Forest Systems
25(1), e048, 10 pages (2016)
eISSN: 2171-9845
http://dx.doi.org/10.5424/fs/2016251-07036
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)

RESEARCH ARTICLE

OPEN ACCESS

Effects of temperature, pH and carbon and nitrogen sources on growth of *in vitro* cultures of ectomycorrhizal isolates from *Pinus heldreichii* forest

Jelena Lazarević [1]*, Dragana Stojičić [2], Nenad Keča [3]

[1] *University of Montenegro, Biotechnical Faculty, Podgorica, Montenegro* [2] *University of Niš, Faculty of Sciences and Mathematics, Niš, Serbia* [3] *University of Belgrade, Faculty of Forestry, Belgrade, Serbia*

Abstract

Aim of study: This study aims to provide basic information about physiological characteristics of isolates of *Lactarius deliciosus* (L.) Gray, *Russula sanguinaria* (Schumach.) Rauschert, *Suillus collinitus (*Fr) Kuntze, *Suillus granulatus* (L.) Rousell, *Tricholoma batchii* Gulden and *Tricholoma imbricatum (*Fr.) Kumm.

Area of study: The isolates are obtained from *Pinus heldreichii* H. Christ forest in the south-eastern part of Montenegro.

Material and methods: The isolates were molecularly characterised by internal transcribed spacer (ITS) sequencing and *restriction fragment length polymorphism (*RFLP) analysis. The effects of different temperatures (20, 22, 25°C), pHs (4, 4.5, 5.2, 5.8, 6.5, 7.5), and carbon (glucose, sucrose, dextrin, arabinose, xylose and starch) and nitrogen (NH_4^+, NO_3^- and protein) sources on their growth were examined under laboratory conditions.

Main results: The studied factors established significant differences in the development of isolates. Isolates of *R. sanguinaria*, *L. deliciosus* and both *Suillus*, were characterised by faster growth at 22°C, while *Tricholoma* isolates grew faster at 25°C. *S. granulatus*, *S. collinitus* and *T. imbticatum* isolates grew well at lower pH values (4 - 5.2), while *L. deliciosus*, *R. sanguinaria* and *T. bachii* exhibited faster growth at pHs between 5.8 and 6.5. The examined isolates were able to utilize various carbohydrates as carbon sources. The biggest mycelial growth was characterised for sucrose, then glucose, dextrin, arabinose, starch and xylose. They grew on all examined nitrogen sources, while the biggest mycelia growth was achieved on ammonium, followed by nitrate and protein. Those characteristics varied amongst the species.

Research highlights: Information about physiological characteristics of *Tricholoma*, *Lactarius*, *Russula*, as well as *Suillus*, are sparse. Hence, the data obtained in this study could contribute to the understanding of their function in ecosystems.

Keywords: *Lactarius;* Montenegro; physiology; RFLP analysis; *Russula; Suillus*; *Tricholoma.*

Citation: Lazarević, J., Stojičić, D., Keča, N. (2016). Effects of temperature, pH and carbon and nitrogen sources on growth of *in vitro* cultures of ectomycorrhizal isolates from *Pinus heldreichii* forest. Forest Systems, Volume 25, Issue 1, e048. http://dx.doi.org/10.5424/fs/2016251-07036.

Received: 30 Oct 2014. **Accepted**: 21 Jan 2016

Funding: This research was supported by grants from the Ministry of Science and Ministry of Agriculture and Rural Development of Montenegro, project contract nmb 01-406/01-558.

Competing interests: The authors have declared that no competing interests exist.

Correspondence should be addressed to Jelena Lazarević: ena.lazarevic@gmail.com

Introduction

Ectomycorrhizal fungi are important components of soil microbial communities in boreal, temperate and Mediterranean forests, where they play a central role in nutrient cycling processes (Allen *et al*., 1995; Azul *et al*., 2014). Forest trees form ectomycorrhizas with a wide range of soil fungi (Harley & Smith, 1983). The growth of ectomycorrhizal fungi in nature depends on a variety of internal and external factors, such as humidity, pH, soil structure, the various physical and chemical properties of the soil, and the nutrient availability, especially carbon and nitrogen sources (Harley & Smith, 1983; Bowen, 1973). All these factors greatly influence the establishment and growth of ectomycorrhizal fungi both in the field and under controlled laboratory conditions (Sanchez *et al*., 2001). It is also well known that data on decomposition capacity and culture characteristics complement those on the trophic status of some fungi, particularly basidiomycetes (Tedersoo *et al*., 2010).

Suillus, *Tricholoma*, *Lactarius* and *Russula* are frequently observed components of ectomycorrhizal communities in mature Mediteranean, temperate and boreal forests (Azul *et al*., 2014; Smith & Read, 1997; Dahlberg *et al*., 1997). Due to the fact that isolating these species in pure culture is extremely difficult (Smith & Read, 1997; Nygren, 2008), there is a little information about their physiological characteristics. In this paper, we study isolates of *Suillus*, *Tricholoma*, *Lactarius* and *Russula* species from the forest community of *Pinus heldreichii* H. Christ in the south-eastern part of Montenegro, that are, according to the sporocarp production, the most common ectomycorrhizal species in those forests (Lazarević *et al*., 2011). *P. heldreichii* grows at the upper forest line in mountainous regions in the western part of the Balkan Peninsula, exposed to the specific influence of the Mediterranean climate on harsh, xerothermic and poor habitats (Janković, 1960). Mycorrhizal associates are important for its successful growth, survival and regeneration, but not sufficiently studied.

The aims of the study were i) to identify and characterise the obtained isolates using molecular tools and ii) to get some basic information about their physiological characteristics in attempt to get insight about the functional significance of fungal diversity in *P. heldreichii* forests. These results could be also used for the optimisation of vegetative ectomycorrhizal inoculums production under laboratory conditions.

Materials and Method

Site description

Fungi were collected in Kučka Korita (42° 28’ 50.4’’ N; 019° 30’ 04.2’’E) in a *P. heldreichii* forest at 1280 m a.s.l. *P. heldreichii* is a tertiary relict and endemic of the Balkans and the most southern parts of the Apennine Peninsula. It inhabits a forest zone at the upper forest line in a high mountainous region in the western part of the Balkan Peninsula, exposed to the specific influence of the Mediterranean climate (subalpine Meditteranean climate) at altitudes ranging from 1,200 to 2,000 m on limestone soils. Average annual temperatures are 2-4° C, winter temperatures are extremely low (below -30° C). The average annual precipitation is about 2500 mm, but the summer precipitation constitutes only 8% of the total annual precipitation. Long dry periods, with up to 40-70 days without rainfall is characteristic for the summer months in this area. (Hydro-Meteorological services of Montenegro, 1995).

In the locality of Kučka Korita, a *P. heldreichii* forest grows in brownish-red forest soil on limestone (calcocambisol) (Pedological map of Montenegro 1:50.000, Fuštić & Đuretić, 2000). It was necessary to determine the basic ecochemical properties of the soil in the location were the fungi and soil samples were collected. The soil reaction was slightly acidic (pH in H_2O of 6.68; pH in 1N KCl of 6.09). The soil was noncalcerous (0% $CaCO_3$) and very rich in organic matter (content of humus 18%), with very low available P content (0.1 mg P_2O_5 mg/100 g), and medium available K content (19.6 mg K_2O/100 g).

Fungal material

Sporocarps of fungi were collected near a single tree during September and October of 2009. Sporocarps were determined using morphological features (Galli *et al*., 1996; Basso, 1999; Riva, 2003; Roux, 2006).

Isolation from the sporocarps was conducted on modified Melin-Norkrans medium (MMN), containing, per litre, the following: 10 g of glucose, 3 g of malt extract, 1 g of yeast extract, 0.05 g $CaCl_2$, 0.025 g NaCl, 0.25 g $(NH_4)_2HPO_4$, 0.012 g $FeCl_3$, 0.001 g thiamine HCl, 0.5 g KH_2PO_4, 0.15 g $MgSO_4 \cdot 7\ H_2O$, and 15 g of agar (Marx, 1969), amended with 50 ppm Streptomycin (Galenika, Serbia) and Ampicillin (Panfarma, Serbia) and 5 ppm Benomyl (Zorka, Serbia). The pH was adjusted to 5.8 using 1N HCl. The isolates were incubated at 22˚C for 15–20 days in the dark, and maintained onto MMN medium by transfer every three months (Rincon *et al*., 1999). It is possible that certain physiological characteristics of ectomycorrhizal fungi, especially those relating to their enzymatic activity, are altered as a result of *in vitro* cultivation (Cairney, 1999; Buscot *et al*., 2000). Therefore, almost all cultures used were of the same age and were examined immediately after isolation.

Effect of temperature, pH, carbon and nitrogen sources on mycelial growth

Mycelium of each mycorrhizal fungus was initially grown on MMN medium at 22°C for 40 days. Agar plugs 7 mm in diameter, cut from the actively growing margin of subcultures, were aseptically placed on nutrient medium. The effects of different temperatures and carbon sources on the growth of the fungi were studied on solid nutrient medium in 10 cm diameter Petri dishes in 2 x 5 repetitions per treatment, which makes total number of 180 Petri dishes in experiment with temperature (3 temperatures x 6 species) and 360 Petri dishes in experiment with different carbon sources (6 carbon sources x 6 species).

The effects of different temperatures were studied on MMN nutrient medium. Fungal cultures were incu-

bated in the dark at 20°C, 22°C and 25°C. The different carbon sources - sucrose, dextrin, starch, arabinose and xylose (10 g/l) - were added to basic MMN medium (minus glucose), the pH adjusted to 5.8 and the temperature set at 22°C. In both experiments set in Petri dishes, colonies were observed and colony diameters were non-destructively measured after 7, 14, 21 and 28 days. The radial growth of mycelia was slow (0.25-0.95 mm/day) and equable, hence for final calculation colony diameter achieved after 28 days were take in statistic analysis.

The effects of different pHs and nitrogen sources on mycelial growth were studied in solution in sterile plastic cups (75 ml in volume) on 35–40 ml of liquid nutrient medium. This experiment was set up in 3 x 5 repetitions per treatment, which makes total number of 580 cups in experiment with different pH (6 pHs x 6 isolates) and 270 cups in experiment with different nitrogen sources (3 nitrogen sources x 6 isolates). In some cups, mycelia did not show any increase, or contamination happened, so up to 10 repetitions per treatment were used for further statistic analysis.

The effects of different pH values (4, 4.5, 5.2, 5.8, 6.5 and 7.5) were studied on MMN solution (basic MMN medium minus agar). Isolates were grown 28 days at 22°C, and measured once. Growth was estimated by mycelial dry weight measured after 24 h drying time at 65°C.

The isolates were grown on nutrient medium wherein nitrogen (N) was present as NH_4^+, NO_3^- or protein. Various nitrogen sources, $(NH_4)_2HPO_4$ (NPK Inžinjering, Srbija), $Ca(NO_3)_2$ (Centrohem, Srbija) and Albumin bovin-BSA (Albumin, Fraction V, Merck, EU), were assayed. The nitrogen sources (120 mg N/l) were added to MMN solution, minus $(NH_4)_2HPO_4$, malt and yeast extract. In order to obtain a suitable ratio of C: N-20: 1, 6 g l^{-1} glucose was added (Dames *et al*., 1999; Daza *et al*., 2006). Isolates were grown at 22° C during 60 days, because the total amount of developed mycelia was lower due to the decreased amount of available nutrients (removed malt and yeast extract) in solution. Mycelial dry weight was measured after 24 h of drying at 65°C.

Molecular analysis

In order to identify the obtained isolates, genomic DNA was extracted from fresh mycelium grown in liquid MMN medium with the DNeasy Plant Mini Kit (Qiagen Ltd., USA).

The internal transcribed region (ITS) of the ribosomal DNA was amplified using the basidiomycete-specific primer pair ITS1-F and ITS4-B (Gardes & Bruns, 1993). The polymerase chain reaction (PCR) mixture contained 0.1 ng of DNA, 18.5 µl of sterile ultrapure water, 2 µl of each primer (10 pmol/µl) and 25 µl of Kapa Taq Ready Mix (Kapa, USA). Amplification conditions were based on Nieto & Carbone (2009): initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. A negative control was included.

The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen Ltd., USA), and sequencing was performed by Macrogen Inc. (Seoul, Korea), utilizing an ABI 3730 XL automated sequencer (Applied Biosystems, USA). Sequencing was performed in both directions, and raw sequence data were analysed using BioEdit version 7.0.5.2 (Hall, 1999).

Database at both GenBank (Benson *et al*., 2005) and the UNITE (Kõljalg *et al*., 2005) (updated August 2014.) were used to determine the identity of sequences. Comparison of sequences for the ITS region was performed with BLAST-Blastn ver. 2.2.26 (Zhang *et al*., 2000).

Alu I, *Hinf* I, *Mbo*I, *Bsu*R (*Hinf* III), *Eco*RI and *Rsa*I enzymes (Fermentas, EU) were used for restriction fragment length polymorphism (RFLP) analysis, using the modified protocols of El Karkouri *et al*. (2002), as follows: 15 µl aliquots of the amplified ITS products were mixed with 1 µl (10 u) of enzyme, 2 µl of buffer and 2 µl of deionised water, and incubated at 37°C for 12 hours. Restriction digest products were separated by electrophoresis on 2% agarose gels. The gels were stained with Midori green (Nippon Genetics, EU) and visualised using UV light. DNA ladder 100 bp (Nippon Genetics, EU) was used for fragment length estimation.

Statistical analysis

Differences in the mycelial growth, emerged due to effect of cultivation condition, were determined by two-way analysis of variance (ANOVA) with "species" and "cultivation condition" (temperature, pH, carbon sources, nitrogen sources) as independent factors, followed by Tukey HSD multiple range test ($p<0.01$) using Statistica 12.0 (StatSoft, Inc., Tulsa, Oklahoma).

Results

Identification and molecular characterisation

Lactatrius deliciosus (L.) Gray, *Russula sanguinaria* (Schumach.) Rauschert, *Suillus granulatus* (L.)

Rousell, *Suillus collinitus* (Fr.) Kuntze*, Tricholoma batschii* Gulden and *Tricholoma imbricatum* (Fr.) Kumm were determined according to the morphological features of fruit bodies. Identification of isolates was confirmed by ITS region sequencing (Table 1). RFLP patterns also confirmed identity of fungal culture, and they could be useful for fast identification of fungal cultures in further work.

Effect of temperature, pH, carbon and nitrogen sources on mycelial growth

Isolates exhibited particular specificities according to growing conditions. Their growth on artificial media was slow. Significant effects of "species" and "cultivation condition" as well as interactions among both factors on the mycelial growth were revealed by two-way ANOVA (Table 2).

S. collinitus and *T. imbricatum* behave similarly within same range of temperatures (p=0.919). Colony diameters of isolates grown at different temperatures after 28 days were shown on Figure 1. Mycelial growth in *R. sanguinaria* was the slowest, while mycelial growth of *L. deliciosus* was relatively fast. Isolates of *R. sanguinaria*, *L. deliciosus* and both *Suillus*, were characterised by faster growth at 22°C, while *Tricholoma* isolates grew faster at 25°C (p<0.001) (Figure 1). Despite the fact that radial growth of *L. deliciosus* was better at 22°C than at 25°C, and mycelial mass was better developed at 25°C. At 25°C, mycelia exhibited a darker orange colour, typical of *L. deliciosus* sporocarps, as also noted with the different carbon sources.

The examined isolates were tolerant to various pH values. There were no differences in growth between *S. collinitus* and *T. batchii* within same range of pH (p=0.60). We found significant differences in mycelial growths among examined isolates on pH 5.8 (p<0.01) and pH 5.2 (p<0.01), while there were no differences in other pHs. Dry weight yields of isolates grown at different pHs in liquid MMN media are shown on Figure 2. There are shown that *S. granulatus*, *S. collinitus* and *T. imbticatum* isolates grew well at lower pH values (4, 4.5, 5.2), while *L. deliciosus*, *R. sanguinaria* and *T. bachii* exhibited faster growth at pHs between 5.8 and 6.5 (p<0.01).

The examined isolates were able to grow on different carbon sources. *L. deliciosus* and *S. collinitus* behaves similarly within same carbon sources (p=0.62). *S. granulatus* exibits the best mycelial growth on different carbon sources, while *R. sanguinaria* exibits the weakest one. Mycelia growth is also influenced by sources of carbon. Similar effect on mycelial growth exibit the pairs: glucose and dextrin (p=0.124), arabinose and starch (p=0.059). The biggest mycelial growth was characterised for sucrose, then glucose, dextrin, arabinose, starch, and finally xylose.

Table 1. Identification of tested isolates based on molecular evidence and fragment sizes observed after enzymatic restriction digestion of amplified ITS PCR products

ITS sequence			BLAST (gb GenBank and in u UNITE)			RFLP fragment size (bp)					
GenBank accesion no	**Species**	**Lng (bp)**	**Closest accession**	**Id bp (%)**	**G bp (%)**	***Bsu*R**	***Hin*fI**	***Alu*I**	***Mbo*I**	***Ecor*I**	***Rsa*I**
JQ685712	*Russula sanguinaria*	750	u-UDB011190	721/724 (99%)	0%	NRS	400 305	460 285	340 250 220	485 300	NRS
JQ685722	*Lactarius deliciosus*	793	gb-FJ858744	788/793 (99%)	3/793 (0%)	480 320	360 290 107	490 285	280 260 140	500 340	NRS
JQ685727	*Suillus granulatus*	802	gb-AY898617	794/799 (99%)	2/799 (0%)	460 240	190 140 100	670 75	280 240	NRS	NRS
JQ685733	*Suillus collinitus*	735	u-UDB011905	602/608 (99%)	5/608 (0%)	460 245	200 130	670 100	230 207 136	NRS	NRS
JQ685729	*Tricholoma batschii*	766	u-UDB011579	756/766 (98%)	8/766 (1%)	NRS	390 340	500 170	540 190	470 335	NRS
JQ685731	*Tricholoma imbricatum*	758	u-UDB011627	756/758 (99%)	1/758 (0%)	NRS	360 320	420 165	510 250	480 360	NRS

Lng-length; Id-identity; G-gaps; NRS-No restriction sites.

Table 2. Mean squares (MS), degrees of freedom (d.f.), F and p values from two-way ANOVA for ectomycorrhizal fungi isolates cultured under different growing conditions

Growing condition	Source of variation	d.f.	MS x10^3	F	p
Temperature	Species	5	9.18	206.08	< 0.001
	Temperature	2	2.01	113.07	< 0.001
	Species x Temperature	10	2.62	29.46	< 0.001
	Error	156	0.009		
pH	Species	5	12.35	844.33	< 0.001
	pH	5	1.92	130.96	< 0.001
	Species x pH	25	2.97	40.62	< 0.001
	Error	318	0.003		
Carbon sources	Species	5	24.38	128.95	< 0.001
	Carbon sources	5	43.14	228.15	< 0.001
	Species x Carbon source	25	36.35	38.45	< 0.001
	Error	340	0.038		
Nitrogen sources	Species	5	37.36	5093.81	< 0.001
	Nitrogen sources	2	2.55	869.99	< 0.001
	Species x Nitrogen source	10	2.70	183.88	< 0.001
	Error	162	0.001		

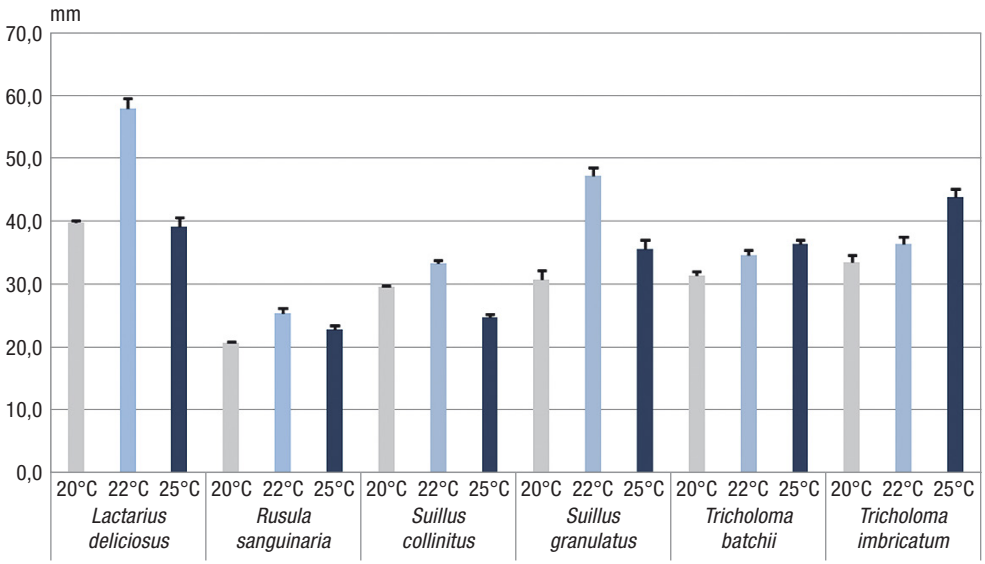

Figure 1. Colony diameter in mm (Mean+SE) of isolates grown at different temperatures on solid MMN media, after 28 days.

Colony diameter of isolates on MMN media containing different carbon sources are shown on Figure 3. In relation to particular ectomycorrhizal species, the growth of *L. deliciosus* was faster on glucose and sucrose, but almost equally good on arabinose. *L. deliciosus* showed little growth on xylose, and grew very poorly on dextrin. If developed on glucose, the mycelia were denser and ray-radial in form. Radial growth of *R. sanguinaria* was faster on glucose and dextrin than on sucrose and arabinose, while it almost did not grow on xylose. However, mycelial mass seemed to be better developed and more compact on glucose and sucrose than on the other carbon sources. Mycelial development of *Suillus* isolates was better on glucose, although its radial mycelial growth was faster on sucrose, dextrin, and for *S. collinitus*, also on starch. Growth of *S. granulatus* on starch was much weaker than that of *S. collinitus*. Both *Suillus* isolates grew very weakly on arabinose and xylose. Similarly, *T. imbricatum* showed better mycelial development on glucose, with better radial growth of mycelium on dextrin and sucrose. Mycelial growth of *T. batchii* was better on glucose, dextrin and arabinose than on starch and sucrose. Both *Tricholoma* isolates exhibited very weak growth on xylose.

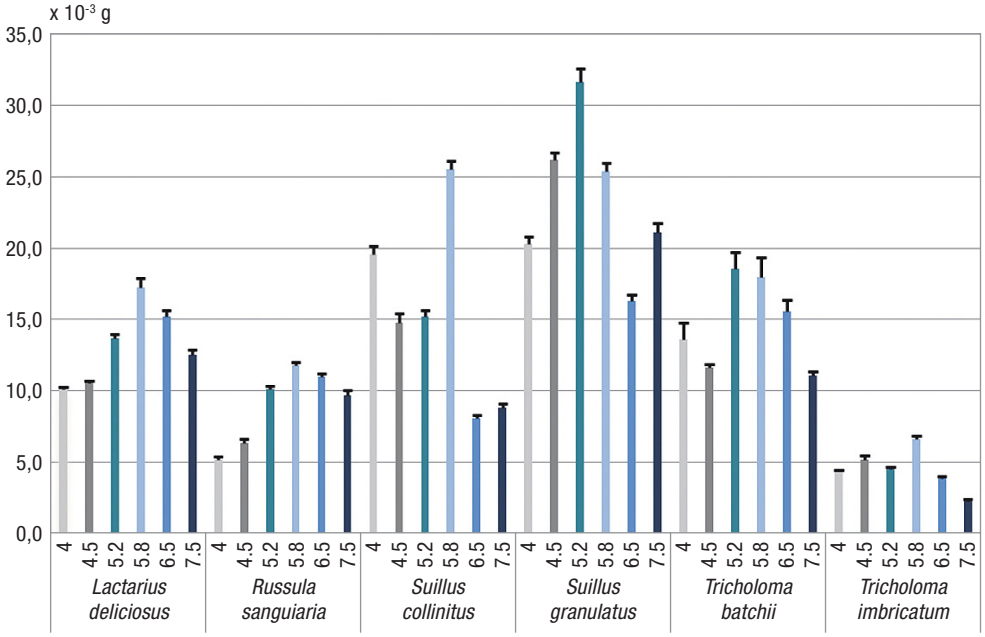

Figure 2. Dry weight yields in 10^{-3}g (Mean+SE) of isolates grown at different pHs in liquid MMN media, after 28 days.

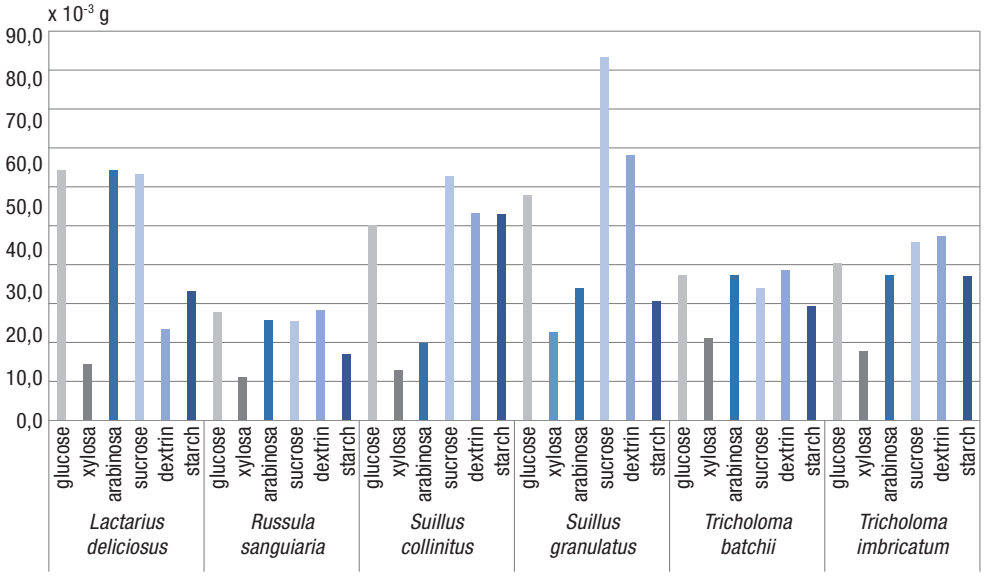

Figure 3. Colony diameter in mm (Mean+SE) of isolates on MMN media containing different carbon sources, after 28 days

The examined isolates could have grown on ammonium, nitrate and protein as source of nitrogen. Here, isolates of *S. collinitus*, *S. granulatus*, than *T. bachi* and *T. imbricatum* exibit the best mycelial growth, while *L. deliciosus* exibit the weakest. The biggest mycelial growth were characterised for ammonium, then nitrate, while mycelial growth on protein was the weakest one.

Dry weight yields of isolates grown on liquid MMN media containing different nitrogen sources are shown on Figure 4. In particular, the growth of *L. deliciosus* was better on organic nitrogen sources, although it was good also on ammonium and nitrate. It was noted that the colour of *L. deliciosus* mycelia on NO_3^- was a more intense orange and also that on ammonium, *L. deliciosus* mycelia were more compact, while on NO_3^-, mycelia were more airy. The growth of *R. sanguinaria* was better on nonorganic nitrogen sources ($p<0.01$). On NH_4^+, *R. sanguinaria* mycelia were more compact. *Suillus* isolates used all examined nitrogen sources equally. The growth of *Tricholoma* isolates was faster on nonorganic nitrogen sources ($p<0.01$). On NO_3^-, more intensive production of pigments was observed with *T. batchii*.

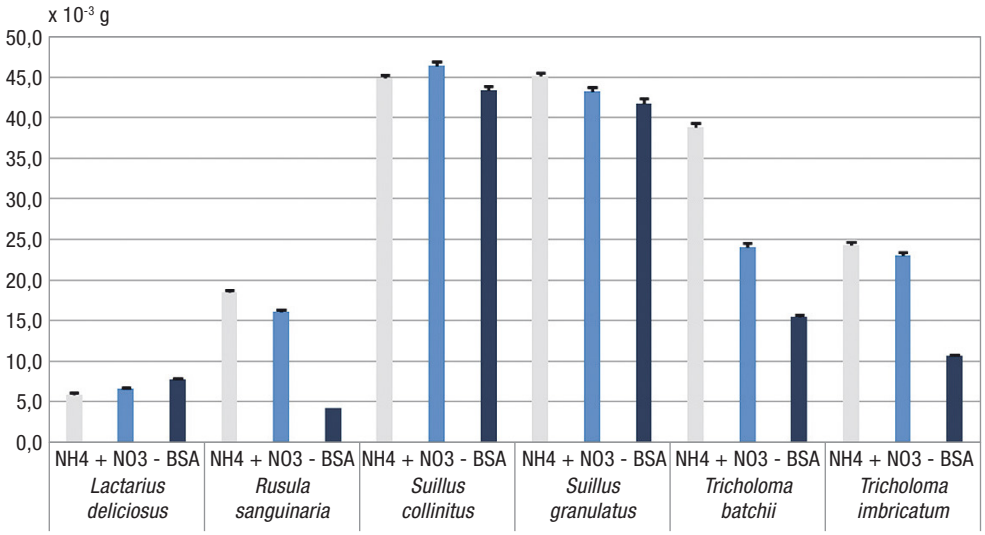

Figure 4. Dry weight yields in 10^{-3} g (Mean+SE) of isolates grown on liquid MMN media containing different nitrogen sources, after 60 days.

Discussion

Most soil ectomycorrhizal fungi are considered to grow well at moderate temperatures between 11°C and 28°C (Hutchison, 1990), however there are differences in the growth of isolates of various ectomycorrhizal fungi in relation to the environmental temperature (Hutchison, 1990; Dames *et al*., 1999). Ectomycorrhizal mycelia are very sensitive to temperature variations (Marx *et al*., 1970; Sanchez *et al*., 2001), and if the soil becomes colder, the metabolic activity of both the fungi and root could decrease, having negative consequences on mycelial growth and availability of nutrients to the fungus. The tested *R. sanguinaria*, *S. granulatus* and *S. collinitus* isolates grew faster at 22°C, while *T. imbricatum*, *T. batchii* and *L. deliciosus*, seem to be more termophilic and grew faster at 25°C. It was also noted that at different temperatures, mycelia, especially those of *L. deliciosus*, exhibited different pigmentations, probably due to different enzymatic activities. It was noted that *S. collinitus* and *T. imbricatum* grow similarly within same range of temperatures, what was consistent with previous observation that they appear in the *P. heldreichii* forests simultaneously (Lazarević *et al*., 2011), but not really differently than the other common species of fungi in those forests. Here obtained data could rather suggest optimal temperature conditions for growing of mycelia in laboratory.

It is well known that fungi are acidophilic, but there is little information about the influence of pH on mycelia growth in the laboratory (Sanchez *et al*., 2001). The examined isolates were tolerant to different pH values, but all of them grew more rapidly at pH 5.8, consistent with the pH values measured at the sampling locality. *L. deliciosus*, *R. sanguinaria*, *Suillus sp*. and *Tricholoma sp. (T. bachii)* typically appear in open forest and grassy habitats, such as the one described here (unpublished results). On the other hand, *T. imbricatum*, besides appearing in such habitats, also frequently grows in denser *P. heldreichii* forests, where there is a greater accumulation of fallen needles (mor humus), providing a more acidic environment. *Suillus sp*. is widespread on many different microhabitats inside *P. heldreichii* forests, where the soil pH could differ locally. This is consistent with the results obtained in the present study regarding growth at different pH values. Moreover, it was shown that *S. collinitus* and *T. batchii* grow similarly within the same range of pH in laboratory.

One of the key issues regarding the ecology of ectomycorrhizal fungi is whether they have the ability to use organic nitrogen. In forest soil, nitrogen is largely in the form of organic compounds, which are unavailable for direct plant uptake. Therefore, organic nitrogen can not be utilized without transformation and translocation by neither ectomycorrhizal fungi nor other soil organisms (Keller, 1996; Lilleskov *et al*., 2002). Since ectomycorrhizal fungi are a component of conifer forests, which are characterised by low mineral nitrogen content, it is considered that their ability to use protein nitrogen is widespread (Finlay *et al*., 1992; Lilleskov *et al*., 2002; Nygren, 2008,). Growth on protein substrata is the best method of documenting the ability of

ectomycorrhizal fungi to use organic food sources (Read & Perez-Moreno, 2003). Ectomycorrhizal fungi have the ability to degrade proteins; this ability varies considerably among the different species (Abuzinadah & Read, 1986; Finlay *et al*., 1992; Lilleskov *et al*., 2002; Nygren, 2008). The isolates examined in this study were able to use organic nitrogen sources.

The major inorganic nitrogen sources in forest soils are ammonium and nitrate, which typically comprise less than 0.01% of the total nitrogen in forest soils (Aguilera *et al*., 1993; Keller, 1996). Generally, forest soils contain more ammonium than nitrate and ammonium is assumed to be the main form in which inorganic nitrogen is taken up by mycorrhizal and nonmycorrhizal tree roots (Keller, 1996). Ectomycorrhizal fungi grow well on ammonium (Abuzinadah & Read, 1986; Finlay *et al*., 1992). This is because ectomycorrizal fungi grow in acid environments with a high accumulation of organic matter, where ammonium is the main source of inorganic nitrogen (Dames *et al*., 1999). On the other hand, it was postulated that in calcareous soils, nitrogen is present primarily as a nitrate (Lapeyrie *et al*., 1987), which, on the local scale, depends mainly on pH. The ability to use nitrate is examined in a small number of ectomycorhizal fungi, and it is shown as extremely variable among the different species, but also within species (Nygren, 2008). Growth of the isolates tested in the present study was weaker on nitrate then on ammonium, with the exception of *L. deliciosus*, which grew better on nitrate. This result is consistent with the view that ectomycorrhizal fungi in general can also use nitrate and ammonium, although some differences between the species are apparent, which may reflect their distribution (Keller, 1996).

The isolates tested in this study were able to use ammonium, nitrate and protein nitrogen as food sources. Although the bigger mycelial growths were measured on ammonium, then nitrate and protein, their abilities to use different forms of nitrogen are quantitative rather than qualitative. As evident in a large number of species, the ability of a fungus to grow on a variety of nitrogen sources clearly indicates the strategy that all of the available nitrogen has to be adopted (Nygren, 2008). Differences in nitrogen utilization are important ecophysiological markers of ectomycorrhizal fungi (Keller, 1996; Nygren, 2008). The capability to use nitrate and proteins may be a better adaptation of species to specific soil types or a prerequisite for selective exploitation of nitrogen sources by individual strains of a given species (Keller, 1996).

In ectomycorrhizal symbiosis, the plant must provide a carbon for the fungus. Therefore, another important question is: From which substrates the fungus can utilize the carbon? What sources can the fungus independently use as carbon? Carbohydrate dependency of mycorrhizal fungi has been tested by their ability to grow on media containing specific carbon sources (Hampp & Schaeffer, 1999). It is considered that, with few exceptions, ectomycorrhizal Basidiomycota do not have enzymes that can degrade cellulose and lignin (Hutchison 1990; Tedersoo *et al*., 2010), although in this respect, final conclusions have not yet been drawn. However, some ectomycorrhizal fungi, such as *Tricholoma aurantium*, *Amanita muscaria*, *Rhizopogon luteolus*, *Rhizopogon roseolus* and *Cenococcum geophylum* (Trojanowski *et al*., 1984), are able to degrade lignin and cellulose, so they act as successful competitors to saprotrophs (Koide *et al.*, 2008; Dames *et al*., 1999). Due to this ability, some ectomycorrhizal fungi are able to survive after lengthy dry periods or harmful disturbances in the ecosystem (Dames *et al*., 1999). The examined isolates were capable of growing on polysaccharides, such as dextrin and starch, indicating the presence of enzymes that are able to carry out their decomposition to glucose. Except for *S. collinitus*, the isolates were successful in using monosaccharides, such as arabinose, while *S.granulatus* and *Tricholoma* isolates were grown even on xylose. This is consistent with indication that some mycorrhizal fungi are able to use carbon, necessary for growth, from organic (nonhost) sources (Trojanowski *et al*., 1984).

This study on the physiological properties of certain ecologically important ectomycorrhizal fungi from mature *P. heldreichii* forests provides novel basic information about these fungi. Cultures were obtained from sporocarps, and were collected near a single tree during a single season. Based on this, it was assumed there are differences among their physiological functions as a prerequisite for selective exploitation of different nutrients from the soil. The observations suggest that the differences in nitrogen utilization and also their dependency on carbohydrates could be important ecophysiological markers of ectomycorrhizal fungi (Buscot *et al*., 2000). Capabilities of using different nitrogen and carbon sources, at different pH levels may allow species to adapt to specific soil conditions, but it is still unknown to what extent the function of different ectomycorrhizal species can be considered to be the same. Recent comparative studies have shown a great diversity in physiological function both within and between species (Cairney, 1999). It is likely that many of these fungi fulfil broadly similar ecological functions and that some degree of functional redundancy exists within ectomycorrhizal fungal communities (Allen *et al.*, 1995; Cairney, 1999).

Future research should be directed toward the study of the enzymatic activity of ectomycorrhizal fungi. In this way, it will be possible to obtain a more complete

picture of the characteristics of fungi and to understand why the diversity of fungi is beneficial to the diversity of plants, as well as the precise role of fungal species in symbiosis.

References

Abuzinadah RA, Read DJ, 1986. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants, Vol.I, Utilisation of peptides and proteins by ectomycorrhizal fungi. New Phytol 103: 481-493. http://dx.doi.org/10.1111/j.1469-8137.1986.tb02886.x

Allen EB, Allen MF, Helm DJ, Trappe JM, Molina M, Rincon E, 1995. Patterns and regulation of mycorrhizal plant and fungal communities. Plant Soil 170: 47-62. http://dx.doi.org/10.1007/BF02183054

Aguilera LM, Griffiths RP, Caldwell BA, 1993. Nitrogen in ectomycorrhizal mat and non-mat soils of different age Douglas-fir forests. Soil Biol Biochem 25:1015-1019. http://dx.doi.org/10.1016/0038-0717(93)90148-5

Azul AM, Nunes J, Ferreira I, Coelho AS, Verissino P, Trovao J, Campos A, Castro P, Freitas H, 2014. Valuing nature ectomycorrhizal fungi as a Mediterranean forestry component for sustainable and inovative solutions. Botany 92 (2): 161-171. http://dx.doi.org/10.1139/cjb-2013-0170

Basso MT, 1999. Lactarius Pers. in Fungi Europ., Alassio, Italy. 847 pp.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL, 2005. GenBank. Nucleic Acids Res 33: D34-D38. http://dx.doi.org/10.1093/nar/gki063

Bowen GD, 1973. Mineral nutrition of ectomicorrhizae. In: Ectomycorrhiza, their ecology and physiology; Marx GC, Kozlowsky TT (eds). pp: 151-205.Academic Press, London, Great Britain.

Buscot F, Munch JC, Charcosset JY, Gardes M, Nehls U, Hampp R, 2000. Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems. FEMS Microbiol Rev 24: 601-614. http://dx.doi.org/10.1111/j.1574-6976.2000.tb00561.x

Cairney JWG, 1999. Intraspecific physiological variation: implications for understanding functional diversity in ectomycorrhizal fungi. Mycorrhiza 9: 125-135. http://dx.doi.org/10.1007/s005720050297

Dahlberg A, Jonsson L, Nylund J-E, 1997. Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in oldgrowth Norway spruce forest in south Sweden. Can J Bot 75: 1323-1335. http://dx.doi.org/10.1139/b97-844

Dames JF, Starker CJ, Sholes MC, 1999. Ecological and anatomical characterisation of some Pinus patula ectomycorrhizas from Mpumalanga, South Africa. Mycorrhiza 9: 9-24. http://dx.doi.org/10.1007/s005720050258

Daza A, Manjon JL, Camacho M, Romero de la Osa L, Aguilar A, Santmaria C, 2006. Effect of carbon and nitrogen sources, pH and temperature on in vitro cultures of several isolates of Amanita caesarea (Scop.:Fr.) Pers. Mycorrhiza 16: 133-136. http://dx.doi.org/10.1007/s00572-005-0025-6

El Karkouri K, Martin F, Mousain D, 2002. Dominance of the mycorrhizal fungus Rhizopogon rubescens in a plantation of Pinus pinea seedlings inoculated with Suillus collinitus. Ann For Sci 5: 197-204. http://dx.doi.org/10.1051/forest:2002006

Finlay RD, Forestegard A, Sonnerfeldt AM, 1992. Utilisation of organic nitrogen sources by ectomycorrhizal fungi in pure culture and in symbiosis with P. contorta Dough.ex Loud. New Phytol 120: 105-115. http://dx.doi.org/10.1111/j.1469-8137.1992.tb01063.x

Fuštić B., Đuretić G, 2000. Soils of Montenegro; University of Montenegro, Biotechnical institute, Podgorica, Montenegro 626 pp. [in Serbian]

Galli R, Mazza R, Consiglio G.1996. Le russule, Atlante pratco-monografico per la determinazione delle russule. Edinatura, Milano, Italy. 480 pp.

Gardes M, Bruns TD, 1993. ITS primers with enhanced specificity for basidiomycetes –application to the identification of mycorrhizae and rusts. Molec Ecol 2: 113-118. http://dx.doi.org/10.1111/j.1365-294X.1993.tb00005.x

Hampp R, Schaeffer C, 1999. Mycorrhiza carbohydrate and energy metabolism. In:Mycorrhizal structure, function,molecular biology and biotechnology; Varma A, Hock B (eds) . pp. 273-303. Springer-Verlag, Berlin, Germany. http://dx.doi.org/10.1007/978-3-662-03779-9_12

Hall TA, 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41: 95-98.

Harley JL, Smith SE, 1983. Mycorrhizal symbiosis. Academic Press, London and New York. pp. 483.

Hydro-Meteorologic service of Montenegro 1995. Hydrometeorological base for spatial plan of Republic of Montenegro (report), Montenegro, Hydro-Meteorological service of Montenegro, Sector for meteorology, Podgorica. [in Serbian]

Hutchison LJ, 1990. Studies on the systematics of ectomycorrhizal fungi in axenic culture.II. The enzimatic degradation of selected carbon and nitrogen compounds. Can J Bot 68: 1522-1530. http://dx.doi.org/10.1139/b90-194

Janković M.M., 1960 . Betrachtungen uber gegenseitigen Bezichungen der Molika (Pinus peuce) und Panzerkiefer (Pinus heldreichii) sowie auch Uber ihre okologishen Eigenschaften, besonders in Bezug auf ihre geologishe Grundlage . Bull Inst Jardin Botaniq'Uni de Beograd 1(2):141-181 [in Serbian]

Keller G, 1996. Utilization of inorganic and organic nitrogen sources by high-subalpine ectomycorrhizal fungi of Pinus cembra in pure culture. Mycol Res 100 (8):989-998. http://dx.doi.org/10.1016/S0953-7562(96)80053-0

Kõljalg U, Larsson K-H, Kessy Abarenkov K, Nilsson HR, Ian J, Alexander IJ, Eberhardt U, Erland S, Høiland K, Kjøller R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo L, Vrålstad T, Ursing MB, 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi, New Phytol 166:1063–1068. http://dx.doi.org/10.1111/j.1469-8137.2005.01376.x

Koide RT, Shumway DL, Xu B, Sharda JN. 2007. On temporal partitioning of a community of ectomycorrhizal fungi. New Phytol 174: 420–429. http://dx.doi.org/10.1111/j.1469-8137.2007.02000.x

Laperyrie F, Chilvers GA, Bhem CA, 1987. Oxalic acid synthesis by mycorrhoizal fungus Paxillus involvutus (Batch.ex. Fr.). New Phytol 106: 39-146.

Lazarević J, Perić B, Perić O, 2011. Ectomycorrhizal fungi in Montenegro- diversity and distribution, Mycol Monten XIV: 85-115.

Lilleskov EA, Hobbie EA, Fahey TJ, 2002. Ectomycorhizal fungal taxa differing in response to nitrogen deposition also differ in pure culture organic nitrogen use and natural abundance of nitrogen isotopes. New Phytol 154: 219-231. http://dx.doi.org/10.1046/j.1469-8137.2002.00367.x

Marx DH, 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic onfections. I. Antagonism of ectomycorrhizal fungi to root pathogenic fungi and soil bacteria, Phytopathology 59: 153-163.

Marx DH, Bryan WC, Davey CB, 1970. Influence of temperature on aseptic synthesis of ectomycorrhizae by Telephora terestis and Pisolithus tinctorius on loblolly pine. For Sci 16: 424-431.

Nieto MP, Carbone SS, 2009. Characterisation of juvenile maritime pine (Pinus pinaster Ait) ectomycorrhiyal fungal community using morphotyping, direct sequencing and fruitbodies sampling. Mycorrhiza 19: 91-98. http://dx.doi.org/10.1007/s00572-008-0207-0

Nygren C, 2008. Functional diversity in nutrient acquisition by ectomycorrhizal fungi. Doctoral thresis, Faculty of Natural Resources and Agricaltural Sciences, Swedish Univrsity of Agricultural Sciences, Uppsala, Sweden.

Read DJ, Perez-Moreno J, 2003. Mycorrhiza and nutrient cycling in ecosystem-a journey toward relewance? New Phytol 157: 475-492. http://dx.doi.org/10.1046/j.1469-8137.2003.00704.x

Rincon A, Alvarez I, Pera J, 1999. Ectomycorrhizal fungi of Pinus pinea L. in northeastern Spain. Mycorrhiza 8: 271-276. http://dx.doi.org/10.1007/s005720050245

Riva A, 2003. Trcholoma (Fr.) Staude. Eduzioni Candusso, Alasio, Italy. 620 pp.

Roux P, 2006. Mille et un champignons, Sainte-Sigolene, France. 1224 pp.

Sanchez F, Honrubia M, Torres P, 2001. Effects of pH, water stress and temperature on in vitro growth of ectomycorrhizal fungi from Mediterranean forests. Cryptog Mycol 22(4): 243-258. http://dx.doi.org/10.1016/S0181-1584(01)01076-4

Smith S, Read DJ, 1997. Mycorrhizal symbiosys. Academic press, London UK, pp: 605.

Tedersoo L, May TW, Smith ME, 2010. Ectomycorrhizal lifestile in fungi: global diversity, distribution and evolution of phylogenetic lineages. Mycorrhiza 20: 217-263. http://dx.doi.org/10.1007/s00572-009-0274-x

Trojanowski J, Haider K, Huttermann A, 1984. Decomposition of 14C labelled lignin, holocellulose and lignocellulose by mycorrhizal fungi. Arch Mycrobiol 139: 202-206. http://dx.doi.org/10.1007/BF00402000

Zhang Z, Schwartz S, Wagner L, Miller W, 2000. A greedy algorithm for aligning DNA sequences. J Comput Biol 7 (1-2): 203-214. http://dx.doi.org/10.1089/10665270050081478