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ISOZYME ANALYSIS ON SOME WOOD DECAY FUNGI

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SUMMARY

This study was carried out to verify the usefulness of isozyme analysis in identifying species of wood decaying fungi isolated from sporocarps and wood of decayed trees. Twelve different enzymes were analysed by native polyacrylamide gel electrophoresis and seven of them gave positive response. Two staining procedures showed a characteristic profile allowing specific identification for the following species: *Fomes fomentarius, Fomitiporia punctata, Inonotus hispidus*, *Phellinus torulosus*, *Rigidoporus ulmarius* (*Polyporaceae s. l.*).

Key words: Polyporaceae, diagnosis, PAGE.

INTRODUCTION

In urban areas, ornamental trees are weakened by several abiotic stress factors, mainly related to soil compaction, air and soil pollution, etc.. Additional damage to tree trunk and roots may be caused by surface grading, trenching, use of lawnmowers and parking cars close to the trunk. All these kinds of damage alter plant physiology and open entry points for wood decaying fungi that are able to cause a considerable reduction of tree steadiness and lead to sudden branch crashes, mostly during windy days or storms (Tattar, 1989; Butin, 1995). The identification of the fungi causing decay in a tree is essential for risk assessment: in fact, each species has its own behaviour towards the host, as it can attack and alter peculiar part of the tree.

Decay fungi (mostly *Polyporaceae s. l.*) often live inside a tree for a long period without producing sporocarps, rendering specific identification impossible. Therefore, alternative tools for identification are needed.

Identification by mycelial characters or by reaction to phenolic compounds in culture are time consuming, and not always successful, also due to the lack of important species in the keys (Nobles, 1965; Stalpers, 1978).

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Isozyme analysis provides a fairly rapid and inexpensive alternative tool for species identification. The use of this technique is consolidated for taxonomic studies (Bonde *et al.*, 1993) and for identifying some wood decay fungi (Karlsson and Stenlid, 1991; Wahlstrom *et al.*, 1991; Bragaloni *et al.*, 1997).

This study considers various fungal species known to inhabit mainly the lower part of trees and produce heavy wood decay. Different enzymes were analysed by vertical native polyacrylamide gel electrophoresis (PAGE) and the profiles obtained were compared for assessing their usefulness in specific identification.

MATERIALS AND METHODS

Several sporocarps were collected from decayed trees, mainly in urban areas, and identified on the basis of macro- and micromorphological characters (Jülich, 1989; Bernicchia, 1990). Twenty isolates were obtained on 2% malt extract agar (MEA: 2% Oxoid malt extract desiccated and 1.2% Oxoid agar technical) from sporocarps of the following species: *Fomes fomentarius* (Fr.) Kickx, *Fomitiporia punctata* (Karst.) Murrill, *Inonotus hispidus* (Bull.:Fr.) Karst., *Phellinus torulosus* (Pers.) Bourd. & Galz., and *Rigidoporus ulmarius* (Sow.:Fr.) Imazeki. Moreover, three isolates from the collection of CBS, Utrecht, The Netherlands [*Phellinus conchatus* (Pers.:Fr.) Quél., *Phellinus robustus* (Karst.) Bourd. & Galz. and *P. torulosus*] and two unidentified fungal cultures obtained from decayed trees, where no sporocarp had been produced, were collected. All these isolates were investigated (Table 1).

Three replicates per isolate were inoculated in malt extract broth (2 g Oxoid malt extract desiccated and 100 ml of distilled water, in 250 ml flasks) with two mycelial plugs each, cut from a 10-day-old colony on MEA. The liquid cultures were incubated for 14 days on a rotary shaker (100 rpm) at 24±1°C, in darkness. Mycelia were harvested by vacuum filtration, washed three times with sterile distilled water and pulverized in a mortar with liquid nitrogen and 1.0 g of mycelium was mixed with 1.5 ml of buffer (Tris 40 mM, glycine 60 mM, saccharose 10% w/v, DTT 1 mM, pH 8.3). The homogenate was centrifuged at 13,000 *g* for 15 min at 5°C. The supernatant was used for electrophoresis,

Table 1. Investigated isolates and respective hosts.

Species	Host
Fomes fomentarius	*Platanus* x *acerifolia*
Fomes fomentarius	*Quercus cerris*
Fomes fomentarius	*Quercus cerris*
Fomes fomentarius	*Tilia* sp.
Fomitiporia punctata	*Laurus nobilis*
Fomitiporia punctata	*Platanus* x *acerifolia*
Fomitiporia punctata	*Platanus* x *acerifolia*
Fomitiporia punctata	*Robinia pseudoacacia*
Fomitiporia punctata	*Robinia pseudoacacia*
Inonotus hispidus	*Platanus* x *acerifolia*
Inonotus hispidus	*Populus* sp.
Inonotus hispidus	*Sophora japonica*
Phellinus conchatus	*Salix* sp.[a]
Phellinus robustus	*Quercus* sp. [b]
Phellinus torulosus	*Erica arborea* [c]
Phellinus torulosus	*Erica arborea*
Phellinus torulosus	*Pinus pinea*
Phellinus torulosus	*Quercus cerris*
Phellinus torulosus	*Quercus ilex*
Phellinus torulosus	*Robinia pseudoacacia*
Rigidoporus ulmarius	*Aesculus hippocastanum*
Rigidoporus ulmarius	*Platanus* x *acerifolia*
Rigidoporus ulmarius	*Populus* sp.
Unidentified mycelium	*Juglans regia*
Unidentified mycelium	*Ostrya carpinifolia*

[a] CBS isolate No. 100118
[b] CBS isolate No. 100119
[c] CBS isolate No. 100089

Table 2. Enzyme activities tested.

Enzyme	Acronym	E.C. number
Acid phosphatase	ACP	3.1.3.2
Alcohol dehydrogenase	ADH	1.1.1.1
Aspartate aminotransferase	AAT	2.6.1.1
Endopeptidase	ENP	3.4.23.6
Esterase	EST	3.1.1.1
Glutamic dehydrogenase	GDH	1.4.1.2
Lactate dehydrogenase	LDH	1.1.1.27
Malate dehydrogenase	MDH	1.1.1.37
Peroxidase	PRX	1.11.1.7
Phospho-gluco-isomerase	PGI	5.3.1.9
Phospho-gluco-mutase	PGM	5.4.2.2
Shikimate dehydrogenase	SKD	1.1.1.25

which was done in vertical native polyacrylamide gel, using 10% resolving gel and 3.5% stacking gel with Mini-Protean II System® BioRad (Hercules, CA, USA). The protein concentration was previously measured according to Bradford (1976). All steps were carried out at 5°C at constant amperage (14 mA) using Tris-glycine buffer, pH 8.3. Twelve isozyme activities (Table 2) were assayed, according to Wendel and Weeden (1990). Each staining procedure was repeated three times. For comparing different isolates, the relative position of the stained band (R_f value) was determined as the ratio of migration distance from the origin of each band to migration distance from the origin of the dye marker (bromophenol blue).

RESULTS

In all species, the enzymes AAT, ENP, GDH, PGI and PGM had very weak activities, and EST showed intraspecific variability: therefore, they were useless for our purpose. On the other hand, isozyme patterns of ADH, ACP, LDH, MDH, PRX and SKD gave positive responses in species identification (Table 3). In particular, PRX allowed to clearly distinguish most of the species tested (Fig. 1), which showed characteristic banding patterns: *F. fomentarius*, *F. punctata* and *R. ulmarius* did not reveal any intraspecific variability, while *P. torulosus* isolates showed variation in one or two band intensity. The same analysis, applied on the other *Phellinus* species (*P. robustus* and *P. conchatus*) gave distinctive profiles too. SKD activity allowed to separate several species (*P. torulosus*, *I. hispidus* and *R. ulmarius*), showing sufficient polymorphism among the species and consistent patterns for each species. Also in this case *P. robustus* and *P. conchatus* produced distinctive profiles (Fig. 2).

LDH, ACP, ADH and MDH banding patterns showed characteristic electrophoretic phenotypes in *I. hispidus*, *F. punctata*, *R. ulmarius* and *F. fomentarius*, respectively (Fig. 3). The interpretation of the profiles obtained was difficult for the other species. In fact, in some cases, MDH and LDH showed limited interspecific variability, ACP high intraspecific variability and, finally, LDH and ADH showed a poor activity at all.

It is noteworthy that unknown isolates from decayed walnut and hornbeam were identified as *F. fomentarius* by PRX and MDH and as *P. torulosus* by PRX and SKD enzymatic activities respectively.

DISCUSSION

The possibility of identifying a wood decaying fungus in absence of its fruiting body is important for a good management of urban trees, as sporocarps are not frequently present. Moreover, the identification of a basidiocarp is often difficult, even in undisturbed conditions, due to the inconsistency of some identification characters (Wagner and Fisher, 2001) which can lead to misidentification. In this paper, some of the most dangerous and polyphagous wood decaying species on ornamental trees were considered. The enzyme analyses gave consistent results also on isolates from different hosts included in the study for each fungal species. The present research confirms that this technique can be

Table 3. Enzyme activity results: responses (R) and species identification (I).

Enzyme	*Fomes fomentarius*		*Fomitiporia punctata*		*Inonotus hispidus*		*Phellinus torulosus*		*Rigidoporus ulmarius*	
	R	I	R	I	R	I	R	I	R	I
ACP	+	-	+	+	+	-	+	-	+	-
ADH	-	-	-	-	-	-	-	-	+	+
AAT	-	-	-	-	-	-	-	-	-	-
ENP	-	-	-	-	-	-	-	-	-	-
EST	+	-	+	-	+	-	+	-	+	-
GDH	-	-	-	-	-	-	-	-	-	-
LDH	-	-	+	-	+	+	+	-	+	-
MDH	+	+	+	-	+	-	+	-	+	+
PRX	+	+	+	+	-	-	+	+	+	+
PGI	-	-	-	-	-	-	-	-	-	-
PGM	-	-	-	-	-	-	-	-	-	-
SKD	-	-	-	-	+	+	+	+	+	+

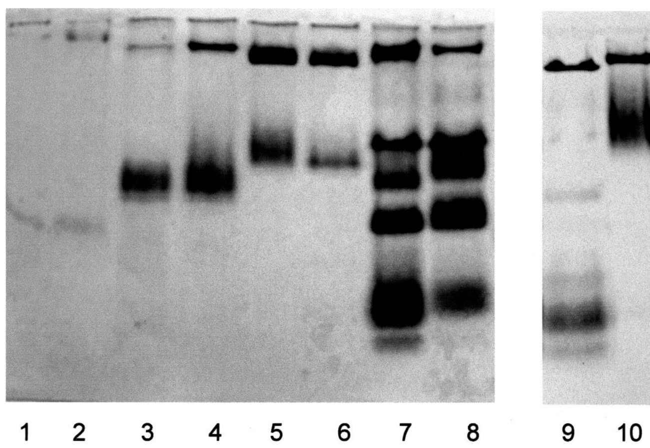

Fig. 1. Polymorphic banding pattern of peroxidase (PRX) isozyme. *F. fomentarius* (lanes 1, 2); *F. punctata* (lanes 3, 4); *R. ulmarius* (lanes 5, 6); *P. torulosus* (lanes 7, 8); *P. conchatus* (lane *9)*; *P. robustus* (lane 10).

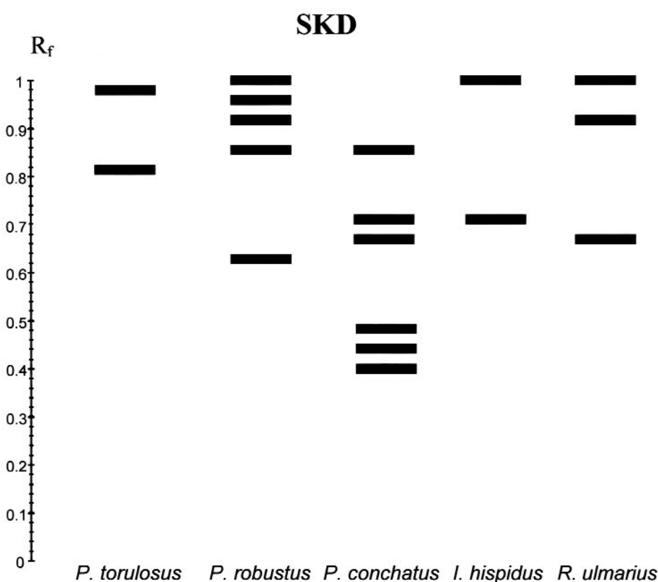

Fig. 2. Shikimate deydrogenase activity (SKD): characteristic electrophoretic phenotypes of *P. torulosus*, *P. robustus*, *P. conchatus*, *I. hispidus*, *R. ulmarius.*

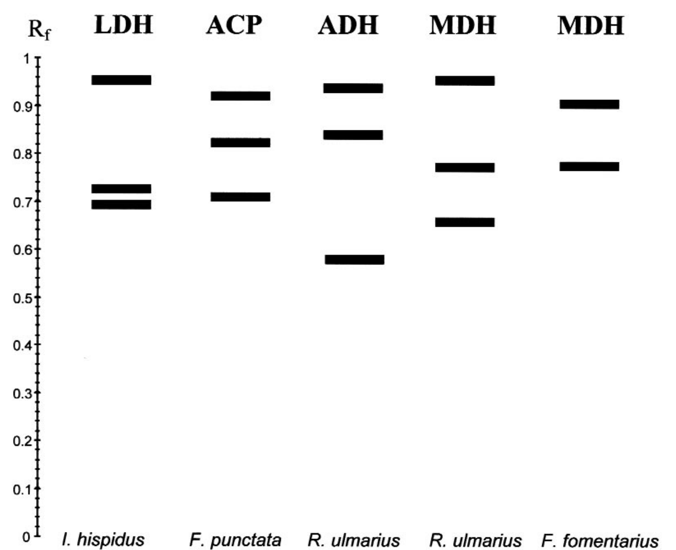

Fig. 3. Characteristic electrophoretic phenotypes of *I. hispidus* obtained by lactate dehydrogenase (LDH), *F. punctata* by acid phosphatase (ACP), *R. ulmarius* by alcohol dehydrogenase (ADH) and malate dehydrogenase (MDH), *F. fomentarius* by malate dehydrogenase (MDH).

used effectively to identify electrophoretic phenotypes of different species of decay fungi. Positive results were obtained for 7 out of 12 of the enzyme activities tested. For each species at least two staining procedures gave a characteristic profile and satisfactory reproducibility, allowing specific identification. This opportunity is even more interesting for the species which have very similar or common mycelial characters. For instance, it is impossible to distinguish between the mycelium of *I. hispidus* and that of *P. torulosus* by the identification key of Stalpers (1978), as the two fungi are placed in the key together. A more extensive investigation is necessary, including other isolates and species frequently observed in urban forestry, in order to obtain a useful tool for practical application. In recent years, several studies

were carried out for the identification of wood decay fungi using DNA-based approaches (Fisher and Wagner 1999; Crivelli *et al*., 2000; Behnweg *et al*., 2002). Further investigations on the effectiveness of different techniques and their combined utilization will be useful for detection and characterization of this important group of fungi.

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