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Cultural characteristics, sexuality and ligninolytic enzyme production of *Trametes cervina*

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Abstract – Cultural characteristics of the wood–inhabiting basidiomycete *Trametes cervina* (Schwein.) Bres. are described. Characteristics of the culture are rather similar to those of the other species of the genus except for the presence of tyrosinase activity that is not reported in other *Trametes* species. Mating compatibility studies of monosporic isolates obtained from sporocarps grown *in vitro* indicated that the sexuality of the species is heterothallic and tetrapolar. Production of three ligninolytic enzymes (laccase, ligninase, manganese peroxidase) was substantially lower in comparison with several strains of different *Trametes* species.

Basidiomycetes / *Trametes* / culture / sexuality / ligninolytic enzymes

Résumé – Le basidiomycète *Trametes cervina* (Schwein.) Bres., qui colonise le bois, a été cultivé. La caractéristique de la culture ressemble à celles des autres espèces du genre à l'exception de la présence d'une activité tyrosinasique qui n`a pas été trouvé chez les autres espèces de du genre *Trametes*. Les études de la compatibilité sexuelle des clones monosporiques qui avait été obtenus des sporocarpes cultivés *in vitro* ont indiqué que cette espèce était heterothallique et tétrapolaire. Les productions des trois enzymes ligninolytiques, laccase, ligninase et Mn peroxidase, ont atteint les taux beaucoup plus bas que chez les autres souches des espèces différentes du genre *Trametes*.

Basidiomycetes / *Trametes* / culture / sexualité / enzymes ligninolytiques

INTRODUCTION

Trametes cervina (Schwein.) Bres. is a rare member of the genus *Trametes*, a typical representative of the polypore genera in Europe. Cultural characteristics of the other species of the genus (*Trametes hirsuta*, *T. ochracea*, *T. versicolor* etc.) are well known (Nobles, 1948; 1965; Stalpers, 1978; David, 1966; Vandendries & Brodie, 1933; Vandendries, 1934), but those of *T. cervina* have not been yet published (according to Ryvarden & Gilbertson, 1994). *Trametes cervina* has mainly a holoarctic distribution – the species is known from Europe, North America and Asia, but it is reported from tropical Africa also (Mswaka & Magan, 1998; Muzariri & al., 2001). *T. cervina* has a scattered occurence in Europe. In central Europe the species is very rare (Jahn, 1983; Kotlaba, 1984), and it is not

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known from the British isles, Nordic countries, Italy and Greece (Ryvarden & Gilbertson, 1994).

Species of *Trametes* produce number of enzymes that degrade lignin and polycyclic aromatic compounds that may have industrial or environmental applications (Collins and Dobson 1995; Itoh & al., 1998; Kirbas & al., 1999; Lee & al., 1999).

This work is focused on the description of cultural characteristics of *T. cervina*, its sexuality and production of important ligninolytic enzymes. The results are compared with those of other *Trametes* species.

MATERIALS AND METHODS

Organism

The *Trametes cervina* strain was obtained from a sporocarp collected from a fallen trunk of *Quercus cerris* in the Czech Republic, Randezvous Natural Reserve by Valtice, district Bǰeclav, 48° 44' 52,34" N, 16° 47' 31,78" E. The exsicate is deposited in the mycological herbarium of the National Museum, Prague (PRM 900574). The heterokaryotic strain was isolated from the tissue of the sporocarp, and the homokaryotic strains from a water suspension of basidiospores obtained from a sporocarp grown in vitro. The cultures were maintained on MEGA medium (malt extract 1%, glucose 1%, Difco agar 1.6%) and incubated at 23 °C. All cultures are deposited in Culture Collection of Basidiomycetes (CCBAS), Prague, Czech Republic.

Cultivation and Fructification

Cultivation of the fungus for sporocarp production was carried out in triplicate in 300-mL Erlenmeyer flasks containing 60 ml of the following media: (1) MEA medium (malt extract 2%, Difco agar 2.5%); (2) MEA medium with 3 cm^3 (6 pieces) of oak wood per flask; (3) N-limited Kirk medium with 2.5% Difco agar (Tien & Kirk, 1988); (4) N-limited Kirk medium with 2.5% Difco agar and (5) 3 cm^3 of oak wood; oak wood (3 cm^3) with 2.5% Difco agar. The flasks were inoculated with four agar plugs (10 mm diameter) cut from a colony grown on MEGA medium in Petri dish and incubated at 23 °C. After 8 weeks of incubation, the flasks were placed in a 4 °C incubator for 4 weeks and then cultivated for 2 weeks at 23 °C. The flasks were observed weekly for the presence of sporocarp primordia.

Static submerged cultivation for growth and enzyme estimation was carried out in 100-ml Erlenmeyer flasks containing 10 mL of N-limited Kirk medium (Tien & Kirk, 1988). The flasks were inoculated with two agar plugs (10 mm diameter) cut from the actively growing margin of a colony on a Petri dish containing MEGA medium. Enzyme activities were measured in the filtrates from three replicate flasks after removing the mycelia every three days. The mycelia were oven dried then weighed.

Identification of the culture

The strain was grown on Petri dishes with MEA medium in the dark at 23 °C and periodically observed by light microscopy. Microscopic preparations were mounted in Congo red (1% solution). Species Codes (abbreviated form) were determined following Nobles (1965) and Stalpers (1978). One week old culture was used for biochemical tests. Tests for laccase, peroxidase and tyrosinase were performed using gallic acid and tannic acid (Nobles 1965) and guaiacol, pyrogallol and *p*-cresol (Stalpers 1978). The intensity of reactions was classified using a four-point scale as follows: negative (–), weak (+), medium (++), strong (+++).

Mating experiments

To determine the sexuality and mating types, reciprocal crosses between sibling monokaryotic isolates were carried out. Each monokaryon was also mated against itself. Single agar plugs of each monokaryon were placed 1 cm apart on MEA medium in Petri dishes and incubated in the dark at 23 °C. After 5–7 days of incubation, a small area at the junction of the two cultures was examined for the presence of clamp connections. Each cross was scored as positive (clamp formation) or negative (clamps not formed). Mating types were then assigned arbitrarily to each monokaryon.

Enzyme assays

Activities of laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) were determined spectrophotometrically by monitoring the absorbance increase at 425 nm (laccase), 310 nm (LiP) or 590 nm (MnP) in the medium filtrates. Laccase activity was determined according to Bourbonnais & Paice (1990) by monitoring the oxidation of ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)). LiP activity was determinated by veratryl alcohol oxidation in the presence of H_2O_2 (Tien & Kirk, 1988). Determination of activity of MnP was based on the method of Ngo & Lenhoff (1980) modified according to Daniel & al. (1994). MBTH (3-methyl-2-benzothiazolinone hydrazone, Sigma) and DMAB (3-dimethylaminobenzoic acid) were oxidatively coupled to give a purple indamine dye product by the action of the enzyme in the presence of added H_2O_2 and Mn^{2+} ions. One unit of enzyme activity (U) was defined as catalyzing the production of one micromole of green or purple dye per milliliter per minute.

Growth estimation

Submerged growth in liquid medium was evaluated by determining the dry mass of mycelia. The mycelia were harvested every three days from the cultivation flasks which were incubated as above, washed with distilled water, dried at 105 °C for 24 hours and weighed. All measurements were done in triplicate.

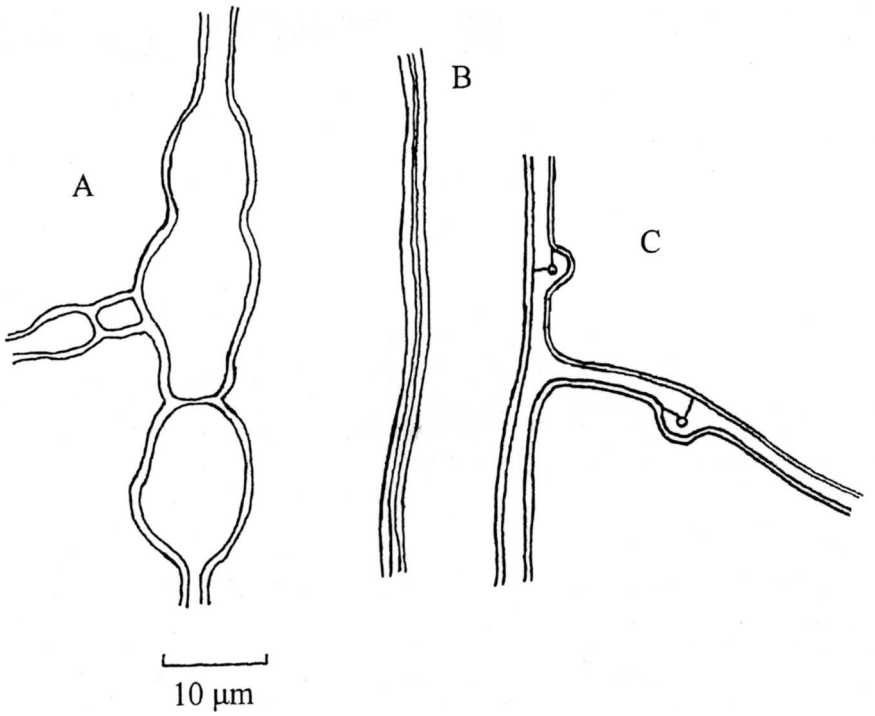

Fig. 1. Cultural characteristics of *Tramestes cervina* : intercalary swellings on generative hypha (A), unbranched skeletal hypha (B), branched generative hypha with clamp connections (C).

RESULTS AND DISCUSSION

Cultural description

The colour of the colony is white; the mat is pellicular, downy, felty to cottony. This appearance of the colony was reached on the 7th day of growth and no further change occurred. Petri dish (90 mm in diam) is covered with mycelium by two weeks. The hyphal system is dimitic; thin-walled branched generative hyphae are (0.9)-1.5-2.5(-5.0) µm wide, thick-walled unbranched skeletal hyphae are 1.5-2.5 µm wide. Clamp connections are very frequent on generative hyphae of the heterokaryotic strain. Intercalary or terminal swellings, 4.5-12 µm wide, are typical for homokaryotic strains. Skeletal hyphae and thick-walled swellings (walls 1.5-2.0 µm wide) occurred after one week of cultivation (Fig. 1.). The dikaryotic status of mycelium was proved by Acridin orange staining according to Hejtmánek & Hejtmánková (1976). The culture did not produce any odour. Reverse reaction absent. Species Codes of *T. cervina* are the following: 2.3.8. 32.(34).36.38.42.54.60 – following Nobles´ system (1965) and 1.2.3.6.(11).13.14.17. 21.24.25.30.39.45.46.52.53.(63).(84).(85).94 – following Stalpers´ system (1978).

Tab. 1. Mating among homokaryons (A-I) of *Trametes cervina*. Alleles were assigned arbitrarily.

	Alleles	*A* *A1B1*	*B* *A1B2*	*C* *A1B2*	*D* *A2B2*	*F* *A2B2*	*H* *A2B1*	*I* *A2B1*
I	A2B1	–	+	+	–	–	–	–
H	A2B1	–	+	+	–	–	–	
F	A2B2	+	–	–	–	–		
D	A2B2	+	–	–	–			
C	A1B2	–	–	–				
B	A1B2	–	–					
A	A1B1	–						

+ , clamp formation; – , clamps not formed

The results of the enzyme tests were as follows: gallic acid ++, tannic acid +, laccase (guaiacol) +, peroxidase (pyrogallol) ++, tyrosinase (*p*-cresol) ++. The presence of tyrosinase activity in *T. cervina* is rather suprising, because activity of this enzyme has not been reported in other *Trametes* species (Stalpers, 1978; Neves & Loguercio-Leite, 1999) or in the closely related genus *Coriolopsis* (ut *Funalia*) (Stalpers, 1978).

Fructification

Fertile sporocarps were formed only on two media of the five tested – on Kirk medium with and without wood pieces. Small clusters of sporocarps 3-50 mm in diam were formed after 14 weeks of cultivation. Other media did not seem to be suitable for sporocarp production in *T. cervina*.

Sexuality

The incompatibility system of *T. cervina* is heterothallic and tetrapolar (Tab. 1.). The results of the mating experiments were expected, because other *Trametes* are tetrapolar also (Ryvarden & Gilbertson, 1994).

Enzyme production and growth

Ligninase was the first enzyme which appeared during the cultivation of *T. cervina*, and it reached its maximum production on the 2nd day of incubation. Laccase and manganese peroxidase had their maximum production on the 9th day. The production of all three enzymes was very low in comparison with the strains of other *Trametes* species *(T. hirsuta, T. ochracea, T. versicolor)* cultivated under the same conditions (Tab. 2.). On the other hand, the overall growth of this species was rather high and comparable with other *Trametes* species. Surprisingly, Muzariri & al. (2001) did not find any activity of these enzymes in another *T. cervina* strain that was cultivated under different conditions. The results of the enzymatic studies confirm the position of *T. cervina* in the white rot genus *Trametes* in contrast to meaning of Kotlaba (1984) who placed it into brown rot genus *Antrodia*.

Tab. 2. Comparison of growth rate and enzyme activity of *T. cervina* with other *Trametes* species.

Strain	*Growth (dry mass, g/L)*	*Maximum enzyme activity, U/L*		
		laccase	*MnP*	*LiP*
T. cervina	8.00	0.42	0.45	0.54
T. hirsuta H1	4.72	9.39	8.87	0
T. hirsuta H3	5.53	86.5	9.12	18.35
T. ochracea Z2	8.44	34.06	18.74	0.9
T. versicolor V1	8.85	148.01	23.5	0
T. versicolor V6	8.11	18.34	9.36	0
T. versicolor V13	6.94	27.39	12.41	0
T. versicolor V14	4.48	18.11	2.66	23.18

In conclusion, our study shows that the culture characteristics of *T. cervina* (except for tyrosinase production) are similar to other members of the genus *Trametes*.

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