sup> mycorrhizal inoculum on the growth and mycorrhizal characteristics of *Quercus robur* seedlings.

**Materials and Methods:** The oak seedlings soaked in an inoculum containing mycorrhizal fungi were planted on six study plots. The non-inoculated seedlings were planted on control plots. Two-year-old plants were evaluated at three of the plots, while 9-to-10-year-old plants were assessed at the remaining three plots. Seedlings' above-ground height, main root length, root dry mass, above-ground dry mass and stem diameter were evaluated. For five plants, mycorrhizal characteristics (the density of active and inactive mycorrhizae and the proportion of active mycorrhizae) were also studied in the laboratory.

**Results and Conclusions:** Inoculated seedlings demonstrated significantly higher proportion of active mycorrhizae in all plots as well as a higher density of active mycorrhizae only on some studied plots when compared to control. Among growth characteristics, inoculated seedlings demonstrated higher values for all evaluated variables. The most striking differences were observed in stem diameter, which was significantly different for all young stands and for one plantation. For the remaining growth characteristics, inoculated and control seedlings displayed significant differences only on some plots. These results showed that artificial mycorrhizal inoculation can be used in forestry practice, but its effects may not always be necessarily positive.

## INTRODUCTION

In consequence of global climate change, forests in Europe will face not only changes in average values for climatic factors, but also higher variations associated with more extreme weather fluctuations, e.g. longer drought periods, storms and floods (1). Oak forests have been damaged by drought in large areas in Europe. Such conditions activate a number of pathogenic fungi (2). A significant threat is *Phytophthora cinnamomi* Rands 1922, an oomycete attacking oak roots in forests, which increases in severity particularly during long periods of drought, higher summer temperatures and long periods of high temperatures combined with short and intense precipitation (3). In Central and Eastern Europe, however, this oomycete will likely not spread significantly

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due to cold winters (4). There is also a threat for the introduction of the allochthonous fungal species, especially *Ceratocystis fagacearum* (Bretz) J. Hunt, imperfect stage *Chalara quercina* Henry, which causes so-called oak wilt in North America and is carried by insect vectors.

The central Morava River alluvial plain (in the eastern Czech Republic) is an area of oak dieback. Permanent decrease in the level of ground water, an initial factor in oak wasting, has probably been accompanied infection honey fungus *Armillaria mellea* (Vahl.) P. Kumm., ultimately leading to oak mortality (own observation). The ash is the second economically crucial tree in the region, which is currently infected by the fungus *Hymenoscyphus fraxineus*, both in the wild and on plantations (5). This has led foresters to abandon the renewal of this tree (own observation). Successful renewal of oak stands is therefore important in future.

Foresters began semi-operational testing of the possibility of supporting seedling vitality by artificially inoculating bare-root seedlings with mycorrhizal fungi before planting (Holuša observ.). Artificial inoculation's main objectives include the improving of seedlings' successful survival, after transplantation and the stimulation of their further efficient growth under stress conditions. Improvement of health also leads to increased resistance against various abiotic influences and harmful biotic agents (6).

GRYNDLER *et al.* (7) draw attention to the fact that research results exhibiting unsuccessful inoculation (i.e. which did not show a beneficial effect from mycorrhizal symbiosis on plant growth and health) are rarely published (e.g. 8). A simple comparison of the number of successful and unsuccessful inoculations in published studies would therefore be considerably confusing for evaluating mycorrhizal inoculation use in practice. In addition, most studies examining the effect of artificial inoculation on tree growth are short-term and frequently involve seedlings grown under artificial conditions. Long-term research including young trees and research on seedlings growing under natural conditions are quite rare (7).

LAZEREVIĆ *et al.* (9) emphasized the need for long-term field observations to compare the effects of various treatments as well as treatments with various ectomycorrhizal fungi on the survival and growth of outplanted seedlings (9). For example, experiments on oaks in containers have been published by MITCHELL *et al.* (1984), LUNT *et al.* HEDGER (2003), NÚÑEZ *et al.* (2006), and HERMANN *et al.* (2004), while data from greenhouses have been published by PRUETT *et al.* (2008) and data from the field by GARBAYE *et al.* CHULIN (1997) (10-15).

The objective of our study was to compare the effect of artificial inoculation of *Quercus robur* L. plants on their growth after 2 and 9-10 years of being planted in nature.

## MATERIAL AND METHODS

The effect of the VAMBAC® mycorrhizal preparation from Biotechnology, a. s. (Czech Republic) on *Quercus robur* seedlings' growth and development was evaluated at six study plots. All plots were situated in one locality in the Upper Moravian Vale near the municipality of Střeň (49°41'19"N, 17°9'25"E, Czech Republic) in *Querceto-Fagetum* forest zone with mesotrophic Cambisols on plain terrain. The plots were 0.7–1 ha, elongated and with north-south orientation. The distance between plots was <1 km. The plots were surrounded by mixed forest stands of various ages.

Each plot was divided lengthwise in the north-south direction into two sections. In 0.3 ha of the first of the two sections the seedlings were treated with the VAMBAC® mycorrhizal preparation after planting (Table 1). The preparation was a mixture of powdery and liquid components, according to production containing isolates of mycorrhizal fungi with fixation powder. The preparation contained 6 genera of ectomycorrhizal fungi (the genera *Cenococcum*, *Hebeloma*, *Laccaria*, *Paxillus*, *Rhizopogon*, and *Scleroderma*) (16). More information about VAMBAC® has not been found out. Since planting was done in forests, the soil was not mechanically agitated. Cambisols were loamy, more than 1 m deep and without stones larger than 1cm.

Seedling roots were thoroughly soaked in the mixture in bundles of 10, and the seedlings were then immediately planted in April. Un-inoculated seedlings were planted in the other 0.3 ha section of the plot as controls. At total, 5,000 seedlings (stem diameter 5 mm, height 18-22 cm, 4 years old) per hectare were planted on the plots. To test the long-term effects of mycorrhizal inoculation, seedling characteristics were determined in two-year-old plantation and in 9-to-10-year-old young stands (Table 1).

During 9–10 October 2009, the oak seedlings' growth characteristics were evaluated in all plots. For plantations, this included measuring stem diameter of control and inoculated seedlings at ground level. For young stands,

**Table 1.** Altitudes and the year of inoculation of oak seedlings in study plots

Plot	Altitude (m)	Year preparation applied
Plantation A	280	2008
Plantation B	250	2008
Plantation C	249	2008
Young stand A	260	2000
Young stand B	250	1999
Young stand C	250	2000

**Evaluation parameters:**

1. Density of active mycorrhizae – calculated as average value of determined number of active mycorrhizal tips associated with 1 cm of root length (cm<sup>-1</sup>)
2. Density of inactive mycorrhizae – calculated as average value of determined number of inactive mycorrhizal tips associated with 1 cm of root length (cm<sup>-1</sup>)
3. Proportion of active mycorrhizae – ratio of active to total mycorrhizal tips (%)
4. Above-ground height – measured from ground to top of the terminal bud (cm)
5. Maximum root length – measured from stem to tip or end of the intentionally modified root section (cm)
6. Root dry mass – average root dry mass (g)
7. Above-ground dry mass – average above-ground part dry mass (g)
8. stem diameter (cm)

only stem diameter was measured. At each plot 50 oak seedlings were evaluated. They were placed in one line in the central part of treated as well as control plot. Weed infestation was strong in all plots, and coverage by grass and other plants was >90 %.

Five treated and five control seedlings from each plantation (30 seedlings in total) were dug out of the ground and then measured for above-ground height (cm), maximum root length (cm) with a folding meter, stem diameter (mm) with a sliding ruler, above-ground dry mass, and root dry mass with a scale measuring grams (g). Seedlings of average height were chosen in the central line of seedling growing 10 m from the edge of plot. Each tenth seedling was studied. From 9-to-10-year-old stands, five soil probes from treated sections and five soil probes from control sections (30 probes in total) were collected using a root drill with internal diameter of 6 cm and depth of 15 cm, equipped with a sawing blade for cutting roots. Probe contents were separated in the laboratory and individual root segments were classified into three size categories according to root diameter (root segments with diameters <1 mm, 1–2 mm, and >2 mm).

Mycorrhizal characteristics were evaluated using the method of identifying all active and inactive mycorrhizal tips (17). The main unit for determining the number of mycorrhizae was a root segment of 5 cm in length and less than 1 mm in diameter. Twenty root segments from each sample were evaluated under stereomicroscope at 40× magnification. Tips with developed hyphal mantle, without root hairs, smooth on the surface and light in colour were classified as “active mycorrhizae”. Tips lacking hyphal mantle and wrinkled surfaces were classified as “inactive mycorrhizae” (18–19). The degree of mycorrhizal colonization was evaluated using two parameters: mycorrhizal tip density and proportion. The density of active and inactive mycorrhizae was calculated as the number of active (inactive) mycorrhizal tips associated with 1 cm of root length. The proportion of active mycorrhizae was calculated as the number of active mycorrhizal tips to all (both active and inactive) mycorrhizal tips (20).

The evaluated parameters of inoculated and control oak seedlings were compared statistically using Statistica 12 software (StatSoft, Inc., Tulsa, OK, USA, 2013). The comparison was done separately for each location, as individual plots differed in seedling age and stand conditions (see Table 1). The data were tested for normality using the Shapiro–Wilk test. Since the evaluated parameters were not normally distributed, the non-parametric Mann–Whitney test was used for their comparison. Dry mass of above-ground plant and the root sections collected on 2-year-old plantation plots was not statistically processed as a result of the weighing procedure whereby all inoculated and control seedling sections for each plot were dried and weighed together and a sufficient quantity of tested samples was therefore not obtained.

The collected seedlings’ root systems (from the whole probe) and above-ground sections were subsequently used to determine dry mass. The samples were dried in a dryer at 105 °C and then weighed on electronic scale (with accuracy 0.01 g).

**RESULTS**

Root dry mass was significantly greater for inoculated seedlings only in 9-to-10-year-old stand B for mass of roots less than 1 mm in diameter (Table 2). In all other cases, no significant differences between inoculated and control seedlings were found for dry mass of total roots or roots with diameter <1 mm, 1–2 mm, or >2 mm (Table 3).

The proportion of active mycorrhizae was significantly greater for inoculated seedlings on all plots (Tables 2, 3). The density of active mycorrhizae was significantly greater for inoculated seedlings in all plantations. In 9–10-year-old stands, the density of active mycorrhizae for inoculated seedlings was significantly greater only in young stand A (Table 3). The density of inactive mycorrhizae was significantly greater for control seedlings in plantation B and young stand C, while on the remaining

**Table 2.** Values of mycorrhizal characteristics and root dry mass of oak plants (mean  $\pm$  SE) in study plots (plantation...2-year-old trees; young stands... 9-to-10-year-old young trees)

Plots	A	A	B	B	C	C
	inoculated	control	inoculated	control	inoculated	control
Plantation						
Density of active mycorrhizae (cm <sup>-1</sup> )	2.0 $\pm$ 0.2	0.9 $\pm$ 0.3	2.3 $\pm$ 0.2	1.3 $\pm$ 0.1	2.2 $\pm$ 0.2	1.1 $\pm$ 0.1
Density of inactive mycorrhizae (cm <sup>-1</sup> )	1.3 $\pm$ 0.1	1.9 $\pm$ 0.2	1.4 $\pm$ 0.2	2.3 $\pm$ 0.2	1.6 $\pm$ 0.2	1.8 $\pm$ 0.1
Total density of mycorrhizae (cm <sup>-1</sup> )	3.3 $\pm$ 0.3	2.8 $\pm$ 0.5	3.7 $\pm$ 0.3	3.6 $\pm$ 0.3	3.8 $\pm$ 0.4	2.8 $\pm$ 0.2
Proportion of active mycorrhizae (%)	61.3 $\pm$ 2.0	31.4 $\pm$ 3.7	61.1 $\pm$ 3.0	35.1 $\pm$ 2.7	57.3 $\pm$ 1.0	36.3 $\pm$ 2.6
Young stands						
Density of active mycorrhizae (cm <sup>-1</sup> )	1.8 $\pm$ 0.1	1.2 $\pm$ 0.0	1.6 $\pm$ 0.4	1.3 $\pm$ 0.2	1.5 $\pm$ 0.1	1.2 $\pm$ 0.2
Density of inactive mycorrhizae (cm <sup>-1</sup> )	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1	1.1 $\pm$ 0.2	1.7 $\pm$ 0.2	0.9 $\pm$ 0.1	1.7 $\pm$ 0.0
Total density of mycorrhizae (cm <sup>-1</sup> )	3.2 $\pm$ 0.2	2.7 $\pm$ 0.1	2.7 $\pm$ 0.6	3.0 $\pm$ 0.5	2.4 $\pm$ 0.2	2.9 $\pm$ 0.2
Proportion of active mycorrhizae (%)	56.7 $\pm$ 2.0	44.3 $\pm$ 1.3	57. $\pm$ 2.85	43.23 $\pm$ 1.87	63.07 $\pm$ 1.08	39.76 $\pm$ 2.82
Root dry mass <1 mm (g)	0.10 $\pm$ 0.01	0.12 $\pm$ 0.02	0.27 $\pm$ 0.02	0.17 $\pm$ 0.02	0.10 $\pm$ 0.02	0.15 $\pm$ 0.02
Root dry mass 1–2 mm (g)	0.44 $\pm$ 0.06	0.28 $\pm$ 0.08	0.24 $\pm$ 0.04	0.38 $\pm$ 0.10	0.18 $\pm$ 0.03	0.09 $\pm$ 0.02
Root dry mass >2 mm (g)	0.49 $\pm$ 0.36	0.12 $\pm$ 0.05	0.54 $\pm$ 0.23	0.75 $\pm$ 0.39	0.31 $\pm$ 0.13	0.06 $\pm$ 0.03
Total root dry mass (g)	1.03 $\pm$ 0.34	0.52 $\pm$ 0.10	1.05 $\pm$ 0.23	1.31 $\pm$ 0.48	0.59 $\pm$ 0.14	0.31 $\pm$ 0.05

**Table 3.** The results of the Mann–Whitney U-test for studied variables (plantation...2-year-old trees; young stands... 9-to-10-year-old young trees; \*...  $p < 0.05$ ; \*\*...  $p < 0.01$ ; \*\*\*...  $p < 0.001$ ).

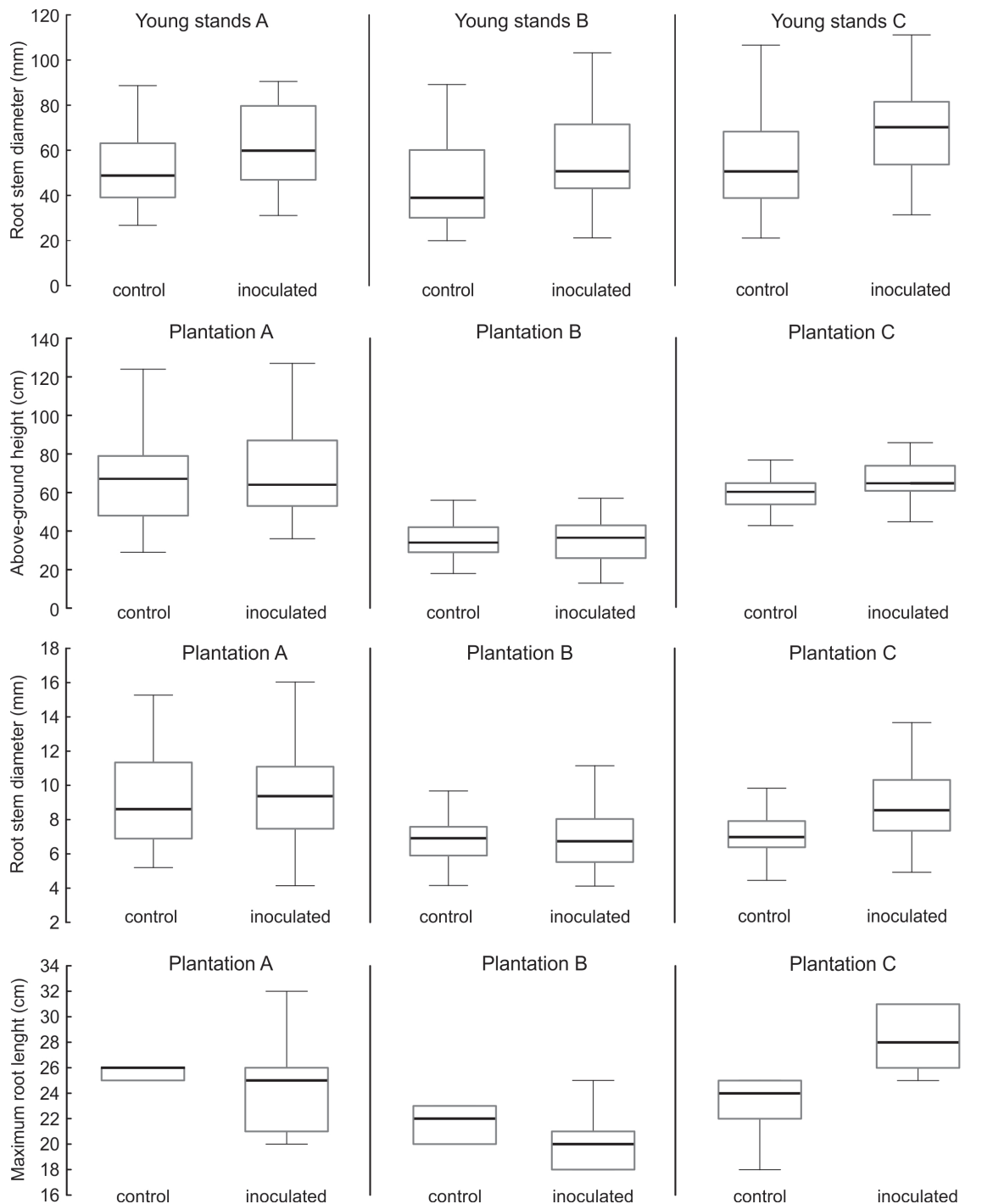
Variable	Plantation	Plantation	Plantation	Young stand	Young stand	Young stand
	A	B	C	A	B	C
Plot	A	B	C	A	B	C
Above-ground height	889.5 n.s.	948.5 n.s.	788**	-	-	-
Stem diameter	814.5 n.s.	913 n.s.	547***	701**	679.5**	469***
Maximum root length	10 n.s.	9.5 n.s.	1*	-	-	-
Root dry mass <1 mm	-	-	-	11.5 n.s.	1.5 *	4.5 n.s.
Root dry mass 1–2 mm	-	-	-	6 n.s.	7 n.s.	4.5 n.s.
Root dry mass >2 mm	-	-	-	10.5 n.s.	12 n.s.	3.5 n.s.
Total root dry mass	-	-	-	6 n.s.	12 n.s.	5.5 n.s.
Proportion of active mycorrhizae	0*	0*	0*	0*	0*	0*
Density of active mycorrhizae	2*	1*	1*	0*	9 n.s.	6 n.s.
Density of inactive mycorrhizae	3 n.s.	1*	8 n.s.	8 n.s.	4 n.s.	0*
Total density of mycorrhizae	6 n.s.	12 n.s.	4 n.s.	3 n.s.	10 n.s.	3 n.s.

plots these differences were not statistically significant (Tables 2, 3).

Above-ground height and stem diameter were significantly higher at inoculated seedlings compared with the control in plantation C, while in plantation A and B these characteristics were not significantly different. In all 9-to-10-year-old stands, stem diameters were greater for inoculated seedlings (Figure 1, Table 3).

## DISCUSSION

Not much data exists on use of VAMBAC®, and it is difficult to compare those that do exist. VON BENNEWITZ and HLUSEK (2006) tested the effect of VAMBAC® and Amalgerol® inoculation preparations on two-year-old apple (*Malus* sp.) trees in plastic containers in sandy clay soil under the climatic conditions of South Moravia. Even though one of the preparations had an



**Figure 1.** Height, stem diameter and root length of inoculated and control oaks in study plots (plantation...2-year-old trees; young stands...9-to-10-year-old young trees; line – median, box plot – 25% and 75% quantiles, whiskers – non-outlier range)

identical name the one in our study, it contained *Glomus* and *Gigaspora* arbuscular fungi as well as the rhizospheric bacteria *Agrobacterium radiobacter* (Beijerinck and van Delden) Conn (21). A VAMBAC® preparation with identical composition, the same as in the our study, was tested by TUČEKOVÁ *et al.* (16) on Norway spruce seedlings

in west-northern Slovakia following a large-scale collapse of spruce pure stands in areas of high infection rate of the genera *Armillaria* and *Heterobasidion*. These fungi had infected newly planted seedlings. Compared to control plants, treated plants had more developed root systems, particularly in fine roots, as well as higher foliage percent-



age and longer needles. However, the inoculum had little effect on the growth of above-ground parts. The authors (16) also separately tested the effects of the VAMBAC<sup>®</sup> preparation and the six individual ectomycorrhizal fungi that VAMBAC<sup>®</sup> contains on spruces in artificial conditions. In such conditions, the best growth characteristics were recorded in seedlings inoculated with the *Rhizopogon* fungus, followed by seedlings inoculated with the *Cenococcum* fungus (16). It is apparent that the VAMBAC<sup>®</sup> preparation may not have a positive effect.

Even when using the same preparation (Ectovit<sup>®</sup>) on the same tree species (*Picea abies* (L.) Karst.) in a single area, different results may be obtained. On stands strongly infected by honey fungus (*Armillaria* sp.), Ectovit-treated spruces compared to untreated trees have significantly greater height, above-ground height, stem diameter, maximum root length, proportion of active mycorrhizae, and rooting rate (22). In another part of the affected area, seedlings' growth characteristics were overall slightly more positive for control seedlings (6). REPÁČ *et al.* (26), meanwhile, did not record any significant effects from inoculation using the Ectovit<sup>®</sup> inoculation preparation on the growth characteristics of either bare-root or containerized seedlings of *P. abies*, *Pinus sylvestris* L., *Larix decidua* Mill., *Fagus sylvatica* L. and *Acer pseudoplatanus* L. (23).

We consider the tree species to be undoubtedly an important factor influencing the result of artificial inoculation. The root colonization by a mycorrhizal fungus showed statistically significant differences among individual oak species (*Quercus robur*, *Q. velutina* Lam., *Q. alba* L.) as well as among species of ectomycorrhizal fungi (*Pisolithus tinctorius* = *P. arrhizus* (Scop.) Rauschert, *Suillus granulatus* (L.) Roussel, *S. luteus* (L.) Roussel, *Thelephora terrestris* Ehrh., *Cenococcum geophilum* Fr.) in a study in containers (10). In addition, a generally positive inoculation effect on seedling growth has been found on *Quercus ilex* L. and *Q. faginea* Lam. in containers (12) and on *Q. rubra* L. in Petri dishes (13). Similarly, the number of ectomycorrhizal morphotypes in *Q. rubra* stands was recorded to be increasing with stand age (24).

It is difficult to select a suitable ectomycorrhizal fungus of artificial inoculation for tree species. The ectomycorrhizal fungus should be capable of easily and quickly creating the host plant ectomycorrhizae which are the most effective for the host. In addition, the ectomycorrhizal fungus needs to be well-adjusted to stand conditions in order to grow well in the soil and withstand stress. From a number of laboratory and field studies, it is apparent that there is no universal symbiont for tree species (25).

Inoculated oaks' growth characteristics compared with those of non-inoculated trees depend also on substrate (10, 12), which is also known in spruce (26) and pine (27). Specifically, interactions between fungus and substrate can be significant (26) and this may explain the differing results at individual locations in the present study.

Because there are only very few results from field experiments, and particularly from studies covering a long period of time, climate conditions have not been described in detail. It is apparent, however, that some studies' ambiguous results may have been caused by a variability in mycorrhizal fungi presence under natural conditions depending on soil and climatic conditions (6). Water has a crucial effect on the growth of ectomycorrhizal fungi's mycelia. Sufficient water is necessary for the growth of mycelia and sporocarps. It is known that drought crucially limits the creation of sporocarp (fructification) (7). On the other hand, inoculation can improve seedlings' water uptake during summer drought periods (12).

In nature, artificially inoculated seedlings are also subject to external competition (7, 28), which probably conceals artificial inoculation's effect on tree growth and mycorrhizal characteristics. Seedlings grown in large nurseries are subject to transplantation stress after being planted in nature. Seedlings which have formed ectomycorrhizae, however, adjust to new stands better and more quickly than non-mycorrhizal seedlings, and their short ectomycorrhizal roots can immediately supply the plant with sufficient nutrients for such period of increased requirements (25). From overall statistical evaluation of a number of studies, it appears that applying an ectomycorrhizal inoculation rarely leads to inhibition of plant growth. Demonstrable stimulation of plant growth occurred in approximately only half of studies (28). The current study support conclusion, that although artificial inoculation may improve survival rate and growth, the results may not always be positive. In the case of using VAMBAC<sup>®</sup>, the ambiguous results may be affected by the fact that, in contrast to artificial substrates, soils on the forested plots contain autochthonous ectomycorrhizal fungi and a wide range of microorganisms able to form symbiotic relationships which can create assumptions that are not investigated. They affect seedling growth, and potentially obscure differences between inoculated and control variants (7). Regarding his experiments with artificial pine inoculation, REPÁČ (26) states that pine seedlings are to a significant degree colonized by original, naturally occurring symbiotic fungi (29). Spontaneous mycorrhiza origin thus occurs also in non-inoculated controls, which recorded a higher average values in some experiments (20).

The experimental evaluation yielded ambiguous results. Possible reasons for the results' ambiguity include (i) inoculum quality, (ii) variability in the natural microorganisms present (primary natural mycorrhization) depending on soil conditions, (iii) the effect of unrepeatable climatic conditions, and (iv) potential microclimatic differences during the test period. On the basis of experimental data evaluation, the method of artificially inoculating seedlings cannot be totally excluded from forestry practice as it apparently leads to higher representation of active mycorrhizae, but forest managers could also consider the costs of such measures.

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