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Growth of pedunculate oak seedlings inoculated with ectomycorrhiza *Laccaria bicolor* in excessively humid substrate conditions

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Abstract

Background and Purpose: Pedunculate oak mortality and the success of regeneration are governed by changes in hydrological conditions. Excessive quantities of water in lowland forest ecosystems lead to site waterlogging. This event is assumed to have an unfavourable effect on forest trees and mycorrhizal symbiosis in the soil. The goal of research was to determine the impact of excessive substrate humidity and inoculation on the growth of pedunculate oak seedlings.

Materials and Methods: Research involved inoculated and noninoculated pedunculate oak seedlings growing in a glasshouse in substrates with two different humidity levels. The inoculum of ectomycorrhizal fungus Laccaria bicolor S238 was made in a mixture of alginate and humus. Seedling diameters at root and at half height and heights of seedlings were analysed.

Results: The height of pedunculate oak seedlings growing in an excessively humid substrate was 50% lower and the diameters at the root smaller in comparison with seedlings growing in favourably humid substrate. No significant differences in the heights of inoculated and non-inoculated pedunculate oak seedlings growing in the same humidity conditions were found. There were significant differences in diameters at the root in excessively humid substrate, but not in normal humidity conditions. No differences were found in diameters at half height.

Conclusion: The growth of pedunculate oak seedlings and the ectomycorrhizal inoculation were considerably impacted by unfavourable hydrological conditions.

INTRODUCTION

Lowland forest ecosystems in Croatia extend along the large rivers of the Sava, Drava, Danube, Kupa and their tributaries. Pedunculate oak (*Quercus robur* L.) as the principal tree species in these ecosystems determines structural and functional relationships in forest communities. The prime ecological factor in these ecosystems is water occurring in the form of precipitation, floodwater, groundwater and surface streamflows. In a combination with micro-relief (micro-elevations and micro-depressions), water influences the occurrence of particular tree species and the growth of varied forest communities *(1)*. Pedunculate oak is a stenovalent species with regard to water as an ecological factor. In other words, it requires special hydrological conditions for optimal growth. It grows best on micro-elevations (locally called «*greda*«) in lowland Posavina, Podravina and Pokuplje areas, where the level of groundwater is close to the soil surface (1 – 3 m) and where floods are absent. It also grows well in micro-depressions (locally called «*niza*«), which are more humid than micro-elevations and are exposed to flooding, although floodwater does not remain for long. However, pedunculate oak is susceptible to water retained on the soil for longer periods (such as, for example, in the lowest micro-depressions). In such conditions narrow-leaved ash (*Fraxinus angustifolia*) and black alder (*Alnus glutinosa*) take over *(2)*.

Lowland forest ecosystems in Croatia have manifested symptoms of instability for a relatively long time. Intensive pedunculate oak mortality has been observed since 1910 *(3)*, while the widespread mortality of lowland elm (*Ulmus carpinifolia*) *(4)* began in the 1930s. More recently, narrow-leaved ash and black alder have also shown signs of mortality *(5, 6, 7, 8)*. Intensive decline and mortality of principal tree species is made worse by problems in natural regeneration of lowland forests *(9)*.

The instability of lowland forest ecosystems is caused by biotic and ecological factors *(6)*. The most important ecological factors include worsened chemical and physical soil properties, the retention of floodwater on the soil and changes in hydrological soil conditions. According to Prpić, waterlogging of pedunculate oak sites is the principal cause of tree mortality *(10)*. A longer presence of floodwater in the forest increases $C0_2$ in water by as much as five times in relation to borderline values, which significantly impacts the stability of lowland forest ecosystems. Rauš and Vukelić detected similar changes in the sites of pedunculate oak forests (site waterlogging) on the basis of changed floral composition in forest communities *(11)*.

The underground part of forest ecosystems is also affected by changes in ecological conditions. Soil fungi play a vital role in forest tree development because they participate in the break-up of organic matter into simpler compounds *(12)*. The most important are mycorrhizal fungi, which form symbiotic communities with higher plants on the roots in the soil, thus securing the necessary nutrients for their development and providing plants with better access to nutrients in the soil *(13)*. Commercially important forest tree species in the temperate and boreal region and about 70% of the species in the tropical region form ectomycorrhiza (ECM). Species of the genus *Quercus* also belong to these groups. Ectomycorrhiza or ectendomycorrhiza is formed by between 5,000 and 6,000 fungi species on over 2,000 plant symbionts. In general, the most common ectomycorrhizal fungi in forest ecosystems are *Pisolithus tinctorius, Laccaria bicolor* and *Hebeloma cylindrosporum (13)*.

The formation of an ectomycorrhizal symbiosis depends on ecological conditions in the soil, which primarily refers to the soil's hydrological conditions and nutrient content. Ectomycorrhizal symbioses in adverse ecological conditions are either reduced or completely absent *(14, 15)*.

Unfavourable hydrological conditions (site waterlogging) in lowland forest ecosystems in Croatia were first observed long ago; yet, their impact on the microbiological soil complex as a constituent part of the ecosystem has not been examined. This research starts from the assumption that excessive water in the soil constitutes an unfavourable ecological factor that occurs with increasing frequency in lowland forest ecosystems. It has a negative effect on the growth of pedunculate oak seedlings and the formation of ectomycorrhiza on the root. Our objective was to assess the impact of excessive substrate humidity on seedling development and inoculation with ectomycorrhiza *Laccaria bicolor*.

MATERIALS AND METHODS

Seedlings were grown from pedunculate oak acorns collected in five localities in Croatia (Velika Gorica, Bjelovar, Veliki Grđevac, Strizivojna and Županja). Fifteen to twenty acorns from each localitiy were sown in a mixture of peat and vermiculite (1:1) and placed in 145 ml plastic containers in a glasshouse. The seedlings were raised under controlled conditions, air temperature (20–30 °C), and air humidity (30–70%) at normal daily light and were watered regularly. The two-month-old seedlings of pedunculate oak were inoculated with the fungus *Laccaria bicolor* S238. The mycelium for the production of the inoculum was obtained from the laboratory INRA Dijon in France. It was in a Petri dish on a MNM nutritive medium for fungus growth (Modified Melin Norkans medium) *(16)*.

To prepare the inoculum we first cultivated a considerable quantity of mycelium. Ten segments of *L. bicolor* mycelium from a nutritive medium of 0.5 cm in diameter were placed in 1 l flasks containing MNM nutritive medium for fungus growth. The flasks were placed on a shaker at a temperature of 25 °C under an artificial source of light. The mycelium in the shaker was cultivated for about 5 weeks.

After producing a sufficient quantity of mycelium we began to prepare the inoculum. We put 100 ml of alginate into a blender. We then added 10 g of fragmented sterilised humus in the alginate (particle diameter 0.4 mm), as well as additional 400 ml of alginate. We stirred the prepared mixture and added 10 g of the fungus *L. bicolor* mycelium. Then, by carefully and quickly turning the blender on and off we fragmented the mycelium and mixed it into the alginate.

We then added 500 ml of sterile distilled water and again stirred everything thoroughly. We poured the prepared mixture through a strainer into a vessel with calcium chloride ($CaCL_2$), on a magnetic blender. As the mixture polymerized, the pellets of inoculum containing the fungus mycelium formed in calcium chloride. The inoculum was rinsed in distilled water, placed in plastic bags and stored in a refrigerator. The whole procedure was conducted in sterile conditions.

We inoculated 40 pedunculate oak seedlings and transplanted them and the same number of non-inocu-

TABLE 1

Diameters at root of pedunculate oak seedlings in different conditions of substrate humidity with and without *Laccaria bicolor* ectomycorrhiza.

Treatments			
Nh-inocul	Nh-noninocul	Exh-inocul	Exh-noninocul
d_0 (mm)			
7,1	4,4	6,0	2,7
6,3	5,0	5,2	4,7
6,8	6,8	5,5	4,7
5,2	5,4	6,1	2,6
5,0	5,1	4,9	3,6
5,6	6,9	3,6	4,0
6,0	5,7	4,9	4,2
7,0	6,4	5,7	5,0
5,3	6,4	4,6	4,4
4,9	5,5	5,6	5,5
6,2	6,2	5,2	4,7
5,7	5,2	5,9	4,3
4,2	5,5	4,4	3,0
7,0	6,3	4,9	5,0
6,7	5,3	4,9	5,0
Average			
5,9	5,7	5,2	4,2

Nh normal humidity,
Exh excessively humidity

lated seedlings in 350 ml plastic containers, which were filled with substrate composed of peat and sand (60:40) and mixed with the inoculum. Sand and peat were autoclaved in a plastic bag at 120 °C for 60 minutes. We added 40 g of inoculum per 1 kg of substrate. During the period of growth in the glasshouse, the seedlings (the inoculated and non-inoculated) were fertilised with a liquid nutrient solution once a week (NPK 20:10:15).

After three months in the glasshouse, 20 inoculated and 20 non-inoculated seedlings were transplanted in two types of substrate humidity (normally humid and excessively humid substrate). Under normally humid conditions, the seedlings and the open bottoms of the vessel holding seedling containers were occasionally watered. Excessive substrate humidity simulated »waterlogged conditions«, in other words, the containers with the seedlings were placed in a water-filled vessel in which the water level permanently topped the surface of the substrate in the containers by several centimetres. Nutrients were added once a week during the seedling growth.

After the inoculated and non-inoculated seedlings had been grown in substrates of varying humidity for two and a half months, they were taken out of the containers and their height (h), diameter at half height ($d_{1/2h}$) and diameter at root (h_0) were measured.

It was a two factorial experiment with four treatments in fifteen repetitions. Analysis of variance (ANOVA) was carried out in Systat 6.0, and the data were processed with the statistics programe SAS* System for Windows ver. 8.2. (SAS 2000).

RESULTS

Seedling diameter at the root

The largest diameters at the root were found in inoculated pedunculate oak seedlings growing in normally humid substrates, whereas the smallest diameters at the root were found in non-inoculated seedlings growing in excessively humid substrates (Table 1).

F-test showed significant differences in diameters at root between treatments (Table 2). According to the factor analysis of variance the highest statistically significant differences were found for factor humidity. There were significant differences for factor inoculation, but at a

TABLE 2

Analysis of variance (ANOVA) for diameters at root of pedunculate oak seedlings in different humidity conditions and inoculation.

Variability	n-1	SS	s^2	$F_{exp.}$	$F_{tab.\ 0.05}$	$F_{tab.\ 0.01}$
Total	59	62,38				
Repetitions	14	12,62				
Treatments	3	26,42	8,81	15,85**	2,84	4,31
Errors	42	23,34	0,56			
Factors:						
Humidity	1	19,61	19,61	35,29**	4,08	7,31
Inoculation	1	4,76	4,76	8,57**	4,08	7,31
Interaction	1	2,05	2,05	3,70 ns	4,08	7,31

*$F_{exp.} > F_{tab.\ 0.05}$
**$F_{exp.} > F_{tab.\ 0.01}$
ns non significant

TABLE 3

Differences of studied treatments least squares means for root collar diameter (d_0, mm). Statistically significant diferences are marked.

Treatment	Treatments	Estimate	Standard Error	DF	t Value	Pr > |t|
Nh-inocul	Exh-inocul	0.6650	0.2699	11	2.46	0.0315
Nh-inocul	Nh-noninocul	0.1087	0.2970	11	0.37	0.7213
Nh-inocul	Exh-noninocul	1.8608	0.2736	11	6.80	<.0001*
Exh-inocul	Nh-noninocul	–0.5563	0.2970	11	–1.87	0.0878
Exh-inocul	Exh-noninocul	1.1958	0.2736	11	4.37	0.0011*
Nh-noninocul	Exh-noninocul	1.7521	0.3011	11	5.82	0.0001*

TABLE 4

Diameters at half height ($d_{1/2h}$) of pedunculate oak seedlings in different conditions of substrate humidity with and without *Laccaria bicolor* ectomycorrhiza.

Treatments			
Nh-inocul	Nh-noninocul	Exh-inocul	Exh-noninocul
$d_{1/2h}$ (mm)			
3,5	2,1	3,7	1,9
3,0	2,5	3,9	2,9
3,1	2,7	2,2	2,6
3,1	2,2	3,0	1,6
2,6	2,5	3,0	2,3
2,5	3,2	2,3	2,5
2,5	2,8	2,6	2,5
2,8	2,5	2,6	2,4
2,5	2,7	2,9	2,4
2,6	2,4	2,4	2,8
3,3	2,6	2,7	2,7
2,3	2,2	2,5	2,5
2,2	2,6	3,5	2,0
2,7	2,7	2,2	3,0
2,5	2,4	2,3	2,6
Average			
2,7	2,5	2,8	2,4

TABLE 5

Heights (h) of pedunculate oak seedlings in different conditions of substarte humidity with and without *Laccaria bicolor* ectomycorrhiza.

Treatments			
Nh-inocul	Nh-noninocul	Exh-inocul	Exh-noninocul
h (cm)			
55,0	28,0	17,0	15,0
46,0	25,0	11,0	20,0
29,0	35,5	22,5	11,0
25,5	27,5	13,0	10,0
41,0	38,0	15,5	19,0
30,0	33,5	16,0	18,0
44,0	41,5	16,5	13,0
48,5	37,0	25,0	21,0
37,0	20,0	18,0	24,0
22,5	20,0	17,5	19,0
43,0	46,5	17,5	19,0
29,0	19,5	16,0	12,0
28,5	38,0	7,0	16,5
50,5	51,5	20,0	15,0
33,5	15,0	13,5	14,0
Average			
37,5	31,8	16,4	16,4

lower level. Interaction (humidity inoculation) was not significant, and factors act as independently. The highest statistically significant differences of least squares means (LSD test in SAS) were observed between different treatments of each factor and between non-inoculated seedlings in different hydrological conditions (Table 3). With regard to hydrological features of the substrate, the diameters at the root in the excessively humid substrate were smaller than the diameters at the root in the normally humid substrate, regardless of inoculation.

There were no significant differences in the diameters between the inoculated and non-inoculated seedlings growing in normally humid substrates. Inoculated seedlings in excessively humid substrates had larger diameters than those of the non-inoculated seedlings in the same conditions of substrate humidity. Differences between them were significant (Table 3). The diameters of the inoculated seedlings growing in the normal substrate humidity were larger than the diameters of the inoculated seedlings growing in an excessively humid substrate, but not statistically significant (Tables 1, 3).

Seedling diameter at half height

Diameters at half height did not differ significantly either in terms of substrate humidity or inoculation (Table 4).

TABLE 6

Analysis of variance (ANOVA) for heights of pedunculate oak seedlings in different humidity conditions and inoculation.

Variability	n-1	SS	s^2	$F_{exp.}$	$F_{tab. 0.05}$	$F_{tab. 0.01}$
Total	59	8759,93				
Repetitions	14	1465,43				
Treatments	3	5219,82	1739,94	35,22**	2,84	4,31
Errors	42	2074,68	49,40			
Factors:						
Humidity	1	4986,82	4986,82	100,95**	4,08	7,31
Inoculation	1	123,27	123,27	2,50 ns	4,08	7,31
Interaction		109,73	109,73	2,22 ns	4,08	7,31

*$F_{exp.} > F_{tab. 0.05}$
**$F_{exp.} > F_{tab. 0.01}$
ns non significant

TABLE 7

Differences of studied treatments least squares means for the height (h, cm). Statistically significant diferences are marked.

Treatment	Treatments	Estimate	Standard Error	DF	t Value	Pr > |t|
Nh-inocul	Exh-inocul	19.8500	2.3700	11	8.38	<.0001*
Nh-inocul	Nh-noninocul	5.4649	2.6078	11	2.10	0.0601
Nh-inocul	Exh-noninocul	20.7126	2.4027	11	8.62	<.0001*
Exh-inocul	Nh-noninocul	-14.3851	2.6078	11	-5.52	0.0002*
Exh-inocul	Exh-noninocul	0.8626	2.4027	11	0.36	0.7264
Nh-noninocul	Exh-noninocul	15.2477	2.6439	11	5.77	0.0001*

Seedling height

Of the tested growth parameters, the greatest significant differences were found in the heights of pedunculate oak seedlings between the factors (Table 6). Inoculated pedunculate oak seedlings growing in normally humid substrates attained the best heights, while seedlings growing in excessively humid substrates had the lowest heights (Table 5).

There were also differences in heights between inoculated and non-inoculated seedlings in normally humid substrates, but not significant. The non-inoculated seedlings were shorter in comparison with the inoculated seedlings in normally humid substrates.

There were significant differences in the height of seedlings growing in differently humid substrates regardless of inoculation. Seedlings in excessively humid conditions were shorter than seedlings in normal humidity conditions. Inoculated and non-inoculated seedlings growing in excessively humid substrates did not differ in heights (Table 7).

Seedlings in normally humid substrate developed a dense root network on the bottom of the vessel outside the container, whereas seedlings growing in excessively humid substrate failed to do so. Not one seedling developed a root outside the container. This indicates an unfavourable effect of excessive water quantity in the substrate on the growth of plants and their roots, as well as on the development of ectomycorrhiza on the plant root.

DISCUSSION

Changes in hydrological conditions with regard to excessive quantities of water in pedunculate oak sites are the principal cause of instability in lowland forests *(6, 9, 10, 11)*. Covering an area of about 200,000 ha, pedunculate oak is the most valuable commercial tree species in Croatian lowland forests. Management problems with this tree species adversely affect overall lowland forest management. In general, the goal of management is to achieve increased quality and quantity of wood mass and other direct and indirect benefits from forests. Management is based on biological and ecological patterns of natural forest development (natural balance), which ensures sustainability of forest ecosystems *(17)*. However, forest ecosystems are also beset by a number of adverse biotic and abiotic factors that exert a negative impact on the development and survival of these ecosystems *(18, 19)*.

One of the main unfavourable ecological factors in lowland forest ecosystems is stagnant flood and surface water on the soil, known as «site waterlogging« *(5, 6, 10, 11)*. It may come as a result of streamflow regulation, construction of forest and other roads and regulation of retention areas. The consequences are intensive decline and mortality of pedunculate oak trees, worsened site conditions (weeds, drainage, waterlogging and compacted soil), as well as failure to regenerate naturally *(9)*. In such conditions, it is necessary to resort to artificial forest regeneration *(17)*. The success of artificial regeneration is often only partial because of adverse site conditions or the occurrence of excessive water in the soil. This is the reason why the growth of pedunculate oak seedlings in natural and artificial regeneration is often restricted.

This research revealed significant differences in the growth of pedunculate oak seedlings in substrates with varying humidity degrees. The greatest differences were found in seedling heights. Seedlings of pedunculate oak growing in normally humid substrates were taller by 52% than seedlings growing in conditions of excessive substrate humidity, regardless of inoculation. In the case of inoculation with ectomycorrhiza, differences between seedling heights in normal and excessive substrate humidity conditions were even greater (54%). Slightly smaller differences were found in diameters at root with regard to different humidity of the substrate. There was a difference in seedling diameter under different conditions of substrate humidity, whereas in the case of inoculation, the inoculated seedlings in normally humid substrate had larger diameters than the inoculated seedlings growing in excessively humid substrates. Unfavourable ecological conditions (excessive substrate humidity) inhibited seedlings.

The effect of ectomycorrhiza on the growth of pedunculate oak seedlings was variable. The heights of inoculated seedlings in normally humid substrates exceeded those of non-inoculated seedlings in the same conditions of substrate humidity, whereas root diameters did not differ. However, in excessively humid substrate conditions the heights of inoculated and non-inoculated seedlings did not differ significantly, while root diameters were larger in inoculated seedlings.

These results suggest that the establishment of optimal ecological conditions in the soil does not benefit only plants, but also other organisms in the ecosystem, primarily micro-organisms or mycorrhizal fungi.

A similar positive effect of ectomycorrhizal inoculation on dry matter weight in the stem and root of black oak seedlings was recorded in a field test in the USA, although seedling growth in a glasshouse was not affected *(13)*. Marx also reported a considerable increase in the height and weight of white oak seedlings and northern red oak inoculated with *Pisolithus tinctorius* ectomycorrhiza in the nursery *(20)*, but poorer success of some individual plants was caused by abundant watering. In another test of *Pisolithus* ectomycorrhiza inoculation, plants in the glasshouse showed better growth, whereas plants inoculated with *Suillus* ectomycorrhiza grew better in the field. According to that research, there are certain specific traits of mycorrhizal species for particular tree species and particular growth conditions *(21)*. Marx also reported better growth of red oak seedlings inoculated with *Pisolithus* ectomycorrhiza in relation to control seedlings and to seedlings with less ECM *(22)*. Garbaye and Churin found a significant improvement in the growth of plants inoculated with *Pisolithus involutus* in two different sites *(23)*.

Castellano gives more attention to the ecology of mycorrhizal fungi when they are used outside their natural range *(24)*. It should also be determined to what extent the plant simbiont, environment, soil and management measures influence the growth and normal functioning of mycorrhizal fungi *(25, 26)*. Inoculation of tree seedlings with mycorrhizal fungi is useful in places where autochthonous fungi are not abundant, where the introduced fungi will tolerate site conditions better, or where the introduced mycorrhizal fungi are more efficient in certain site conditions *(18, 24)*.

The principal factors having an affect on the proportion of fungi in a soil are the soil organic matter, pH value, organic and mineral nutrients, humidity regime, soil aeration, soil temperature, position in the profile, season of the year and vegetation composition *(27)*.

Other environmental factors, such as industrial gasses and pollution with heavy metals, which are the result of human activity, may also adversely affect the development of ectomycorrhiza on forest trees *(13, 21)*. The similar results indicate that changes in ecological conditions may be reflected on inhibition or change in ectomycorrhiza fungi species which occur in specific site conditions, for example, a particular pH value *(26, 28)*. To ensure successful natural or artificial forest regeneration it is necessary to establish optimal ecological and primarily hydrological conditions in the site *(27, 29)*.

The obtained results suggest that the development of seedlings in extreme ecological conditions was more affected by ecological factors and less by ectomycorrhiza, which may be inhibited. There was an almost insignificant formation of ectomycorrhiza on inoculated seedlings growing in excessively humid substrates. Excessive substrate humidity, as an unfavourable factor, had an important effect on both plant growth and ectomycorrhiza development.

These disturbances may be lessened or eliminated, and the stability of pedunculate oak forest ecosystems increased only if favourable ecological conditions for the growth of trees and the development of other plant and animal organisms and micro-organisms are ensured *(30)*.

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