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Оригинални научни рад

THE IMPACT OF DIFFERENT SOURCES OF BASIC NUTRIENTS CONTAINING CARBON, NITROGEN AND PHOSPHORUS ON MYCELIAL MASS PRODUCTION OF WHITE ROT FUNGUS *Stereum hirsutum* (Wild. ex Fr.) S.F. Gray.

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Abstract: Wood decaying fungi can utilize cellulose, lignin and hemicelluloses, causing brown-, soft- or white rot. Among this group extremely important are the species with both parasitic and saprophytic mode of nutrition. Their deteriorating activities can start in stems and continue (or start) in felled timber. The content of available and easy - assimilable nutrients inside xylem sap, for sure plays the most important role in the very first stage of fungal attack. The fungus *Stereum hirsutum* can behave as facultative parasites or parasites of weakened trees**,** but after felling, and during storing of logs in forest or industrial plots, it behaves as a saprophyt. This fungus is widespread and represents the one of the most frequent fungus in forests and storages, playing very important role in wood deterioration. Physiological requirements of fungi are of the highest importance in understanding of mechanism of decaying processes in the wood. The most important factors as like main nutrients as sources of carbon, nitrogen and phosphorus can affect the behaviour of wood decaying fungi. Nutrition of fungi is the main reason for appearance of wood decay, since rotting fungi utilize wood as source of carbon (C), nitrogen (N) and phosphorus (P). Different sources of these elements are important not only for the decaying process but also in the state of mycelial colonisation of the wood before appearance of active decay. Different seasonal status of nutrients in alive plant sap is important in this phase when fungi behave as weakened – tree parasites. The impacts of different sources of C, N and P on production of mycelial mass of *Stereum hirsutum* (Willd. ex Fr.) S. F. Gray., have been investigated *in vitro*. This fungus is one of the most frequent appearing on the Oak weakened trees or felled logs. As a causer of Oak sapwood white rot *S. hirsutum* causes significant damages of wood in forest stands as well as at industrial storages. Among the tested monosaccharides the best carbon source for *S. hirsutum* was mannose, while disaccharide mannitol was the most convenient for Strain 1. and starch for Strain 2. Generally, malt as a complex nutritive substance has been appropriate source of carbon for the both strains of tested fungus. In N - test Strain 1. mainly produced higher amount of mycelia in accordance with incubation period, except in the cases of control group, L – glutamic acid and peptone. Peptone has been the most convenient nitrogen source for Strain 2. Mycelial yield in all tested series containing phosphorus showed lower amount than in the cases of carbon and nitrogen. That means that phosphorus is not extremely necessary for vegetative fungal growth in comparison with carbon and nitrogen which are of major importance. Higher concentration of phosphorus in substrate could inhibit vegetative growth of fungi.

Keywords: *Stereum hirsutum,* nutrition, white rot, carbon, nitrogen, phosphorus

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УТИЦАЈ РАЗЛИЧИТИХ ИЗВОРА ОСНОВНИХ НУТРИЕНАТА КОЈИ САДРЖЕ УГЉЕНИК, АЗОТ И ФОСФОР НА ПРОДУКЦИЈУ МАСЕ МИЦЕЛИЈЕ ГЉИВЕ БЕЛЕ ТРУЛЕЖИ *Stereum hirsutum* (Wild. ex Fr.) S.F. Gray.

Извод: Гљиве трулежнице могу користити целулозу, лигнин и хемицелулозе изазивајући мрку-, меку- или белу трулеж. У оквиру те групе, екстремно су важне врсте и са паразитским и са сапрофитским начином исхране. Њихова деструктивна активност може почети у стаблима и наставити се (или почети) у посеченим стаблима. Садржај доступних и лако асимилабилних нутриената унутар биљног сока, засигурно игра најважнију улогу у најранијој фази напада гљиве. Гљива *Stereum hirsutum* може се понашати као факултативни паразит или паразит слабости, али после сече и за време чувања обловине у шуми или на индустријским стоваришитима, она се понаша као сапрофит. Ова гљива је широко распрострањена и представља једну од начешћих гљива у шуми и на стоваришитима, играјући врло важну улогу у пропадању дрвета.Физиолошки захтеви гљива су засигурно од највећег значаја у разумевању механизма процеса трулежи у дрвету. Најважнији фактори, као што су главни нутриенти као извори угљеника, азота и фосфора, могу утицати на понашање гљива трулежница. Исхрана гљива је главни разлог за појаву трулежи дрвета, пошто гљиве трулежнице користе дрво као извор угљеника (C), азота (N) и фосфора (P) као главних нутриената. Различити извори ових нутриената су важни, не само за процес трулежи, већ и у фази мицеларне колонизације дрвета пре појаве активне трулежи. Различит сезонски статус нутриената у биљном соку живе биљке је такође од највеће важности за гљиве трулежнице које се, у тој фази колонизирања дрвне масе, понашају као паразити слабости дрвета. Утицај различитих извора C, N и P на продукцију масе мицелије гљиве *Stereum hirsutum* (Willd. ex Fr.) S. F. Gray. је испитан *in vitro*. Ова гљива је једна од најчешћих која се појављује на храстовим ослабелим стаблима или посеченим трупцима, понашајући се као факултативни паразит али такође и као сапрофит. Као проузроковач беле трулежи бељике храста, *S. hirsutum* изазива значајна оштећења дрвета у шумским састојинама, као и на индустријским стоваришитима.Међу испитиваним моносахаридима најбољи извор угљеника за *S. hirsutum* била је маноза, док је дисахарид манитол био најпогоднији за Изолат 1. а скроб за Изолат 2. Генерално, малц као као комплексна хранљива материја, био је одговарајући извор угљеника за оба изолата испитиване гљиве.У Н- тесту Изолат 1. је углавном продуковао већу количину мицелије у складу са инкубационим периодом, осим у случајевима контролне групе, Л- глутаминске киселине и пептона. Пептон је био најповољнији извор азота за Изолат 2. Принос мицелије у свим испитиваним серијама које су садржале фосфор, показао је нижу количину него у случају угљеника и азота. То значи да фосфор није крајње неопходан за вегетативни раст у поређењу са угљеником и азотом који су од главног значаја. Већа концентрација фосфора у супстрату могла би да инхибира вегетативни раст гљива.

Кључне речи: *Stereum hirsutum*, бела трулеж, угљеник, азот, фосфор

1. INTRODUCTION

Depending on their enzyme activity, wood decaying fungi can utilize cellulose, lignin and hemicelluloses, causing brown-, soft- or white rot (Schmidt, O., Kerner-Gang, N., 1986.). This group of fungi includes extremely important species with both parasitic and saprophytic modes of nutrition (Rypaček, V., 1966). Their deteriorating activities can start in trunks and continue (or start) in felled logs (Mirić, M., Ivković, S., Rajković, S., Marković, M., 2012).

The content of easily available nutrients in the xylem sap certainly plays the most important role in the very first stage of fungal attack, just before the development of the active decay process.

The oaks are the most important species in Serbia due to their mechanical, physical and esthetical properties (Jovanović, B., 1991; Wagenführ, R., Scheiber, C.H.R., 1974; Ugrenović, A., 1950). *Stereum hirsutum* (Willd. ex Fr.) S. F. Gray. frequently attacks oak wood after felling, but also physiologically weakened or injured trunks (Butin, H., Kowalski, T., 1983). This fungus is widespread in Europe, Asia and North America.

Nutritional requirements of carbon, nitrogen and phosphorus originating from different organic and inorganic sources were investigated by using liquid nutritive media. The tested fungus has been known to attack wood through debarked spots or fresh wounds caused by wood boring insects and/or other injuries (Grosser, D., 1985). The fungus *Stereum hirsutum* can act as a facultative parasite or a parasite of weakened trees (Mirić, M., Schmidt, O., 1992), but after felling and during the storing of logs in forest or industrial storage yards, it acts as a saprophyte (Mirić, M., Ivković, S., Vuković, M., 2012). *S. hirsutum* is widespread and represents one of the most frequent fungi in forests and storages, playing a very important role in wood deterioration.

The aim of this article was to discover the impact of different nutritive sources of C, N and P on the growth of *Stereum hirsutum,* which is important for the understanding of the mechanism of decaying processes in oak wood.

2. MATERIAL AND METHODS

Nutritional requirements were investigated in liquid media convenient for the measurement of mycelial mass yielded after 30, 60 and 90 days of incubation. The basal medium (according to Beever, 1969.) was prepared as follows: glucose: 25 g; L – glutamic acid: 2.72 g; KH_2PO_4: 1g; $MgSO_4$ x $7H_2O$: 0.5g; $CaSO_4$ x $2H_2O$: 0.3g; solution of oligo elements: 1.0 ml (0.5 ppm B from $Na_2B_4O_7$; 0.5 ppm Mn from $MnSO_4$; 0.05 ppm Zn from $ZnSO_4$; 0.02 ppm Cu from $CuSO_4$; 0.02 ppm Mo from Na_2MoO_4; Thiamine – HCl: 100 μg; Fe – EDTA: 6 ppm;) filled up to 1 l with distilled water. The value of pH was 5.8 and had been adjusted with 1M NaOH or 1M HCl.

Certain substances which provided the necessary amount of carbon, nitrogen and phosphorus were excluded from test – series and replaced with tested substances – sources of certain nutrients. In C – test, the necessary quantity of nitrogen in the basal medium was obtained from L – glutamic acid, while in N – test, the necessary quantity of carbon was obtained from glucose. The appropriate quantities of the tested sources of nutrients were calculated based on molecular and atomic weights of substances and elements, so that all test–series provided exactly the same concentration of nutritive elements: carbon: 10 g/l; nitrogen: 225 mg/l (just Dl – phenylalanine: 450 mg/l) and phosphorus: 0.5 g/l.

Sterilisation was done using the autoclave standard procedure (20 min. /121 °C), except for urea which was filtered (R max = 0.2 μ) because of its thermolability. The test was run in 300 ml Erlenmeyer flasks each containing 50 ml of the medium. Inoculation was done under aseptic conditions with dikaryotic mycelia of 2 tested strains (1 = German strain and 2 = domestic Serbian strain) previously developed in Petri dishes on a malt (2%) – agar (2%) medium. Two geographically different

strains of fungi were tested in order to discover whether some differences in growth would appear in the same test conditions.

After 30, 60 and 90 days of incubation, the developed mycelia were harvested by vacuum – filtration, dried to oven-dry mass and measured. We tested 10 sources of carbon, 8- of nitrogen and 4- of phosphorus (as shown in the result tables).

3. RESULTS AND DISCUSSION

Carbon – test

The obtained results are shown in Table 1.

Table 1 The yield of mycelia of *S. hirsutum* on different carbon sources after 30, 60 and 90 days (mg)

Табела 1. Принос мицелије *S. hirsutum* на различитим изворима угљеника после 30, 60 и 90 дана (mg)

fungus *S.hirsutum*	Incub. (days)	Source of carbon									
		control	**glucose**	**mannose**	**galactose**	**sorbose**	**maltose**	**cellobiose**	**mannitol**	**starch**	**malt**
Strain 1 (Germ.)	30	12	142	216	26	16	119	176	200	188	200
	60	17	159	234	75	25	198	214	258	206	223
	90	12	142	212	97	23	182	199	308	184	199
Strain 2 (Serb.)	30	9	138	213	24	24	96	105	95	322	216
	60	19	238	364	62	64	260	193	196	391	333
	90	9	225	286	53	71	186	245	254	361	300

The control group had the lowest mycelial yield, probably due to the presence of some 0.97 g/l of C in the basal medium (which couldn`t be totally excluded). As much as 90 % of mycelial mass increased for 60 days after the incubation and it declined after that period. The reason might be in the deficiency of nutrients after two months of fungal nutrition, and consequently the occurrence of the phenomena of autolytic degradation of hyphae.

Something similar happens in wood. Swift (1978) found that mycelial mass increases up to the weight loss of inoculated wood of some 40%, but declines in the later stages of decay, which was determined by using `Hexosamine` test-method.

The most convenient carbon sources for Strain 1 (German strain) of the fungus were: mannitol, mannose, malt, cellobiose, followed by starch and maltose and finally glucose. For Strain 2 (Serbian strain), starch was the best carbon source, followed by mannose and malt, mannitol, cellobiose, glucose and maltose. Except in the case of mannitol and galactose, Serbian Strain 2 showed better mycelial production in comparison with German Strain 1. Among the tested monosaccharides, mannose was the best source of carbon, while disaccharide

mannitol was the most convenient for Strain 1 and starch for Strain 2. Generally, malt as a complex nutritive substance was the appropriate source of carbon for both strains of the tested fungus.

Nitrogen – test

The obtained results are shown in Table 2.

The control group (no nitrogen source) showed the lowest production of mycelial mass. Strain 1 mainly produced a higher amount of mycelia depending on the incubation period, except in the case of the control group, L – glutamic acid and peptone. The major increments of mycelial mass were mainly reached after 60 days of incubation, but the growth then slowed down up to the 90th day. Peptone was the most convenient nitrogen source for this strain.

Table 2 The yield of mycelia of *S. hirsutum* on different nitrogen sources after 30, 60 and 90 days (mg)

Табела 2. Принос мицелије *S. hirsutum* на различитим изворима азота после 30, 60 и 90 дана (mg)

Fungus *S.hirsutum*	Incub (days)	Source of nitrogen								
		control	**potassium nitrate**	**ammonium sulphate**	**L-valine**	**L-glutamic acid**	**DL- phenyl alanine**	**L-asparagine**	**urea**	**peptone**
Strain 1 (Germ.)	30	18	27	94	74	76	77	107	87	226
	60	42	84	128	99	116	97	143	131	203
	90	38	129	132	115	106	117	144	161	192
Strain 2 (Serb.)	30	43	66	108	137	147	68	139	143	206
	60	58	154	209	283	253	141	260	244	283
	90	68	334	260	351	217	104	303	308	244

The Serbian strain (2) of *S. hirsutum* showed certain differences compared to the German one. The yields of the produced mycelia were in almost all cases higher than in the case of Strain 1. The mycelial mass increased with the time of incubation with just a few exceptions. The most convenient sources of nitrogen for this strain were: L – valine, potassium nitrate, urea and L – asparagine, peptone, ammonium sulphate and L – glutamic acid.

Phosphorus – test

The obtained results are shown in Table 3.

The mycelial yield in all the tested series containing phosphorus showed lower amounts than in the cases of carbon and nitrogen. These facts led us to the conclusion that phosphorus is not as necessary for the vegetative fungal growth as carbon and nitrogen which are of major importance. Nevertheless, considering the role that phosphorus has in the metabolic processes and in the transfer of energy (Vučetić, J., 1985), one could suppose that its deficiency in the natural substrate (wood), could badly affect the fungal ability to decompose polioses,

cellulose and lignin, i.e. to reduce fungal enzyme activity (Mirić, M., Ivković, S., Marković, M., 2010). Both strains attained good growth in the presence of potassium phosphate and calcium phosphate. Sodium- and ammonium phosphate were not convenient sources of phosphorus since the test series showed lower mycelial yields than the control series containing no phosphorus.

Table 3 The yield of mycelia of *S. hirsutum* on different phosphorus sources after 30, 60 and 90 days (mg)

Табела 3. Принос мицелије *S. hirsutum* на различитим изворима фосфора после 30, 60 и 90 дана (mg)

Fungus *S.hirsutum*	Incubation (days)	Source of phosphorus				
		control	**potassium phosphate**	**sodium phosphate**	**calcium phosphate**	**ammonium phosphate**
Strain 1 (Germ.)	30	54	51	44	73	17
	60	56	177	61	95	12
	90	60	97	50	126	75
Strain 2 (Serb.)	30	67	175	67	158	12
	60	89	186	89	147	18
	90	132	193	73	162	75

Mainly, fungi don't have significant phosphorus requirements when the vegetative growth is concerned (Rayner, A. D. M., Boddy, L., 1988). This is in accordance with the data obtained by Muntaňola–Cvetković, M. (1987) that the content of phosphorus is 3-4 times higher in spores than in mycelia, which leads us to the conclusion that this element might have a more important role in the production of reproductive organs. Nevertheless, fungi consume some 30 – 50 % of phosphorus from the substrate in the first 24 hours (Vučetić, J., 1985). Robinson, P. M. (1978) states that if it is available from potassium phosphate, phosphorus is consumed very fast during the mycelial growth.

It can be concluded that the concentration of phosphorus used in the test series of 0.5 g/l might act inhibitory being too high.

A comparison of the state and quantitative content of carbon, nitrogen and phosphorus in all the presented tests clearly shows the following: C- and N- tests used 228 mg of phosphorus on 1 liter of basal medium, while in all the tested combinations it was always 10 g/l C and 225 mg/l N. The same quantities of C and N were also used in the phosphorus test, only the amount of phosphorus in the test series was twice higher. The obtained results, if compared, point to the higher production of mycelial mass if phosphorus was used in the concentration of 228 mg/l of the substrate. These facts clearly show that an increase in the concentration of phosphorus in the substrate could inhibit the vegetative growth of fungi.

There are some differences in the obtained results between the two different geographical strains of the fungus. In order to prove that it was the same fungus species tested, the protein content of the tested strains was tested. It can be concluded that the differences in mycelial behaviour of the German strain is a consequence of the presence of the specific protein whose molecular mass is

bellow 36000. To prove its presence, we analyzed the mycelial protein content by polyacrylamide gel electrophoresis method. Given that the other proteins on the `film` were identical, it was concluded that there were two different strains of the same species of *Stereum hirsutum* fungus.

4. CONCLUSIONS

The most convenient carbon sources for Strain 1 (German strain) of the fungus were: mannitol, mannose, malt, cellobiose, starch, maltose and finally glucose. For Strain 2 (Serbian strain) the best carbon source was starch, followed by mannose and malt, mannitol, cellobiose, glucose and maltose. Except in the case of mannitol and galactose, Strain 2 showed better mycelial production than German Strain 1. Among the tested monosaccharides, mannose was the best carbon source, while disaccharide mannitol was the most convenient for Strain 1 and starch for Strain 2. Generally, malt as a complex nutritive substance was an appropriate source of carbon for both strains of the tested fungi.

In N – test, Strain 1 mainly produced higher amounts of mycelia depending on the incubation period, except in the cases of control group, L – glutamic acid and peptone. The major increments of the mycelial mass were mainly reached after the incubation of 60 days, but the growth then slowed down up to the 90th day of incubation. Peptone was found to be the most convenient nitrogen source for this strain. Serbian Strain of *S. hirsutum* had a higher mycelial yield than German Strain in almost all the cases. The mycelial mass increased with the time of incubation with just a few exceptions. The most convenient sources of nitrogen for this strain were: L – valine, potassium nitrate, urea and L – asparagine, followed by peptone, ammonium sulphate and L – glutamic acid.

The mycelial yield in all the tested series containing phosphorus showed lower amounts than in the cases of carbon and nitrogen. This fact led us to the conclusion that phosphorus is not as necessary for the vegetative fungal growth as carbon and nitrogen which are of major importance. Both strains attained good growth in the presence of potassium phosphate and calcium phosphate. Sodium – and ammonium phosphate were not convenient sources of phosphorus since the test series showed lower mycelial yields than the control series containing no phosphorus. Thus, a higher concentration of phosphorus in the substrate could inhibit the vegetative growth of fungi.

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УТИЦАЈ РАЗЛИЧИТИХ ИЗВОРА ОСНОВНИХ НУТРИЕНАТА КОЈИ САДРЖЕ УГЉЕНИК, АЗОТ И ФОСФОР НА ПРОДУКЦИЈУ МАСЕ МИЦЕЛИЈЕ ГЉИВЕ БЕЛЕ ТРУЛЕЖИ *Stereum hirsutum* (Wild. ex Fr.) S.F. Gray.

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Сажетак

Циљ рада био је да се разоткрије утицај различитих хранљивих извора угљеника, азота и фосфора на развој мицелије гљиве *Stereum hirsutum* што је значајно са аспекта разумевања механизма процеса труљења храстовог дрвета. Нутритивни захтеви за угљеником, азотом и фосфором пореклом из различитих (како органских тако и неорганских) извора, испитани су коришћењем хранљивих подлога које су обезбеђивале принос масе мицелије. Испитивања су спроведена на течним базалним подлогама (према Бееверу), при чему су одређене супстанце које су обезбеђивале неопходну количину угљеника, азота и фосфора, искључиване из тест-серија и замењиване тестираним супстанцама – изворима одређених нутриената. У С - тесту, неопходну количину азота у базалној подлози обезбеђивала је Л - глутаминска киселина, док је у N - тесту, неопходну количину угљеника обезбеђивала глукоза. Одговарајуће количине испитиваних извора нутриената обрачунате су на основу молекулских и атомских маса једињења и елемената, тако да је у свим тест серијама обезбеђена потпуно иста концентрација хранљивих елемената и то: угљеника: 10 g/l; азота: 225 mg/l (само Дл- фенилаланина: 450 mg/l) и фосфора: 0.5 g/l.

Стерилизација је спроведена по стандардној процедури (20 мин. /121 °C) сем за уреу која је филтрирана у асептичним условима при карактеристикама гуч - филтера Р мах. = 0.2 μ због своје термолабилности. Затим је испитивање вођено у Ерленмајер – боцама од 300 ml које су садржале свака по 50 ml подлоге. Инокулација је урађена у асептичним условима са дикарионом мицелијом два географски различита изолата (1 = немачки- и 2 = домаћи српски изолат) претходно развијеним у Петри посудама на хранљивим подлогама састава 2% малца и 2% агара.

После 30, 60 и 90 дана инкубације, развијена мицелија је пожњевена вакуум - филтрацијом, осушена до апсолутно суве масе и измерена. Испитано је 10 извора угљеника, 8 извора азота и 4 извора фосфора. Најповољнији извор угљеника за немачки изолат гљиве био је манитол, док је за српски изолат то био растворљиви скроб, са највећим приносом продуковане мицеларне масе.

Пептон је био најбољи извор азота у течној подлози за немачки изолат. У свим испитаним комбинацијама српски изолат је имао већу продукцију масе мицелије него немачки изолат, са највећим приносом у подлогама које су садржале Л- валин, калијум нитрат, уреу и Л-аспарагин.

Фосфор је имао мањи утицај на продукцију масе мицелије у поређењу са угљеником и азотом. У нижим концентрацијама фосфор је изазивао раст мицелије, али само уз неопходно присуство угљеника и азота. Резултати истраживања су показали да фосфор није крајње неопходан за вегетативни раст гљиве у поређењу са угљеником и азотом који су били од великог значаја. У вегетативној фази развоја мицелије, већа концентрација фосфора могла би изазвати инхибиторан ефекат.

Између немачког и српског изолата *Stereum hirsutum* постојале су разлике у продукцији масе мицелије код свих испитаних извора нутриената.

Може се закључити да би за ове разлике могао бити одговоран специфични протеин молекуларне масе испод 36000 у мицелији немачког изолата. Његово присуство забележено је анализом садржаја протеина у мицелији методом полиакриламид - гел електрофорезе. Како су остали протеини на филму били идентични, очито је да се ту ради о два различита изолата (соја) исте врсте гљиве - *Stereum hirsutum*.