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Enzymatic activities and decay characteristics in some wood-rotting Basidiomycetes from Cameroon and determination of the time-dependent activity of syringaldazine in spot tests

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Abstract – A comparative study of the detection capacity of enzymatic activities in 22 wood-rotting Basidiomycetes from Cameroon using substrates of various chemical sensitivities in spot tests was undertaken for the first time with a thorough investigation and accurate determination of the reaction time margin of syringaldazine. Syringaldazine is one of the rare substrates with a fading shift in colour in positive tests, but until now its reaction time margin in spot tests from mycelial cultures has been inaccurate. In spite of some cases of convergent results, the laccase specific substrates α-naphtol and syringaldazine generally showed more or less important differences in their ability to detect this enzyme with a highly varying time-dependent detection level according to substrate and species. Positive tests with syringaldazine, interpreted for the first time using a colour chart, could be recorded between 1s to about 120 mn and in some cases up to 180 mn according to species, with maximum laccase activity recorded between about 5 to 30 mn. α-naphtol reactions took longer and our results enabled us to refine the range of this reaction time from about 30 mn to 72 H with maximum laccase activity recorded between 1H to several days after the test, thereby demonstrating for both substrates enormous differences in the time-dependent shift in colour and sustainability during laccase detection. Tincture of guaiac and guaiacol used to detect unspecific polyphenol oxidases showed the same inconsistencies trend as α-naphtol and syringaldazine in spite of some cases of similar detection level. 14 species that tested positive with tincture of guaiac were identified as white rot fungi, whereas 8 testing negative were classified as brown rotters. Various levels of laccase, tyrosinase and peroxidase activities were recorded in the 22 species, and led to the determination of 5 taxonomic groups based on polyphenol oxidases.

enzymatic activities / syringaldazine / wood decay / Basidiomycetes / Cameroon

Résumé – Une étude comparative de la capacité de détection de l'activité enzymatique de 22 espèces de Basidiomycètes du Cameroun causant la pourriture du bois est faite pour la première fois en utilisant des substrats de diverses sensibilités chimiques avec une détermination précise du temps de réaction de la syringaldazine. En effet, ce substrat est

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non seulement l'un des rares dont le changement de couleur en réaction positive se décolore plus ou moins rapidement, mais aussi son temps de réaction dans les tests à taches colorées sur cultures mycéliennes était jusqu'alors imprécis. En dépit de quelques résultats convergents, α-naphtol et la syringaldazine qui sont des substrats spécifiques pour la laccase ont révélé des différences plus ou moins importantes dans leur capacité à détecter cette enzyme avec des variations importantes dans leurs niveaux de détection en fonction du temps et des espèces. Alors que pour les tests à la syringaldazine, interprétés pour la première fois à l'aide d'une charte des couleurs, les résultats positifs pouvaient être enregistrés entre 1s à 120 mn après le dépot des gouttes du substrat sur le mycélium et exceptionnellement jusqu'à 180 mn selon les espèces, avec l'activité maximum des laccases observée aux environs de 5 à 30 mn ; α-naphtol a quant à lui présenté un temps de réaction beaucoup plus long que nos résultats permettent d'affiner entre 30 mn et 72H avec l'activité maximum des laccases enregistrée entre 1H jusqu'à plusieurs jours après le tests. Ces résultats ont ainsi démontré pour ces deux substrats de grandes différences dans le temps et la durabilité des réactions colorées lors de la détection des laccases. La tincture de guaiac et le guaiacol utilisés pour détecter les polyphenol oxidases non spécificiques ont montré des différences semblables dans leurs capacités de détection, malgré quelques résultats convergents. 14 espèces testées positives à la tincture de guaiac ont été identifiées comme des espèces causant la pourriture blanche et 8 testées négatives étaient des espèces à pourriture brune. Divers niveaux d'activité de la laccase, tyrosinase et peroxydase ont été enregistrés chez les 22 espèces et ont permis la détermination de 5 groupes taxonomiques.

activités enzymatiques / syringaldazine / pourriture du bois / Basidiomycètes / Cameroun

INTRODUCTION

Since the works of Bavendamm (1928) on the determination of extracellular phenoloxidases in some wood-rotting fungi, enzymatic activities in various groups of fungi have been widely studied by several authors using species from various parts of the world. Besides their natural role in wood degradation, thereby contributing to the clearing of our forests, some fungal oxidative enzymes are used in biopulping processes by paper making industries, for waste water treatment and soil remediation, in food industries and in production of laundry detergents. However, in spite of its rich fungal biodiversity and forest ecosystems, very little research has been undertaken into fungal enzymatic activities in species from tropical Africa. Among the few noteworthy papers are those of Boidin & Lanquetin (1983, 1995), Mossebo (2002) and Mswaka & Magan (1998). Focus in this geographical area has mainly been on taxonomic and systematic issues, given the numerous still undescribed species in the tropics.

Various methods are used to determine the existence and assess the level of extracellular oxidases activity in fungi. These techniques include among others, agar-plate assays (Bavendamm 1928; Boidin 1951, 1983, 1995; Nakasone 1990) and spot tests on mycelial cultures and sporocarps (Harkin *et al.* 1973, 1974; Käärik 1965; Marr 1979, 1986; Mossebo 2002; Nobles 1958, Stalpers 1978). Spot tests on mycelial cultures are particularly useful in polypores or other species with fruitbodies showing colours other than white, as in most of the species investigated in our study. In fact, considering the difficulties of directly performing spot tests in vivo on fruitbodies on which the substrate shift of colour, which is the main assessment criteria in spot tests, can hardly be observed, since it is masked by the sporocarps' natural colours, these spot tests performed on mycelium in

culture (mostly white) are much easier to interpret, and therefore constitute one of the best and most reliable ways to assess enzymatic activities in these species. Concerning laccase in particular, the only available detection substrates are α-naphtol and syringaldazine, the latter being the best laccase-identifier according to Marr (1979). When studying extra-cellular laccase in *Coriolus versicolor* (*Polyporus versicolor*) (L.) Quél, Harkin (1973) who first introduced syringaldazine remarked that : "A single small drop of azine solution on the culture surface immediately evokes an extremely intense purple color. Here and with some other fungi, the color fades within a few minutes and the culture continues to grow. With other organisms, the color fades very slowly, but growth continues". A year later, when carrying out the same investigations on sporophores of other species, the same author (Harkin, 1974) further observed that : "Wherever laccase is present, a pink through red to purple color appeared. The rate of color formation and the color intensity suggested the amount of active enzyme present. With very high laccase activities, almost immediate purple coloration occurred, but the color faded again gradually. Generally the color was stable. If no color developed, the absence of laccase was indicated". During our preliminary spot tests assays, we made the same observations, but we also noticed that contrary to most other spot tests substrates where the shift in colour does not fade, key parameters for the determination of positive tests with syringaldazine, such as the shift in colour occurrence, its intensity, sustainability and decolouration, were highly time-dependent and varied enormously with species. For most other substrates (α-naphtol, p-cresol, guaiac, guaiacol, H_2O_2/pyrogallol) parameters including PH range, shift in colour, reaction time etc…have already been determined by various authors. For syringaldazine, however, neither Harkin (1973, 1974), nor subsequent authors using this substrate have given an accurate time margin for the recording of positive tests or for the laccase activity level according to time-dependent shifts in colour intensity before decolouration. Accordingly, this study also investigated the above- mentioned parameters for a better utilization of syringaldazine as the best laccase identifier. Comparative levels of activity of other enzymes involved in decay caused by some wood-rotting Basidiomycetes from Cameroon and tropical Africa were also investigated, 15 out of the 22 species being investigated for the first time.

MATERIALS AND METHODS

22 species of wood-rotting Basidiomycetes belonging to 6 families were investigated for their enzymatic activities and related decay characteristics using spot tests reagents in mycelial cultures. These species were collected in Cameroon and mostly determined by Ryvarden (pers. comm.) and at the Royal Botanic Gardens, Kew.

Mycelium was grown for 7 to 14 days on Malt Extract Agar (15g Agar Agar (Merck), 20g Malt Extract (Merck), 1L distilled water) in 9 cm petri dishes either from spore dilution or explants from sporocarp context. The identification of oxidising enzymes and their level of activity was undertaken with common substrates used for drop tests at the edge of actively growing mycelia according to Käärik (1965) and Stalpers (1978), guaiacol being applied differently according to Tichy *et al.* (1962) (cit. by Kreisel & Schauer 1987). Tincture of

guaiac and guaiacol were used as non-specific polyphenol oxidase reagents, the first being prepared by adding 0.5 g of gum guaiac powder (Merck) to 30 ml of 95% ethanol and the second as 1% solution of guaiacol (Merck) in distilled water. The response to tincture of guaiac was assessed according to Nobles (1958). Here the rapid appearance of blue colour indicated a positive result that also determined white rot fungi; no change or tardy appearance of pale blue colour indicated a negative result and thereby brown rot fungi. Laccase substrates were α-naphtol (Fluka) and syringaldazine (Sigma-Aldrich) used according to Käärik (1965) and Stalpers (1978) for the first and Harkin *et al.* (1973, 1974) for the second. Activity of both substrates in the 22 fungal species was compared, and, given the specificities of syringaldazine, the time-dependent reaction of this substrate in mycelial cultures was closely monitored. P-cresol (Merck) and H_2O_2/Pyrogallol (Merck) were respectively used to measure the oxidising activity of tyrosinase and peroxidase. Control tests were undertaken either with distilled water or 95% ethanol according to which one was used as solvent in the substrate.

Results of spot tests with most substrates (α-naphtol, p-cresol, guaiacol, H_2O_2/pyrogallol) were regularly recorded at 3H, 24H and 72H, but guaiac test results were assessed according to Nobles (1958), and on the basis of our preliminary assays, time-margins between 1s and 180 mn were tested for syringaldazine as presented in Tables 3 & 4. The commonly used code + and – indicated positive and negative results and the assessment scale +, ++, +++, ++++ (scored respectively 1, 2, 3, 4 for the construction of Figs 1, 2, 3 & 4), derived among others from Nobles (1958), Boidin *et al.* (1951, 1983, 1995) and Nakasone (1990), indicated the shift in colour intensity of the reagent. In this respect and considering particularly syringaldazine, this shift in colour intensity is correlated to the level of enzyme (laccase) activity as postulated by Harkin *et al.* (1973, 1974). Referred to the colour chart of Kornerup & Wanscher (1978), our assessment scale for syringaldazine gave the pattern presented in Tab. 1 with a gradual increase in colour shift intensity ranging from white for negative tests to purple through purplish white, purplish pink and purplish red for positive tests.

Similar assessment scales as that in Tab. 1 were designed when using other spot test substrates. Figs 1, 2, 3 & 4 compare the enzymes' activity level in various species and a tentative taxonomic value of the enzymatic activities recorded is proposed as well as a grouping of species according to their time-dependent reaction to syringaldazine.

Table 1. Assessment scale of the shift in colour of syringaldazine in spot tests

Rating of colour shift intensity (i)	– (0)	+ (1)	++ (2)	+++ (3)	++++ (4)
Correspondence in the colour chart (Kornerup & Wanscher 1978)	14A1 15A1	14A2 14A3	14A4	14A5 14A6	14A7 14A8 (15A7) (ii) (15A8) (ii)

Negative test (-) / positive tests: (+) weak, (++) medium, (+++) strong, (++++) very strong;
(i) Cases of intermediate results (shown in brackets in Tabs. 2 & 3) were observed
(ii) Colours observed only exceptionally in a very few species

RESULTS

Enzymatic activity and taxonomic grouping

The maximum level of enzymes oxidising activity of each of the 22 species is presented in Tab. 2 where it appears that tincture of guaiac and guaiacol together detected polyphenol oxidase activities in 21 out of the 22 species tested, *Trametes menziezii* being the single species showing no sign of enzymatic activity. At specific level, laccase was identified at various levels of activities in the above-mentioned 21 species, but tyrosinase and peroxidase were present or absent in some of these species according to the following 5 taxonomic groupings:

Group I . Species with laccase, tyrosinase and peroxidase activities
Earliella scabrosa, Lentinus sajor-caju, Lentinus velutinus, Phellinus gilvus, Trametes cotonea, Trametes elegans
Group II. Species with laccase and tyrosinase, but no peroxidase activity
Perenniporia tephropora, Gymnopilus aureobrunneus
Group III. Species with laccase and peroxidase, but no tyrosinase activity
Coriolopsis polyzona, Flavodon flavus, Gymnopilus russipes, Lentinus squarrosulus, Lentinus villosus, Microporus affinis, Nothopanus hygrophanus, Steccherinum ethiopicum,
Goup IV. Species with laccase, but no tyrosinase and no peroxidase activity
Lentinus cladopus, Lentinus tuber-regium, Polyporus tenuiculus, Pycnoporus sanguineus, Trametes lactinea
Group V. Species with no laccase, no yrosinase and no peroxidase activity
Trametes menziezii

Wood-decay characteristics and comparative detection level of enzymatic activities by various substrates

The response to tincture of guaiac led to the identification of 14 white- and 8 brown rot fungi as indicated on Tab. 2. Figs 1 & 2 respectively compare the

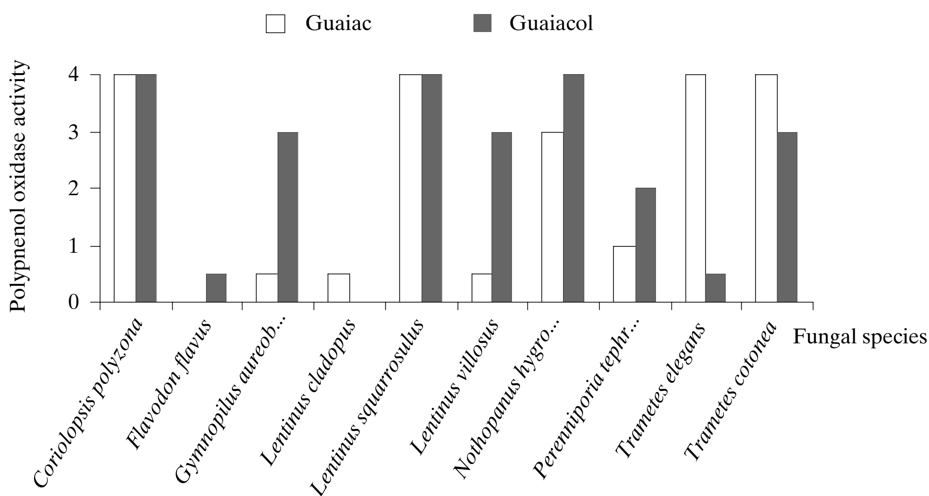

Fig. 1. Maximum detection level of polyphenol oxidases by tincture of guaiac and guaiacol in some wood-rotting Basidiomycetes

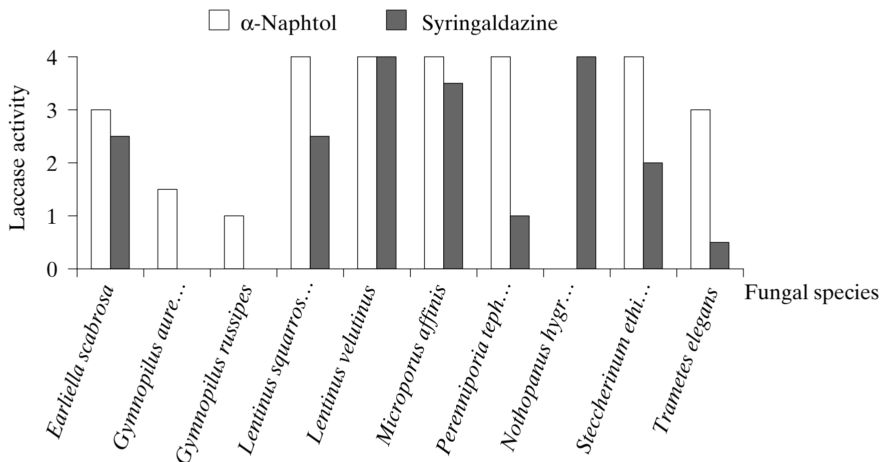

Fig. 2. Maximum detection level of laccase by α-Naphtol and syringaldazine in some wood-rotting Basidiomycetes

level of activity of unspecific polyphenol oxidases detected by guaiac and guaiacol in a sample of species, and that of laccase detected by α-naphtol and syringaldazine in another sample. For most of these species, these figures show more or less substantial discrepancies in the enzyme detection level depending on the substrate used, the most conspicuous differences being observed when a specific substrate detected a certain level of enzymatic activity in a species and the other gave no response for the same enzyme. Examples are provided (Fig. 1) by *Flavodon flavus* and *Lentinus cladopus* for polyphenol oxidases detected respectively by tincture of guaiac and guaiacol. Elsewhere on Fig. 2, *Gymnopilus aureobrunneus* and *Nothopanus hygrophanus* showed the same trend for laccase detected by syringaldazine and α-naphtol. Based on these results, it is clearly necessary to use several substrates with different chemical sensitivities when using spot tests to investigate the existence of an enzyme and its activity level in a fungal species.

Comparative time-dependent reaction of α-naphtol and syringaldazine

Tab. 3 shows the time-dependent detection capacity and laccase activity level depending on which of both substrates was used. Except for *Nothopanus hygrophanus, Gymnopilus aureobrunneus* and *Gymnopilus russipes* where laccase was only detected by a single substrate, either syringaldazine or α-naphtol, this enzyme was detected by both substrates in 18 species, but at highly varying levels (Tab. 3). Only *Trametes menziesii* showed no sign of laccase activity. As mentioned earlier and illustrated in Figs 3 & 4, syringaldazine reacted quite differently from α-naphtol which is the other laccase-specific substrate. Whereas for a sample of species, Fig. 4 shows a steady increase of this enzyme activity measured by the increasing intensity and sustainable shift in colour of α-naphtol, that never fades and indefinitely remains stable at maximum coloration, Fig. 3 also in its first part shows an increasing shift in colour of syringaldazine for the same sample of species, but immediately followed by a more or less rapid fading of the colour, usually until total decolouration of the mycelium in samples that originally tested positive. As presented in Tab 3 & 4, the reaction time margin of

Table 2. Maximum enzymatic activity level detected by various substrates in mycelial cultures and characteristics of wood-decay

Fungal species	*Polyphenol oxidases and type of rot*			*Laccase*		*Tyro-sinase*	*Pero-xidase*
	guaiac	*type of rot*	*guai-acol*	*α-naphtol*	*syringal -dazine*	*p-cre sol*	*H_2O_2/ pyrogallol*
Lentinaceae							
Lentinus cladopus Lév.	(+) (**i**)	B	–	++	+(+)	–	–
Lentinus sajor-caju (Fr.) Fr.	+++	W	++++	++	++	++	++
Lentinus squarrosulus Mont.	++++	W	++++	++++	++(+)	–	++++
Lentinus tuber-regium (Fr.) Fr.	(+) (**i**)	B	+(+)	+++	++	–	–
Lentinus velutinus Fr.	++++	W	++++	++++	++++	++++	++
Lentinus villosus Klotzsch	(+) (**i**)	B	+++	+++	++	–	++
Polyporaceae							
Coriolopsis polyzona (Pers.) Ryv.	++++	W	++++	++++	+++	–	++
Eearliella scabrosa (Pers.) Gilbn. & Ryv.	++++	W	++	+++	++(+)	+(+)	++
Flavodon flavus (Kl.) Ryv.	–	B	(+)	++	+	–	(+)
Microporus affinis (Nees & Blume : Fr) Kunth	+++(+)	W	++	++++	+++(+)	–	++
Perenniporia tephropora (Mont.) Ryv.	+	W	++	++++	+	++	–
Polyporus tenuiculus (Beauv.) Fr.	–	B	(+)	++	(+)	–	–
Pycnoporus sanguineus (Fr.) Murr.	(**ii**) +++	W	++	(**ii**) ++++	(**ii**) ++	–	–
Trametes elegans (Fr.) Fr.	++++	W	(+)	+++	(+)	+++	++
Trametes cotonea (Pat. & Har.) Ryv.	++++	W	+++	++	++	+	+(+)
Trametes lactinea Berk.	++(+)	W	–	+++ (+)	++++	–	–
Trametes menziesii (Berk.) Ryv.	–	B	–	–	–	–	–
Hymenochaetaceae							
Phellinus gilvus (Schw.) Pat.	++++	W	++++	+++	+++(+)	++++	++++
Steccherinaceae							
Steccherinum ethiopicum Maas Geesteranus	++++	W	+(+)	++++	++	–	++(+)
Tricholomataceae							
Nothopanus hygrophanus (Mont.) Singer. ex. Pegler	+++	W	++++	–	++++	–	+(+)
Cortinariaceae							
Gymnopilus aureobrunneus (Berk. & Curtis) Murr.	(+) (**i**)	B	+++	+(+)	–	+++	–
Gymnopilus russipes Pegler	(+) (**i**)	B	+++	+	–	–	(+)

B : Brown rot , W : White rot
(**i**) : Species showing a faint late-appearing blue, suggesting erratic or very limited production of white rot enzymes in favour of those causing brown rot
(**ii**) : Reaction occurred only on the reddish part (showing micro-fructifications) of mycelium in culture, never observed on the white mycelium

Table 3. Time-dependent laccase activity detected by syringaldazine in comparison with α-naphtol in mycelial cultures

Fungal species	*Laccase activity*								
	α-naphtol			*syringaldazine*					
	3 H	*24 H*	*72 H*	*5 mn*	*15 mn*	*30 mn*	*60 mn*	*120 mn*	*180 mn*
Lentinaceae									
Lentinus cladopus Lév.	++	++	++	+(+)	+	(+)	–	–	–
Lentinus sajor-caju (Fr.) Fr	++	++	++	++	+(+)	+	(+)	–	–
Lentinus squarrosulus Mont.	++++	++++	++++	+++	++(+)	++	+	(+)	–
Lentinus tuber-regium (Fr.) Fr.	+++	+++	+++	–	+	++	+	–	–
Lentinus velutinus Fr.	++++	++++	++++	+++	+++	++	+	(+)	tr
Lentinus villosus Klotzsch	+++	+++	+++	+++	+++	++	(+)	–	–
Polyporaceae									
Coriolopsis polyzona (Pers.) Ryv.	++(+)	++++	++++	+++(+)	+++(+)	+++	++	(+)	tr
Eearliella scabrosa (Pers.) Gilbn. & Ryv.	+++	+++	+++	++	++(+)	++	(+)	–	–
Flavodon flavus (Kl.) Ryv.	(+)	++	++	+	+	(+)	(+)	–	–
Microporus affinis (Nees & Blume : Fr) Kunth	+(+)	++++	++++	++	+++	+++(+)	+(+)	(+)	tr
Perenniporia tephropora (Mont.) Ryv.	+++	++++	++++	++	+	+	(+)	–	–
Polyporus tenuiculus (Beauv.) Fr.	+	++	++	(+)	(+)	+	(+)	–	–
Pycnoporus sanguineus (Fr.) Murr.	+++	++++	++++	(+)	++	++	++	+	–
Trametes elegans (Fr.) Fr.	++	+++	+++	tr	(+)	(+)	(+)	(+)	(+)
Trametes cotonea (Pat. & Har.) Ryv.	++	++	++	++	++	+	(+)	–	–
Trametes lactinea Berk.	++(+)	++++	++++	++++	++++	+++(+)	+(+)	(+)	–
Trametes menziesii (Berk.) Ryv.	–	–	–	–	–	–	–	–	–
Hymenochaetaceae									
Phellinus gilvus (schw.) Pat.	+	++	+++	+(+)	+++	+++(+)	++(+)	++	(+)
Steccherinaceae									
Steccherinum ethiopicum Maas Geesteranus	++++	++++	++++	++	++	+++	+(+)	(+)	tr
Tricholomataceae									
Nothopanus hygrophanus (Mont.) Singer. ex. Pegler	–	–	–	++	+++(+)	++++	++++	+	tr
Cortinariaceae									
Gymnopilus aureobrunneus (Berk. & Curtis) Murr.	–	+	+(+)	–	–	–	–	–	–
Gymnopilus russipes Pegler	–	–	+	–	–	–	–	–	–

tr : traces of shift in colour

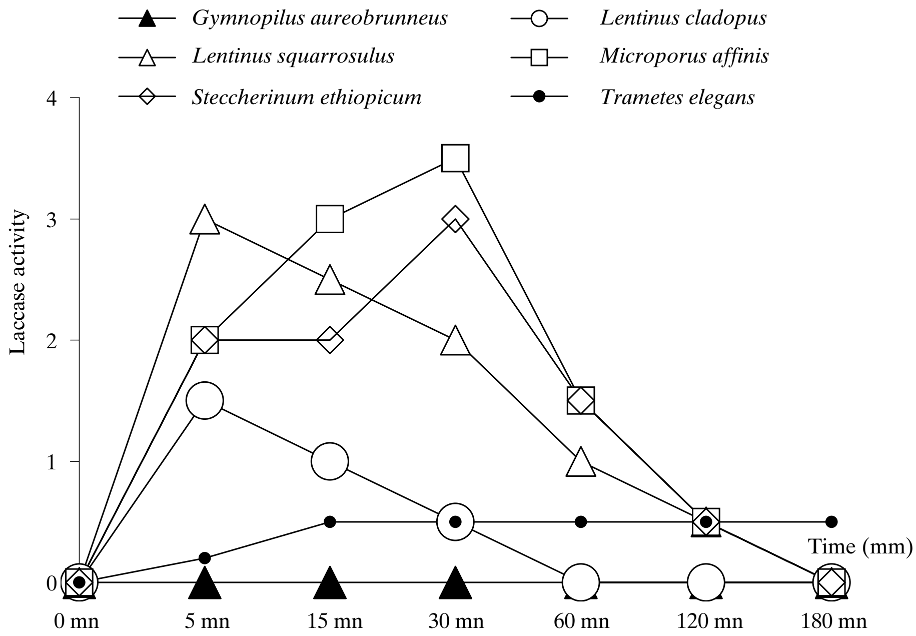

Fig. 3. Time-dependent reaction of syringaldazine during detection of laccase in mycelial cultures of wood-rotting Basidiomycetes

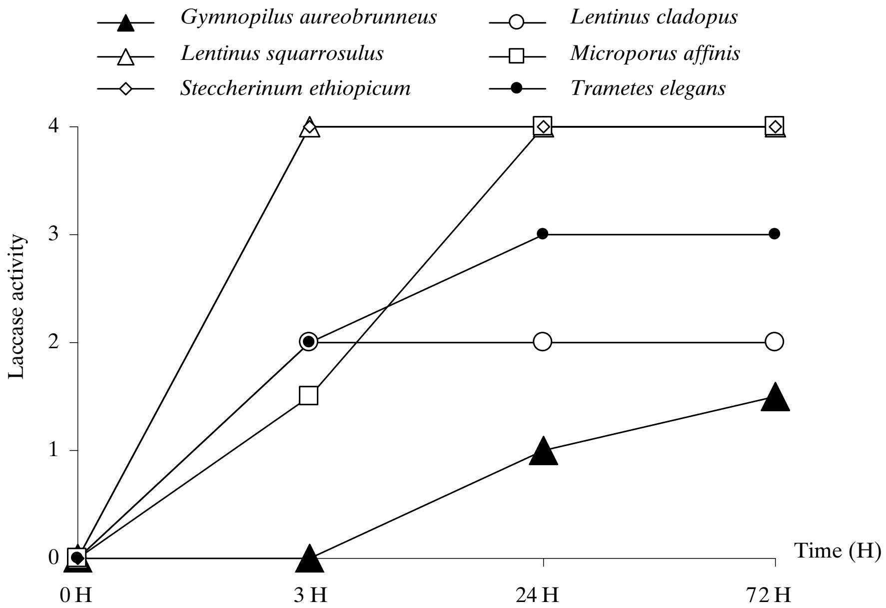

Fig. 4. Time-dependent reaction of α-Naphtol during detection of laccase in mycelial cultures of the same species of wood rotting Basidiomycetes as on Fig. 3

Table 4. Grouping of fungal species according to their time-dependent reaction to syringaldazine

Groups according to approximate time (T_1) for the shift in colour appearance	*Sub-groups according to approximate time (T_2) for total coloration fading*	
- I - Early reacting species ($T_1 \approx$ 1s to 5 mn)	I.1. Slow fading colouration ($T_2 \approx$ 60 mn)	
	Lentinus cladopus	
	I.2. Very slow fading colouration ($T_2 \geq$ 120-180 mn)	
	Coriolopsis polyzona	*Perenniporia tephropora*
	Earliella scabrosa	*Phellinus gilvus*
	Flavodon flavus	*Polyporus tenuiculus*
	Lentinus sajor-caju	*Pycnoporus sanguineus*
	Lentinus squarrosulus	*Steccherinum ethiopicum*
	Lentinus velutinus	*Trametes cotonea*
	Lentinus villosus	*Trametes lactinea* (**i**)
	Microporus affinis	
	Nothopanus hygrophanus	
- II - Slow reacting species (5 mn < $T_1 \leq$ 15 mn)	II.1. Slow fading colouration ($T_2 \approx$ 60 mn)	
	II.2. Very slow fading colouration ($T_2 \geq$ 120-180 mn)	
	Lentinus tuber-regium	
	Trametes elegans	
	NB: no species was identified in the sub-group II.1	
- III - Negative group (no shift in colour observed)	*Gymnopilus aureobrunneus*	
	Gymnopilus russipes	
	Trametes menziesii	

(**i**) The shift in colour in this species appeared instantaneously at contact of syringaldazine drops with the mycelial culture

syringaldazine varied from 1s in very early reacting species as *Trametes lactinea* where the shift in colour occurred instantaneously at drop contact with mycelium, to about 180 mn in long-lasting positive tests such as in *Trametes elegans* and *Phellinus gilvus*, but the maximum laccase activity was recorded between about 5 to 30 mn and the time to total decolouration in samples tested positive with this substrate varied from approximately 60 mn for the shortest living positive tests as observed for instance in *Lentinus cladopus*, to about 120 mn for the longest as in *Earliella scabrosa, Lentinus squarrosulus, Lentinus villosus, Perenniporia tephropora, Pycnoporus sanguineus, Trametes cotonea* etc… Some positive tests (Tab. 3) as in *Lentinus velutinus,*

Coriolopsis polyzona, Microporus affinis, Nothopanus hygrophanus, Phellinus gilvus, Steccherinum ethiopicum and Trametes elegans still showed traces of the pink to purple colour up to about 180 mn when the shift in colour had totally faded in most other species. As presented in Tab. 3 and Fig. 3, the maximum laccase activity was recorded from about 5 mn for the early reacting species as *Lentinus cladopus* and *Lentinus squarrosulus*, to 15 mn for the slow reacting such as *Trametes elegans*, and 30 mn for the very slow reacting species as *Microporus affinis* and *Steccherinum ethiopicum*. Our results therefore show a clear difference between on the one hand the total reaction-time of syringaldazine ranging from the first second of the test to about 120 mn and 180 mn in some few cases and during which positive results could be recorded, and on the other hand the time for maximum laccase activity varying from just about 5 mn to 30 mn.

Concerning α-naphtol, Tab. 3 shows that 9 out of the 20 species tested positive showed their maximum laccase activity 3H after the test was launched, and all the 11 remaining only about 24H later as for *Coriolopsis polyzona*, *Microporus affinis* and *Trametes elegans*. We also observed that some early reacting species reached this maximum activity just 1H after the test began.

Compared with the reaction-time of syringaldazine, Fig. 4 also first shows a steady increase in the enzymatic activity measured by the shift in colour intensity that, contrary to that of syringaldazine (Fig. 3), never fades and remains indefinitely stable for all species tested positive, thereby enabling the recording of positive results hours or even several days after the test.

DISCUSSION

Detection level of polyphenol oxidases by tincture of guaiac and guaiacol and determination of type of wood-decay

Concerning unspecific polyphenoloxidases as detected by guaiac and guaiacol, these substrates showed according to species, more or less important differences in their ability to detect these enzymes as presented in Tab. 2 and Fig.1. Agerer *et al.* (2000) also noticed such differences when studying extra-cellular oxidase activities in some ectomycorrhizal and saprophytic fungi, which he explained by the high heterogeneity of phenoloxidases which are composed of many different phenolic compounds with different oxidising capabilities according to the substrate (guaiac, gallic acid agar, tannic acid agar) used.To explain these differences, Cairney & Burke (1998) evoked the possible role of iron-radicals in culture media that could cause "false positive" reactions though their findings were later on amended by Agerer *et al.* (2000).

Tincture of guaiac provides one of the best illustrations of the above-mentioned inconsistencies according to species. Here, the cases of *P. tenuiculus* and *T. menziesii* based on our results compared to those of previous studies are worth mentioning. These two species which were taxonomically determined by Ryvarden (pers. comm.) both tested negative with guaiac (Tab. 2), thereby determining brown rot. The same species tested positive in other studies, and were consequently identified as white rotters as reported by Mswaka & Magan (1998) for *T. menziesii* and Gilbertson & Ryvarden (1987) reporting works of other authors for *P. tenuiculus*. However, concerning the specific case of *T. menziesii* of which 2 different strains from Cameroon tested negative with guaiac (absolutely no shift in colour was observed with the first strain and very faint traces of blue appeared about an hour after putting tincture of guaiac drops on mycelium of the second strain, meaning unequivocally a negative result), our results are rather in correlation with an additional cross-check experiment carried out by Mswaka & Magan (1998), who in a phenoloxidase and lignin biodegradation assay noticed the inability of *T. menziesii* to degrade lignin, the general rule being that fungi that cause white rot are able to degrade lignin. The authors (Mswaka & Magan 1998) however recognized the contradiction between their result on lignin biodegradation assay with *T. menziezii* and their guaiac test, the assay correlating with our guaiac test result on this species.

Besides the common explanations (heterogeneity of phenolic compounds in phenoloxidases, possible "false positives" due to iron-radicals etc.) usually

given by authors for such controversial results and concerning the specific case of tincture of guaiac, we noticed that these differences in guaiac test results could also arise from various interpretations of two important parameters that determine positive and negative results, namely the precise moment at which the shift in colour to blue occurs during drop-test on mycelial cultures, and the blueing intensity observed. In fact Nobles (1958) who first introduced this substrate mentioned that the presence of phenoloxidases in mycelial cultures is determined by a rapid blueing and that a faint late-appearing blue should be regarded as a negative result. However, except for clearly positive tests identified by a rapid blueing occurring during dropping on mycelium, or clearly negative tests recorded when absolutely no shift in colour occurs no matter how long the time, the author did not precisely indicate at which time-limit and which blueing intensity the intermediate results should be considered positive or negative. Considering that tincture of guaiac reacts quite differently on mycelial cultures as far as time, blueing intensity and fungal species are concerned, and furthermore that no additional investigation has so far been carried out to clarify these guaiac test-parameters, this has undoubtedly led to cases of misinterpretations by different authors of what should be considered positive or negative to guaiac. This could therefore be the explanation for the above-mentioned controversial results concerning guaiac tests in some species.

However, still concerning polyphenoloxidase activities identified by guaiac, the results recorded in some of our strains are in agreement with those of previous studies carried out by other authors. To be noted here is the case of *Trametes elegans* that tested positive with this substrate as did the collection of Mswaka & Magan (1998). The positive guaiac test of *Irpex stereoides* investigated by the same authors and the same result recorded in our collection of *Irpex (Flavodon) flavus* is in agreement with the remarks of David (1980) and Gilbertson (1980), who among several authors noted a correlation between morphologically defined genera and their type of rot, though Ryvarden (1991) later on pointed out the existence amongst some genera of both white rot and brown rot species, as illustrated on the one hand by our results on *Trametes* species, where *T. elegans*, *T. cotonea* and *T. lactinea* tested positive with guaiac, and *T. menziesii* testing negative, and on the other hand by the differences recorded in guaiac and guaiacol tests in our *Lentinus* species as presented in Tab. 2. Considering the *Lentinus* species investigated and *L. tuber-regium* in particular, the negative guaiac test result recorded in this species is very likely, since contrary to other *Lentinus* species, it never grows on wood in its natural environment, but rather on a sclerotium substrate resembling cocoyam tubers. Nevertheless, about the determination of the type of rot, if the large majority of white and brown rot species (Tab. 2) where clearly identified due to a rapid or total absence of reaction to tincture of guaiac, the suspected brown rot species (Tab. 2 (i)) due to a faint late-appearing blue still need some confirmation through additional tests either on different strains of the same species collected elsewhere or in different countries, or lignin biodegradation tests as those carried out by Kirk & Kelman (1965), Sundman & Näse (1971) and Mwsaka & Magan (1998). It is however worth mentioning that in spite of their reliability, these tests could not clarify the type of rot caused by some species investigated by these authors. About the above-mentioned intra-generic differences in the oxidising activity level of enzymes of some species, they could be explained by the varying capability of these species to form oxidases as postulated by Agerer *et al.* (2000), who observed the same differences in the genus *Cortinarius*, where *C. infractus* tested strongly positive with guaiac and *C. odorifer* consistently negative. The

author found the same trend in the genus *Amanita*, where *Amanita* subg. *Amanitopsis* and *Amanita* subg. *Amanitopsis* tested respectively positive and negative with the same substrate.

Our results on *Pycnoporus sanguineus* testing positive with guaiac are in agreement with those of Mswaka & Magan (1998), but it is worth noting here that for tincture of guaiac, as well as for syringaldazine and α-naphtol, we observed the shift in colour that determined positive tests only on the red to reddish (colour of mature fructifications : 9A7, 9A8, 9B8, 9C8, 10A8, 10B8, 10C8 in Kornerup & Wanscher 1978) parts of the mycelial cultures showing micro-fructification growths and never on the white part of the mycelium, where colour change usually occurs in most species. This phenomenon, peculiar to this species and never mentioned before in any other study, could be explained by the phenoxazin-3-one pigments such as cinnabarin, cinnabarinic acid and tramesanguin already known to be produced in fructifications of this species as reported by Sullivan & Henry (1971) and that could be involved in complex reactions leading to the oxidation of the above mentioned substrates.

Time-dependent reaction of syringaldazine and α-naphtol

On time-dependent reaction of syringaldazine and α-naphtol, whereas for syringaldazine Harkin (1973, 1974), who first introduced this substrate gave no accurate data on its reaction-time in positive tests, Käärik (1965) and Stalpers (1978) advocated readings at 3H, 24H and 72H for α-naphtol and Marr (1979) recorded results at 0 mn, 5 mn, 15 mn and 30 mn for all substrates used, including syringaldazine, α-naphtol, P-cresol, tincture of guaiac, guaiacol, phenol, L-tyrosine and pyrogallol.

Concerning α-naphtol in particular, our findings as far as the most relevant time margin for recording results is concerned are closer to those of Käärik (1965) and Stalpers (1978), the 0 to 30 mn recording time advocated by Marr (1979) being barely enough for the first signs of colour shift to appear in most species testing positive. The maximum laccase activity detected by α-naphtol was recorded between approximately 1 to 24 H in most of our samples, however with the possibility of recording the highest activity hours or even several days after the test, given the non-fading nature of the shift in colour observed in this substrate.

In regard to syringaldazine, our results compared to those of Marr (1979) have brought more accuracy for a better utilization of this substrate particularly concerning its reaction time margin in spot tests that varies between 1s to about 120 mn and exceptionally 180 mn for some species (Tab. 3 & 4), the maximum laccase activity being recorded from about 5 mn for early and fast reacting species, till maximum 30 mn for the late and slow reacting. Still in regard to syringaldazine, our results establish a straight relationship between the remarks (cf. Introduction) of Harkin *et al.* (1973, 1974) and the accurate reaction time concerning the recording of positive results and the level of enzymatic activity depending on time and colour shift intensity that we have also related for the first time to the colour chart of Kornerup & Wanscher (1978).

Laccase activity detected by α-naphtol and syringaldazine

As the substrates for unspecific polyphenol oxidases, α-naphtol and syringaldazine also showed more or less important differences in their ability to detect laccase as presented in Tab. 2 and Fig. 2. In spite of some few cases where

these two substrates detected laccase at the same level, these inconsistencies could be explained, as for unspecific polyphenol oxidases, by the possible role of iron radicals in culture media and the varying capacities of investigated species to produce laccase that probably oxidises α-naphtol and syringaldazine in different manners. This last hypothesis is supported by the existence of multiple forms of laccases as postulated by several authors including Blaich & Esser (1975), who in a study carried out on 15 wood-fungi by analytical isoelectric focussing (IEF) noticed that most of these fungi exhibit a pattern of 6 to 10 extra- and intracellular laccases divided into 2 groups, laccase A complex reacting at PH 3-4 and laccase B complex at PH 5-8, with quantitative differences in their ability to oxidize various phenolic substrates. Marr (1979) further reported multiple forms of laccases and tyrosinases, each form (isoenzyme) exhibiting a characteristic substrate-specific pattern.

In conclusion, in addition to the determination of decay characteristics of 22 wood-rotting Basidiomycetes from Cameroon, 15 of which were tested for the first time, the accurate determination of the reaction time of syringaldazine in spot tests with a link established between on the one hand the colour shift intensity measuring the enzymatic activity level and on the other hand the colour chart of Kornerup & Wanscher (1978), this study also shows the relevance in spot tests of using several substrates with different chemical sensitivities for a reliable detection of enzymatic activity in wood-rotting fungi.

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