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Enhanced production of laccase in the fungus *Trametes versicolor* by the addition of xenobiotics

Christian Mougin*, Albert Kollmann & Claude Jolivalt

Unité de Phytopharmacie et Médiateurs Chimiques, Institut National de la Recherche Agronomique, Route de Saint-Cyr, 78026 Versailles Cedex, France

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Abstract

Agrochemicals, industrial compounds and their transformation products have been assayed for their ability to enhance laccase production in liquid cultures of *Trametes versicolor*, when added at the concentration of 0.5 mM. After 3 days of treatment, enzymatic activity in the culture medium was increased 14-fold by 4-*n*-nonylphenol and 24-fold by aniline. Laccase activity was enhanced 10-fold by oxidised derivatives of the

herbicide diquat, 17-fold by N,N'-dimethyl-N-(5-chloro,4-hydroxyphenyl)urea and 22-fold by 9-fluorenone.

Introduction

Many white-rot fungi, including *Trametes versicolor*, produce extracellular copper-containing phenol oxidases, named laccases (EC 1.10.3.2). The two main possible biological functions ascribed to fungal laccases are first their involvement in lignin degradation, together with others ligninolytic enzymes such as peroxidases, and second their role in fungal virulence as key agent in pathogenesis against plant hosts (Gianfreda *et al*. 1999). In addition, laccases exhibit *in vivo* other functions that are the basis of some industrial applications. For example, in *Aspergillus nidulans*, laccases are act on pigment formation in fungal spores (Smith *et al*. 1997). Some fungi also secrete laccases to remove either potentially toxic phenols released during lignin degradation or toxins produced by others organisms. As a consequence, the enzyme has potential applications in the dye, paper or textile industries, as well as for the degradation of various xenobiotics, which are recognised as environmental pollutants (Rama *et al.* 1998, Jolivalt *et al*. 1999, Mougin *et al.* 2000).

Laccases have been detected and purified as constitutive or inducible isoenzymes from many fungi (Gianfreda *et al.* 1999). The stimulation of the laccase production with respect to the culture medium composition has also been investigated: for example, Filazzola *et al.* (1999) reported the influence of xenobiotics on the catalytic activity of constitutive forms of laccases. Metal ions and several organic molecules have also been assayed for their ability to enhance the production of the inducible form of laccases

(Farnet *et al.* 1999, Lee *et al*. 1999, Scheel *et al*. 2000, Crowe & Olsson 2001). Gallic and ferulic acids were used, mainly because of their structural analogy with lignin model compounds (Gianfreda *et al*. 1999). Moreover, 2,5-xylidine has often been used to increase enzyme production in laboratory experiments by stimulating the expression of an inducible form of laccase (Bollag & Leonowicz 1984). The substrate range of laccase includes some potential pollutants of the environment. Unfortunately, very little data are available concerning the ability of these xenobiotics of environmental interest to interact with laccase production.

The major concern of this work is to evaluate the effect of compounds from agronomic or industrial origin on laccase production, by measuring total extracellular activity. Most of these chemicals are persistent pollutants in the environment. Natural products are also considered for comparison.

Materials and methods

Chemicals

Oxidised analogs of phenylurea herbicides were synthesised according to Jolivalt *et al.* (1999). Benzo[a]pyrene quinones were produced as previously published (Rama *et al*. 1998). ε-Viniferin was a generous gift of Actichem (Perpignan, France). All other xenobiotics were from commercial origin: Sigma, Lancaster (Strasbourg, France) and CIL (Ste-Foy-la-Grande, France).

Microorganism and culture conditions

Trametes versicolor ATCC 32745 was grown on a culture medium described by Lesage-Meesen *et al.* (1996), containing maltose and ammonium tartrate as carbon and nitrogen sources. A mycelial mat on agar plugs (10 mm diam) was inoculated into 10 ml of the culture medium in a 150 ml Erlenmeyer flask. Cultivation was carried out statically in the dark at 25°C for 4 days. After this time, concentrated solutions of xenobiotics in organic solvents were added to the cultures to give 0.5 mM (100 µl solution per Erlenmeyer flask). Aliquots were assayed for laccase activity after a further 2 or 3 days incubation.

Laccase activity measurements

Laccase production was assessed by measurement of enzymatic oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS) at 420 nm ($\varepsilon = 3.6 \times 10^4$ cm^{-1} M^{-1}) according to Wolfenden & Wilson (1982). The reaction mixture contained 20 µl extracellular fluid and 980 µl 1 mM ABTS in 0.1 M KH_2PO_4/citric acid buffer (pH 3.0) at 30°C. The buffer solution was saturated with air by bubbling prior to the experiment. One unit of enzyme activity is defined as the amount of enzyme that oxidises one µmol ABTS in one min.

Results

Constitutive laccase activity was 0.13 ± 0.02 unit ml^{-1} and 0.16 ± 0.02 unit ml^{-1} in *T. versicolor* cultures after 2 and 3 days (Table 1). It was increased 35-fold in the presence of 2,5-xylidine used as a positive control. Then, numerous xenobiotics were evaluated for enhancing enzymatic activity. The following compounds gave a significant (more than 5-fold) increase: resveratrol, ε-viniferin, diquox, 4OH5ClF, 4-*n*-nonylphenol, 9-fluorenone and aniline. ATAB, a cationic surfactant, was highly toxic and dramatically reduced fungal growth. Nevertheless, it stimulated laccase production 5-fold after 3 days of treatment, and was a potent enhancer after 8 days with a 20-fold stimulation (data not shown).

Discussion

The results presented above clearly show that xenobiotics of environmental interest and natural products can stimulate laccase production. As a general rule, the greatest effects were observed after 3 days of treatment. One first group of active chemicals includes compounds that are substrates of the laccase, namely resveratrol, ε-viniferin, 4-*n*-nonylphenol and aniline. A second group comprises chemicals without any own activity, whilst their transformation products (*via* chemical or biological processes) are inducers. Such active derivatives are diquox (a mixture of phototransformation products obtained from diquat), 4OH5ClF and 9-fluorenone (oxidised derivatives of monuron and fluorene, respectively). Our results establish for the first time that metabolites of xenobiotics (*i.e.* 9-fluorenone) can stimulate the production of fungal laccases involved

in the biotransformation of their own parent compounds. However, such behaviour can not be generalised since compounds belonging to one of the previous categories, namely BaP as a laccase substrate and BaPQ as its corresponding transformation product, were both without any enhancing effect. Finally, a last group of chemicals includes compounds, which are neither substrates of the enzyme nor enhancers. It is the case of paraquat, which is without effect on laccases of *T. versicolor* whereas it was a potent inducer in *Rhizoctonia solani* cultures (Crowe & Olsson 2001).

Some conclusions can be drawn from our results. Additional efforts must be devoted to the optimisation of culture conditions to enhance the sensitivity of the fungal response to the treatment with xenobiotics. It may be important to modify the composition of the culture medium to decrease the amounts of chemicals required to induce a significant enzyme induction. In such conditions, laccases could be used as biomarkers to assess environmental contamination. In that context, a current axis of research of our group concerns the evaluation of mRNAs transcript abundance in response to xenobiotic treatment, as well as the identification of the isoforms produced.

Laccase-producing fungi have also been reported to be useful tools for xenobiotic removal in liquid effluents as well as in soil bioremediation (Gianfreda *et al.* 1999, Jolivalt *et al.* 2000). Our results show that the resulting transformation products themselves are likely to induce biological effects either on degrading or non-target organisms. Therefore, a complete characterisation of these compounds is necessary for a complete assessment of the remediation processes.

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Table 1. Effect of xenobiotics on laccase activity after 2 and 3 days of treatment. The concentration of solvent was 100 µl per flask and each chemical was 0.5 mM. Results are expressed as the mean ± SD, n = 3.

Xenobiotic	Laccase activity (unit ml^{-1})	
	After 2 days	After 3 days
Controls		
water	0.13 ± 0.02	0.16 ± 0.02
xylidine	4.63 ± 0.40	5.69 ± 0.21
*Solvent*s		
acetone	0.10 ± 0.01	0.22 ± 0.07
ethanol	0.12 ± 0.01	0.23 ± 0.06
DMSO	0.08 ± 0.02	0.17 ± 0.01
Natural products		
resveratrol	0.99 ± 0.68	1.05 ± 0.54
ε-viniferin	1.35 ± 0.03	1.46 ± 0.14
Agrochemicals		
atrazine	0.61 ± 0.19	0.87 ± 0.33
γ-HCH	0.46 ± 0.01	0.64 ± 0.01
PCP	0.25 ± 0.14	0.49 ± 0.29
diquat	0.36 ± 0.06	0.62 ± 0.03
diquox	0.47 ± 0.09	1.61 ± 0.84

Table 1 (continued).

paraquat	0.13 ± 0.02	0.44 ± 0.20
paraquox	0.14 ± 0.02	0.43 ± 0.06
fenuron	0.05 ± 0.02	0.25 ±0.15
2HF	0.26 ± 0.08	0.50 ± 0.20
4HF	0.50 ± 0.01	0.59 ± 0.09
monuron	0.06 ± 0.03	0.11 ± 0.04
2OH5ClF	0.43 ± 0.09	0.70 ± 0.16
2OH4ClF	0.13 ± 0.05	0.15 ± 0.03
4OH5ClF	1.06 ± 0.08	2.70 ± 0.23
ivermectin	0.18 ± 0.04	0.32 ± 0.06
8,9-Z-I	0.18 ± 0.05	0.33 ± 0.06
10,11-Z-I	0.36 ± 0.04	0.49 ± 0.09
Industrial compounds		
nonylphenol	1.75 ± 0.08	2.23 ± 0.08
isomers	0.55 ± 0.09	0.78 ± 0.04
octylphenol	0.61 ± 0.16	0.85 ± 0.43
Tween 80	0.07 ± 0.03	0.27 ± 0.07
ATAB	0.41 ± 0.07	0.70 ± 0.15
fluorene	0.45 ± 0.25	0.68 ± 0.32
9-fluorenone	2.32 ± 0.18	3.49 ± 0.22
BaP	0.28 ± 0.16	0.21 ± 0.04

Table 1 (continued).

BaPQ	0.16 ± 0.05	0.18 ± 0.04
aniline	1.58 ± 0.25	3.82 ± 0.80
phtalic acid	0.10 ± 0.01	0.30 ± 0.06
bipyridyl	0.15 ± 0.01	0.33 ± 0.10

Abbreviations are: DMSO, dimethyl sulfoxide; γ-HCH, γ-hexachlorocyclohexane; PCP, pentachlorophenol; diquox and paraquox, transformation products of diquat and paraquat; 2HF, N,N'-dimethyl-N-(2-hydroxyphenyl)urea; 4HF, N,N'-dimethyl-N-(4-hydroxyphenyl)urea; 2OH5ClF, N,N'-dimethyl-N-(5-chloro,2-hydroxyphenyl)urea; 2OH4ClF, N,N'-dimethyl-N-(4-chloro,2-hydroxy phenyl)urea; 4OH5ClF, N,N'-dimethyl-N-(5-chloro,4-hydroxyphenyl)urea; 8,9-Z-I and 10,11-Z-I, isomers of ivermectin; isomers, isomers of nonylphenol; ATAB, alkyltrimethyl-ammonium bromide; BaP and BaPQ, benzo[a]pyrene and benzo[a]pyrene quinones.