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Intraspecific Diversity within *Ganoderma lucidum* in the Production of Laccase and Mn-Oxidizing Peroxidases During Plant Residues Fermentation

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Abstract Comparison of the potential for laccase and Mn-oxidizing peroxidases synthesis by ten strains of *Ganoderma lucidum*, originating from different worldwide areas, during solid-state fermentation of selected plant raw materials was the aim of this study. The great intraspecific variability in the production of analyzed enzymes as well as the dependence of the enzyme activity on plant raw materials were reported. The strain HAI 957 was the best laccase producer in the presence of corn stem, as a unique carbon source (129.46 U/L). The highest level of Mn-dependent peroxidase activity was noted after wheat straw fermentation by *G. lucidum* HAI 246 (78.64 U/L), while the maximal versatile peroxidase production (59.72 U/L) was observed in strain HAI 957 in the medium with oak sawdust.

Keywords *Ganoderma lucidum* · Laccase · Mn-dependent peroxidase · Versatile peroxidase · Plant raw materials

Introduction

Ganoderma lucidum (W. Curt.: Fr.) P. Karst. is a medicinal mushroom species that belongs to the group of white-rot fungi. It produces extracellular ligninolytic enzymes: laccase (Lac), lignin peroxidases, and Mn-oxidizing peroxidases [Mn-depending peroxidase (MnP) and versatile peroxidase (VP)], which participate in modification and degradation of lignin and structurally similar aromatic compounds into low-molecular-weight components [1–4]. Due to the mentioned facts, *G. lucidum* could be a participant in various biotechnological processes, among which biotransformation of raw plant materials takes important place.

Lignocellulose is the major component of plant biomass, which represents the most abundant renewable organic resource. Thus, world annual generation of agricultural

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lignocellulose residues is considerable, 123×10^6 tons per year [5]. Since approximately half of that amount is used neither for food and feed nor for textile and paper production, the plant raw materials present as significant environmental pollutant, which could be bioconverted to several valuable products [6].

Physiological demands for ligninolytic enzyme production vary among white-rot species, even among strains of a species [7]. The factors that significantly affect enzyme production are cultivation type (submerged or solid state), carbon and nitrogen sources and concentrations, presence or absence of different inducers, medium pH, temperature, agitation, cultivation period, etc. [8–10]. According to the results of numerous studies, various agricultural and forestry residues present better substrates for enzyme production than glucose or other simple saccharides [9, 11–13]. These residues contain significant amount of soluble carbohydrates and inducers of enzyme synthesis [14–17] and therefore appear as prospective substrates for bioconversion into fungal biomass and ligninolytic enzymes.

The aim of this study was the research of intraspecific diversity within *G. lucidum sensu lato* based on the production of Lac, MnP, and VP under conditions of solid-state fermentation of selected plant raw materials.

Material and Methods

Organisms and Cultivation Conditions

Ten *G. lucidum* strains, collected from different worldwide areas, were objects of this study (Table 1). The cultures were obtained from the culture collection of the Institute of Evolution, University of Haifa (HAI), Israel and from the National Agricultural Research Foundation–Institute of Kalamata (Ik), Greece and preserved on malt agar medium in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade.

The inoculum preparation was composed of few steps: (1) inoculation of 100 mL of synthetic medium (glucose, 10.0 g/L; NH_4NO_3, 2.0 g/L; K_2HPO_4, 1.0 g/L; $NaH_2PO_4 \times H_2O$, 0.4 g/L; $MgSO_4 \times 7H_2O$, 0.5 g/L; yeast extract, 2.0 g/L; pH6.5) with 25 mycelial discs (Ø 0.5 cm, from 7-day-old culture from malt agar); (2) incubation at room temperature (22±2°C), on a rotary shaker (160 rpm), for 7 days; (3) washing of obtained

Table 1 Investigated *Ganoderma lucidum* strains and their origin.

Scientific name of species	Strain code	Origin of strain
G. lucidum (Curt.: Fr.) Karst.	HAI 447	Israel, Tel Aviv, park, on *Quercus* spp.
	HAI 109	CCBA (922)
	HAI 158	China
	HAI 246	USA, New York, on deciduous tree
	HAI 611	Ukraine, Kiev, A.S. Buchalo (922)
	HAI 626	Germany, Stuttgart, Botanical garden
	HAI 957	China
	Ik-1	Greece
	Ik-2	Czech Republic
	Ik-3	USA

biomass (three times) by sterile distilled water (dH_2O); (4) biomass homogenization with 100 mL of sterile dH_2O in laboratory blender.

Analyzed plant residues were wheat straw, corn stem, oak sawdust, and grapevine sawdust. The length of plant particles was about 0.5 cm. Solid-state fermentation was carried out at 25 °C in 100-mL flasks containing 2 g of analyzed plant residue soaked with 10 mL of the synthetic medium modified by the presence of NH_4NO_3 in one of two tested nitrogen concentrations (10 and 20 mM) and pH5.0. In this way, prepared substrates were inoculated with 3 mL of the homogenized inoculum. Samples were harvested after 7 days of cultivation, and the enzymes were extracted by stirring of samples with 50 mL of dH_2O for 10 min at 4 °C. The obtained extracts were separated by centrifugation (4 °C, 3,000 rpm, 15 min), and the supernatants were further used for measurements of the Lac and Mn-oxidizing peroxidase activity as well as total protein content.

Three repeats for each analyzed strain, plant residue, and nitrogen concentration were prepared in order to decrease the statistical error.

Enzyme Activity Assays

Lac activity was assayed spectrophotometrically using 50 mM ABTS (ε_{436}=29,300 $M^{-1}cm^{-1}$) as a substrate in 0.1 M phosphate buffer (pH6.0). The reaction mixture contained buffer, ABTS, and sample (V_{tot}=1 mL).

Mn-oxidizing peroxidase activities were determined with 3 mM phenol red (ε_{610}= 22000 $M^{-1}cm^{-1}$) as a substrate, in a buffer with the following content: succinic acid disodium salt, albumin from bovine serum, and DL-lactic acid sodium salt (pH4.5). The reaction mixture (V_{tot}=1 mL) contained buffer, sample, 2 mM H_2O_2, and phenol red, with or without 2 mM $MnSO_4$ (for MnP and VP, respectively). Reaction was stopped with 2 M NaOH.

Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 μmol of substrate per minute. An UV-160 A Spectrophotometer (Shimadzu) was used for these assays.

Determination of Total Proteins

The amount of total proteins was performed by means of a standard curve obtained from solutions containing bovine serum albumin at known concentrations (0.00, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, and 0.07 mg/mL), Bradford's reagent (0.2 mL), and sufficient water to complete a final volume of 1 mL. The mixture contained 0.80 mL of the sample and 0.20 mL of Bradford's reagent, and absorbance was measured at 595 nm after reaction at room temperature for 5 min. Total protein content is shown in milligram per milliliter [4].

Results

The tested *G. lucidum* strains produced Lac, MnP, and VP in all selected plant raw materials and both nitrogen concentrations, after 7 days of solid-state cultivation. The results have documented the great intraspecific diversity in the production of the analyzed enzymes within *G. lucidum*, under the same cultivation conditions (Figs. 1, 2, and 3).

Laccase Production

The best Lac producer was strain HAI 957 in the all selected substrates, except in grapevine sawdust medium, where the strain Ik-1 was the best producer (Fig. 1). In strain HAI 957,

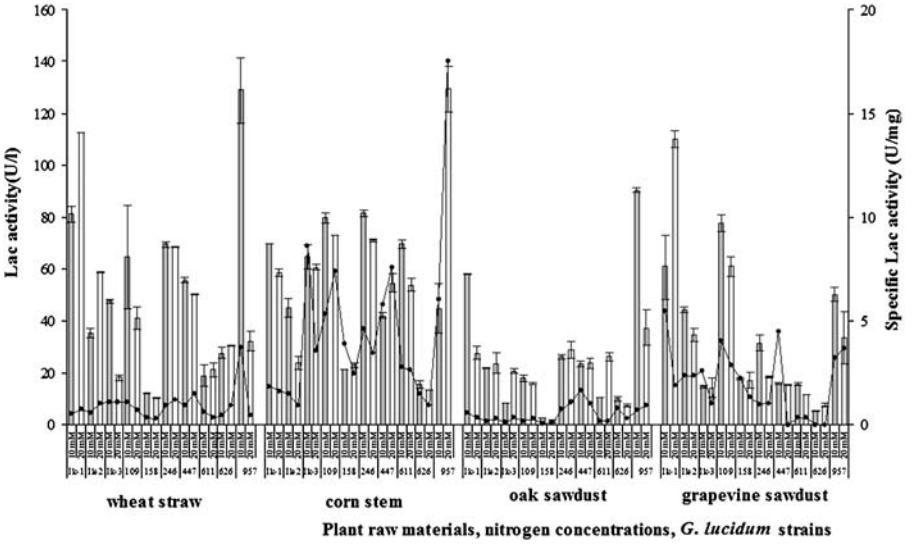

Fig. 1 Effect of selected plant raw materials and nitrogen concentrations on Lac activity. *Gray bar* 10 mM nitrogen concentration, *white bar* 20 mM nitrogen concentration, *circle* specific activity (data represent mean value of activities of three different samples. Variations are given as standard errors)

the maximum Lac activity (129.46 U/L) was noted in the corn stem medium with nitrogen concentration of 20 mM, while at nitrogen concentration of 10 mM, it was about threefold lower (44.90 U/L). Wheat straw was also a good substrate for Lac synthesis at nitrogen concentration of 10 mM (129.01 U/L), while at 20 mM nitrogen concentration, Lac activity

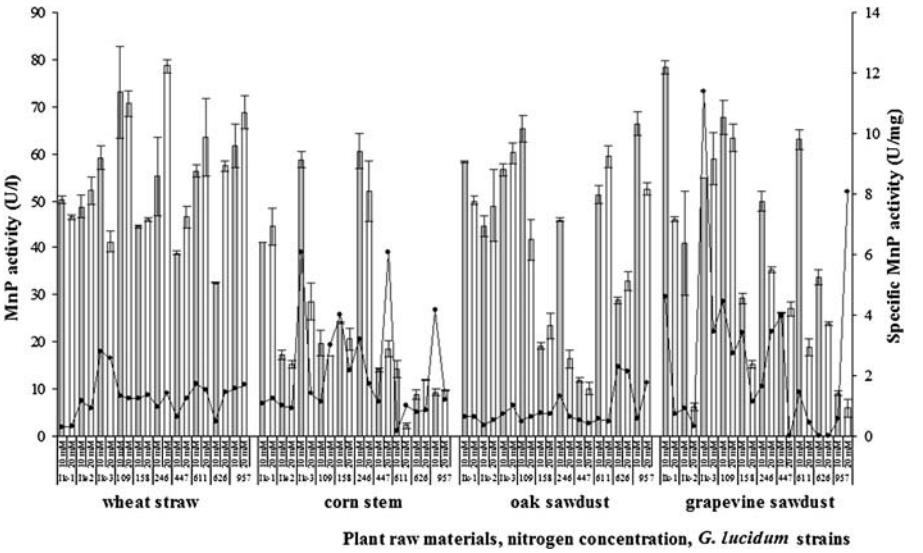

Fig. 2 Effect of selected plant raw materials and nitrogen concentrations on Mn-dependent peroxidase activity. *Gray bar* 10 mM nitrogen concentration, *white bar* 20 mM nitrogen concentration, *circle* specific activity (data represent mean value of activities of three different samples. Variations are given as standard errors)

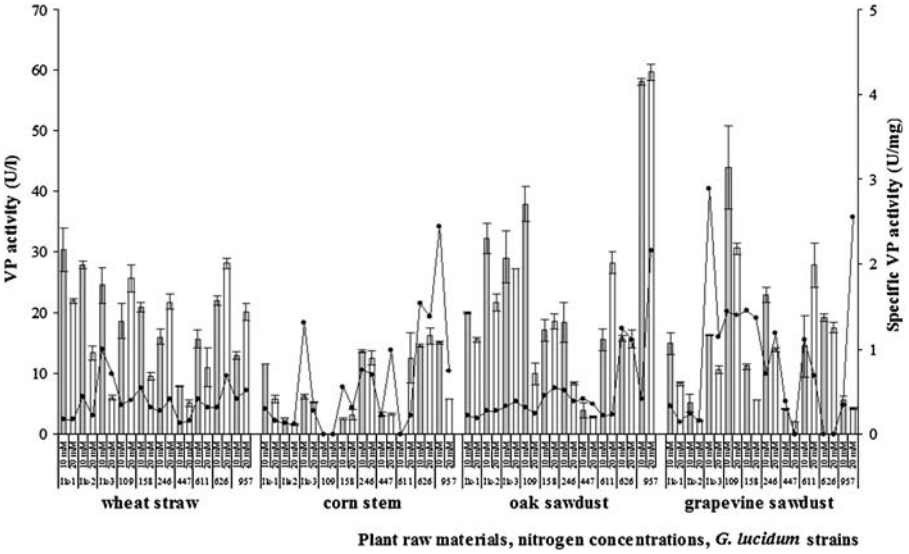

Fig. 3 Effect of selected plant raw materials and nitrogen concentrations on versatile peroxidase activity. *Gray bar* 10 mM nitrogen concentration, *white bar* 20 mM nitrogen concentration, *circle* specific activity (data represent mean value of activities of three different samples. Variations are given as standard errors)

was even fourfold lower (32.20 U/L). Strain Ik-1, which was the best producer in 20 mM nitrogen-enriched grapevine sawdust medium (110.01 U/L), was also a good producer during cultivation in the other three substrates (Fig. 1). The weakest producers were strains HAI 158, in oak sawdust and wheat straw media (1.45 and 10.40 U/L, respectively), and HAI 626, in grapevine sawdust and corn stem substrates (5.17 and 13.40 U/L, respectively). In these cases, the influence of nitrogen concentration was minor (Fig. 1).

According to the specific Lac activity, corn stem was the best substrate, where strain HAI 957 achieved the maximum value (3.74 U/mg), while oak sawdust was the worst medium, with the obtained activity of 0.08 U/mg, in HAI 158 (Fig. 1).

Mn-Dependent Peroxidase Production

The highest level of MnP activity was noted in the strains HAI 246 (78.64 U/L) and Ik-1 (78.35 U/L) after cultivation in 20 mM nitrogen-enriched wheat straw and 10 mM nitrogen-enriched grapevine sawdust substrate, respectively. MnP production in both strains was lower at another tested nitrogen concentration (Fig. 2). Low MnP production was noted in strain HAI 957 only during cultivation in corn stem (9.26 U/L) and grapevine sawdust substrates (5.80 U/L), while the lowest activity was obtained in strain HAI 611 in corn stem medium enriched with 20 mM nitrogen (2.28 U/L).

All analyzed substrates, except corn stem, were good for the enzyme production, while wheat straw was the optimal one, where activity level did not fall below 32.50 U/L. However, specific MnP activity had the highest value in strain Ik-3 after grapevine sawdust fermentation (11.40 U/mg), while the lowest value was noted in HAI 447 and HAI 626 in the same substrate (0.1 U/mg; Fig. 2).

Versatile Peroxidase Production

G. lucidum HAI 957 was emphasized by high values of VP activity at both nitrogen concentrations during oak sawdust fermentation (58.12 and 59.72 U/L, respectively). Significant activity level was noted in strain HAI 109 after cultivation in grapevine sawdust (43.92 U/L) and oak sawdust (37.90 U/L) at the nitrogen concentration of 10 mM. Other tested strains, in all analyzed substrates, showed lower activity (Fig. 3). Strain HAI 447 was generally a bad VP producer, with activity level ranging from 1.93 to 7.96 U/L. However, it should be emphasized that strain HAI 109 during cultivation in corn stem substrate, independent on nitrogen concentration, has not synthesized this enzyme. The same result was reported in strain HAI 611 in the same medium with nitrogen concentration of 10 mM.

The best substrate for VP production was oak sawdust; good ones were also grapevine sawdust and wheat straw, while the worst one was corn stem, as in the case of MnP. The total protein production was highest in Ik-1 during wheat straw fermentation (0.166 mg/mL) and lowest in HAI 626 in grapevine sawdust medium, which was reflected on specific VP activity (Fig. 3).

Discussion

This study showed possibility of usage of enzyme production ability as taxonomic character for strains separation within *G. lucidum sensu lato*. Contribution of the study is also finding the optimal strain degrader of different plant raw materials, which are potential environmental pollutants.

The previous studies have investigated the ability of enzyme production by different mushroom genera. By studying production of MnP in four species of the genus *Pleurotus*, Camarero et al. [18] obtained significant interspecific differences in rate of wheat straw lignin mineralization during solid-state fermentation. Lignin degradation by *Pleurotus pulmonarius* was the highest, lignin degradation by *Pleurotus floridanus* was the lowest, while that by *Pleurotus ostreatus* and *Pleurotus sajor-caju* was between the two mentioned. The inter- and intraspecific diversity in Lac and MnP production during solid-state fermentation of grapevine sawdust and Mandarin peels were also reported by Stajić et al. [7] and Elisashvili et al. [19]. Thus, significant interspecific differences in MnP production were noted between *Coriolus hirsutus* and *Coriolus pubescens*, as well as *Pleurotus salignus* and *P. ostreatus*, while the intraspecific diversity was noted for Lac activity between two strains of *Cerrena maxima* and two strains of *P. ostreatus*. Silva et al. [4], by evaluating Lac and MnP production by four Brazilian strains of *G. lucidum* during submerged fermentation of wheat bran, showed presence of intraspecies diversity. The differences in Lac production by Brazilian strains were much higher (ranged from 0.581 to even 49,519 U/L) than among the strains used in this study (from 1.45 to 129.46 U/L). MnP production was obtained in all analyzed strains of this study, which was not the case with Brazilian strains. The same result was reported by Songulashvili et al. [10] within *Ganoderma aplanatum* and *Trametes versicolor* during Mandarin peel submerged fermentation, as well as by Elisashvili et al. [20] in *Lentinus edodes* and *P. ostreatus* during solid-state fermentation of wheat straw. *G. aplanatum* and *T. versicolor* strains were good Lac producers with high level of intraspecific diversity (190–27,380 U/L and 17,140–20,360 U/L, respectively), while *Lentinula edodes* and *P. ostreatus* strains were much weaker producers, but intraspecific diversities were notable.

Although numerous studies of different white-rot fungi species showed significant participation of Mn-oxidizing peroxidases in lignin degradation, the presence and characterization of VP in *G. lucidum* was mentioned recently for the first time [Stajić et al., unpublished data]. VP production, as well as production of other two tested enzymes, was different among studied *G. lucidum* strains, which is in accordance with the results of Stajić et al. [7] that demonstrated, for the first time, the presence of intraspecific diversity within the genus *Pleurotus*.

Effect of composition of plant raw materials on production of selected ligninolytic enzymes was the object of numerous studies. D'Souza et al. [2] reported interesting and unique results on the effect of substrate type on Lac and MnP production by selected *G. lucidum* strain. Lac activity was fourfold higher in cultures with high nitrogen content than in culture with low nitrogen concentration. It is in accordance with the results of this study because corn stem, which was the optimum substrate for Lac production, is much richer in nitrogen amount (7.9%) than grapevine sawdust (0.7–0.8%) and wheat straw (0.53%) [15–17]. Significant differences in Lac production in *Ganoderma adspersum* during submerged cultivation depending on the nature and composition of plant raw materials were also observed by Songulashvili et al. [9]. The maximum Lac synthesis was noted during cultivation in Mandarin peel medium (34,000 U/L), while it was significantly lower in wheat or corn bran medium (600 and 700 U/L, respectively). In *Phlebia floridensis*, sugarcane bagasse-enriched medium was a better substrate for Lac production than wheat and rice straws [21]. The results of Fenice et al. [22] once again confirmed the influence of medium composition on Lac production. These authors showed that Lac production was stimulated by solid-state cultivation of *Panus tigrinus* in olive mill waste medium due to significant content of phenols in the substrate. Songulashvili et al. [10] also showed effect of numerous plant residues on MnP production in *G. lucidum* HAI 447. In that strain, MnP synthesis was absent during submerged fermentation of corn bran, kiwi fruits, and banana peels, while soy bran, Mandarin peels, and specially wheat bran were good substrates.

According to obtained results, ligninolytic enzyme production could be used as taxonomic character. However, generally, these results have much higher importance for biotechnology because, recently, special attention has been given in finding the best producer of ligninolytic enzymes for degradation of various agricultural and food industry residues, which can often be serious environmental pollutants. The low-molecular-weight degradation products are easily absorbed by fungi, better digested by animals, and could be used in further processing such as in producing food of high nutrition value (mushroom fruiting bodies), feeds, and basic commodities for different industrial purposes.

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