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The duration effect of natural seasoning of *Quercus petraea* Liebl. and *Quercus robur* L. on the diversity of existing fungi flora and some aspects of its ecology

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Summary : Towards the end of natural seasoning, or after contamination of wood piles of 3 years old or more, a great number of species establish themselves on the wood, favoured by the detoxification of the environment and the presence of autolysis residues from the first fungi generation. *A. pullulans*, the main species from natural seasoning is thus in competition with other fungi. These mechanisms affect the quality of natural seasoning. The lytic enzymes are mostly released whilst the synthesis of the heterosidase activities, used for the assimilation of substrates, are heavily suppressed.

Key words : *Quercus sp.*, natural seasoning, fungi, competition, autolysis, *A. pullulans*

INTRODUCTION

Natural oak wood seasoning constitutes a refinement stage essential for cooperage (TARANSAUD, 1976 ; VIVAS *et al.*, 1996). Only barrels made from naturally seasoned wood are capable of amplifying the quality of superior wines (PONTALLIER *et al.*, 1982). Furthermore, the wood should imperatively have a relative humidity (Hr 15 to 18 %) compatible with the impermeability of the barrels in which the liquid is held.

Factors necessary for the natural seasoning of oak are numerous : rain, wind, temperature ranges (MARCHE et JOSEPH, 1972 ; PONTALLIER *et al.*, 1982), as well as the micro-organisms (CHENG et CHANG, 1985 ; VIVAS *et al.*, 1991). They ensure a refinement of the wood characterised by a diminution of the phenolic compound content and by the, more or less complete, elimination or transformation of the astringent and bitter substances (MARCHE and JOSEPH, 1972 ; PONTALLIER, 1981).

Recent studies (VIVAS and GLORIES, 1993a ; VIVAS and GLORIES, 1993b ; VIVAS, 1993 ; VIVAS and GLORIES, 1996) have shown that natural seasoning is accompanied by the development of a limited number of moulds, which appear to be characteristic. The authors isolated : *Aureobasidium pullulans*, which represents 80 % of the

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total population, *Trichoderma harzianum* and *T. koningii* which constitute the main secondary species. Nonetheless, over and above 18 to 20 months of exposure to the air, the fungi flora appears to diversify to the benefit of a good number of other species characteristic of mouldy environments (BOTTON *et al.*, 1982). Fungi diversity thus introduce the phenomenon of competition (VIVAS and GLORIES, 1993a) susceptible to affect the wood refinement process.

Competition between fungi can be brought about in two ways : either by the liberation of an inhibiting substance (DONECHE and MARCANTONI, 1992), or by competing for the nutriments (KONESTCHNY *et al.*, 1988 ; WEGER *et al.*, 1986). Concerning wood, the energy sources are few and difficult to use ; the fungi thus need to produce a certain number of soluble enzymes, notably heterosidases, in order to extract the necessary nutrients. Competition for nutrition is thus constant in this environment. But biological control of fungus by fungicide substances released by other fungi is also conceivable. VIVAS and GLORIES (1993a) showed the repressive effects of a *T. harzianum* filtrate culture with regards to *A. pullulans*.

It therefore seems useful to bring to light, following these different works, the inversion of the fungi flora during a prolonged natural seasoning and to study the influence of some induced ecological relationships, of a competitive nature, on the aptitude of fungi to transform the phenolic heterosides of the wood. The primary observations are presented in this article.

MATERIAL AND METHODS

I - ISOLATION AND CULTURE OF OAK WOOD STAVE FUNGI

Split staves, 30 mm thick, 150 mm wide and 1.05 m long, were distributed in piles throughout a Bordeaux region wood park (Average annual temperature = 12.5°C, total precipitation = 950 mm/year ; average over 50 years). The piles were aligned, each being 50 cm apart along the line with 1 m between each row. The *Q. robur* L. wood came from Limousin and the *Q. petraea* Liebl. from the Allier forests. The samples were taken at random and presented as 2 staves taken per 10 piles of Allier wood and 4 piles of Limousin wood in the process of drying. Each wood sample was sawn up into a test sample of 20 cm and then placed in a sterile bag.

The fungi were taken either from oak wood sawdust representing the first 10 mm of the upper face of the staves, constituting the total flora (spores and mycelia), or from a sterile swab used when cleaning the surface of the staves, representing the spores deposited on the surface and fragments of mycelium.

Culture and purification of the different species were carried out in a MAG solid medium under the conditions described by VIVAS *et al.* (1991). The medium is composed of a malt gelose extract (45 g), agar-agar (15 g), glucose (25 g) and sterilised water (enough for 1000 ml), the medium is autoclaved for 15 min at 115°C. Isolation of the fungi was carried out by mixing 1 g of sawdust from the surface of the staves with 10 ml of sterile water and by an infusion of the stave surface cleaning

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swab in 10 ml of sterile water. The cultures were realised in Petri dishes placed in a dark chamber at 25°C. Purification of the morphologically different strains was realised using sub-cultures of portions of mycelium in the MAG medium. The identifications were performed in collaboration with the Mycology department at the Pasteur Institute (Paris).

II - DEVELOPMENT OF DIVERSE SPECIES OF ISOLATED OAK WOOD FUNGI IN A GELOSE MEDIUM SUPPLEMENTED BY AN AQUEOUS EXTRACT OF UNSEASONED OAK

Each isolated species was cultivated in a MAG medium in Petri dishes at 25°C. The medium is inoculated by depositing a fragment of mycelium from a pure culture in the centre of the Petri dish. The results express the ease with which colonisation occurs in the medium containing a freeze dried water extract of unseasoned oak *Q. petraea* L. (1/5 ; p/v) compared to a control sample in a solid MAG medium on its own. The readings were taken after 5 days of incubation. The coloration of the solid culture is also notable.

When the fungi develop at the same rhythm in the two types of gelose media, we consider that the unseasoned oak constituents do not affect the growth of the mycelia, under the experimental conditions.

III - METHODS OF ESTIMATING COMPETITION PHENOMENON OF DIFFERENT SPECIES

1 - Effect of competitive phenomena on the total exocellular ß-glucosidase activity of the culture medium

The majority of isolated oak wood fungi produce over the course of their development great quantities of soluble exocellular ß-glucosidase (VIVAS and GLORIES, 1993a). This activity enables a fungi to develop in a medium where the only source of energy is a phenolic heteroside (ellagitannins, gallotannins...) (VIVAS *et al.*, 1991 ; VIVAS, 1993b). The glucosidase activity is estimated thanks to *p*-nitrophenyl ß(D)glucopyranosides (pNPG) under the conditions described by DARRIET *et al.* (1988), inspired by TINGLE and HALVORSON (1971).

The non-prolific liquid mediums ($[NH_4]H_2PO_4$: 1 g, K_2HPO_4 : 1 g, KCl: 0.5 g, $MgSO_4$: 0.5 g, $FeSO_4$: 0.005 g, KOH : enough for, pH 3.5, distilled water enough for 1000 ml) are cultured with *A. pullulans* mycelium and tested fungi in the ratio of 1 mg of dry mycelium from each of the two strains for 100 ml of medium. Furthermore the production and preparation of dry mycelium is described (VIVAS *et al.*, 1993b). The control sample contains 2 x 1 mg of *A. pullulans* mycelium. The glucosidase activity is measured after 10 days of culture at 20°C (± 2°C) on an agitation table.

The culture is centrifuged and the supernatant carefully collected. Six ml of the supernatant was mixed with 4 ml of a sodium acetate solution (NaAc 0.05 M ; pH = 5), and then 0.3 ml of pNPG 0.01 M was added. The tubes are incubated for 24 hours at 25°C. The reaction is stopped with 1 ml of sodium carbonate (Na_2CO_3, 1M). The optic density of the supernatant was measured at 400 nm, and the results were reduced to a percentage of the estimated activity at O.D. 400 nm, compared to the pure culture *A. pullulans* control sample.

2 - Effect of competitive phenomena on the mycelium in autolysis phase in a mixed culture

The O.D. measure at 210 nm is due to the fact that the mycelia altered by stress conditions (age of the culture, substrate concentration, competition amongst species) release into the medium endocellular matter absorbed by ultraviolet (GARBAY and LONVAUD-FUNEL, 1990). The measurement conditions are those of GARBAY and LONVAUD-FUNEL (1990).

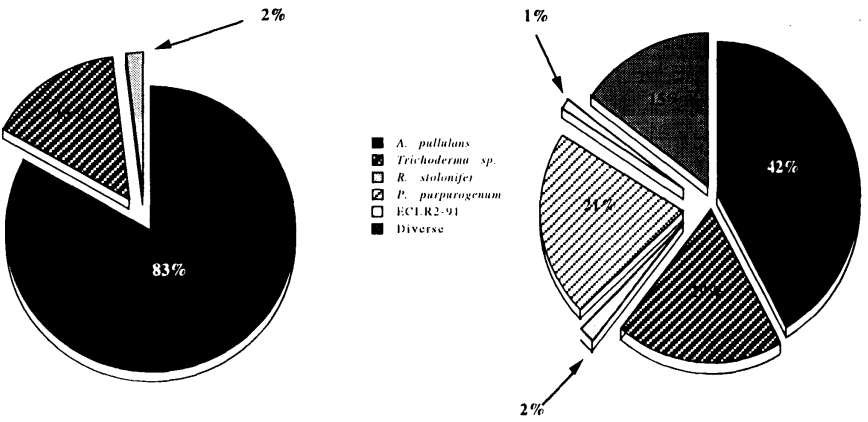
5 ml of a non-prolific liquid medium culture is taken, centrifuged (20000 x g, 15 min) and the O.D. at 210 nm is measured. The value of the optic density is multiplied by 100.

RESULTS

I - DIVERSIFICATION OF THE FUNGI FLORA DURING NATURAL SEASONING

During the first six months in which the oak staves are exposed to the air, *A. pullulans* is the dominant species. After 36 months of seasoning (figure 1), *A. pullulans* remains largely dominant (83 %) but other species appear, principally *Trichoderma sp.* After three years, the flora is not represented by these species alone (figure 1) ; one equally finds on the surface of the wood numerous other fungi, and particularly *Penicillium purpurogenom.*

In a laboratory experiment, we cultivated different species, in a gelose medium supplemented with an aqueous extract of unseasoned oak. The results are grouped in table 1. It is worthy to note that all the strains chosen develop in the gelose control medium. We noticed that only the species found at the beginning of seasoning develop perfectly in the gelose medium containing unseasoned oak extract. On the



12 months of natural seasoning **36 months of natural seasoning**

Figure 1 - Effect of natural seasoning duration on the distribution of total fungi flora on the oaks staves

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other hand, diverse species found in the mixed culture and *P. purpurogenum* after two years of seasoning, establish themselves with difficulty in the presence of unseasoned oak extract. The addition of a sterilised filtrate of an old *A. pullulans* culture in the lytic phase, membrane filtered (porosity of 0.2 µm) and characterised by a strong increase in O.D. at 210 nm, improves the establishment of these species. Over and above this, replacing the unseasoned oak extract with an extract of naturally seasoned oak enabled, all the fungi, a satisfactory development in the gelose medium.

Table 1
Influence of aqueous extract of unseasoned oak on the development of isolated species of oak wood during natural seasoning
The results express the facility with which moulds develop on a gelose medium containing a green oak extract in comparison to the control sample

Tested species	*A. pullulans*	*T. Harzianum*	*T. Koningii*	*R. Stolonifer*	*P. purpurgogenum*	ECLR2-91	Divers*
Development after 5 days	+	+	+	-	-	-	0
Colour in gelose medium control	black	white/green	green	green	white/grey	red	cream
Colour in gelose medium + green oak extract	black	green	green	grey	brown	pink	-

* : *Geotricum sp., Geomyces sp., Penicillium sp., Phialophora sp., Aspergilus versicolor, Alternaria alternata*
0 : undeveloped ; - :weakly developed ; +: normal development

II - Effect of competitive phenomenon on the aptitude of fungi to liberate enzymes capable of hydrolysing the phenolic heterosides of unseasoned wood

In order to study these ecological relationships of a competitive nature, we cultured *A. pullulans* in association with different species. Only one type of activity is measured : the ß-glucosidase, representing the soluble exocellular enzymes capable of liberating glucose from heterosidic structures. We observed that certain fungi like ECLR2-91, which develop slowly, do not affect the activity under study (table 2). Other fungi are, on the other hand, particularly adapted to the culture medium and induce competitive phenomena with varying intensities (*T. harzianum*, *T. koningii* and particularly *P. Purpurogenum*). Competition between the two different species seems to be expressed by an appreciable diminution of the glucosidase activity.

The simultaneous development of several fungi, in a medium which is totally colonised, provokes a phenomenon of competition. One could suppose that the concurrent relationships between different species are due to the accumulation of lytic

Table 2
Effect of competitive phenomena between *A. pullulans* and some species found at the end of natural seasoning on the activity of ß-glucosidase

	A. pullulans			
	A. pullulans	*T. harzianum*	*ECLR2-91*	*P. purporogenum*
ß-glucosidase activity	100	64	107	37

enzymes in the medium, to the detriment of the trophic enzymes. Thus, the beginning of the lysis phase is ealier and the autolysis of the mycelia is more significant than in the pure culture (table 3).

Table 3
Incidence of competition phenomena on mycelia autolysis in a mixed culture

	Autolysis start (days)	D.O. 210 nm x 100	
		40 days	80 days
A. pullulans	36	22	67
T. harzianum	21	48	97
P. purpurogenum	28	35	84

DISCUSSION

The prolonged seasoning of oak wood leads to the development of numerous fungi. Meanwhile, the phenomenon is slow because the wood piles are not in direct contact with the humid ground. The aeration and sunshine slow down the process to a considerable degree (VIVAS, 1993a). Everything happens as though the wood evolves from a refined state to one of decomposition. These observations suggest that natural seasoning should be performed by a limited flora, largely represented by *A. pullulans*, which seems to be best adapted to this function (VIVAS and GLORIES, 1993a). This diversification of the fungi flora may be linked to a modification in the composition of the wood's surface , characterised both by the accumulation of protein-like residues and polysaccharides stemming from the autolysis of the initially established fungi, and in this way, by detoxification of the environment by these same initial fungi (VIVAS and GLORIES, 1993a ; DONECHE and MARCANTONI, 1992). In fact, previous results show that these two factors may be propitious for the secondary colonisation of oak staves. Rain leaching of the staves leads to a significant amount of extractable matter, especially phenolic compounds (VIVAS, 1993b). Nevertheless, the activities of *A. pullulans* and *Trichoderma sp.* allow the destruction of a number of phenolic heterosides (VIVAS *et al.*, 1996). Knowing the toxic effect of phenols on a large number of micro-organisms (SCALBERT and HASLAM, 1987 ; SCALBERT, 1992), one can postulate that, only the moulds possessing an enzymatic genotype adapted to a ligneous substrate, initially, colonise the wood ; these species must be of a lichenous type like *A. pullulans*. In the second phase, the mycelia grow and cover the surface of the staves, the colonies then age and their autolysis begins (MARTINEZ *et al.*, 1983). At this stage, other fungi may colonise the wood, propitious to the detoxification of the surface wood and the accumulation of autolysis residues.

These species are essentially cellulolytic such as *Trichoderma sp.*, they may, inter alia, inhibit the primary fungi by the liberation of an antibiotic, toxin or fungicide (WYLLIE and MORHENMOUSE, 1977), or an exocellular enzyme susceptible to altering the cell walls of the mycelia of the other fungi species which may be present (WOOD, 1951 ; ARTIGUES, 1985). Overlapping of all the reactions seems to be sufficient in order to interpret the modification of the fungi flora of oak wood during natural seasoning.

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Technologically, changing a specific flora, essentially represented by *A. pullulans*, to a varied flora, at the origin of competitive relationships between fungi has the effect of stimulating the liberation of fungicidal and lytic substances to the detriment of a metabolism which is oriented towards transforming the wood's phenolic heterosides. Natural seasoning becomes less efficient for the cooper, because the refinement of the wood by the ligneous fungi is interrupted to the benefit of an alteration of the ultra structure of the wood by cellulolitic fungi. One also notices, under practical conditions, that the proximity of the wood piles, three years old and more, is propitious to the contamination of piles which are next to each other. Diversification of the flora over a significant part of the wood park and the inversion of the fungi flora becomes less premature. These kinds of results are found in wood parks where the renewal level is insufficient. It is noteworthy that the surface of oak wood which has been left outdoors for several years is covered by a very diversified fungi flora, but few collected spores are susceptible to germinating and creating a thallus in this environment. The preliminary discrimination between all the spores in a gelose medium, rich in oak extract, enables the limitation of isolates to those species adapted principally to the wood environment.

Thus, in the same way that numerous biotechnological operations aim at refining a primary substance (beer, wines, cheeses...), natural seasoning should be considered as an optimisation operation, in particular by employing reasonable aspersion rhythms and the use of appropriately selected fungi strains. Our results appear to indicate that *A. pullulans* is the best adapted. Nevertheless, it is appropriate to not exclude other fungi adapted to wood, which may be encountered, during a more exhaustive study of the fungi flora in oak wood parks, throughout the entire French territory.

Conclusion

Open air natural seasoning of oak heartwood is differentiated in particular from artificial seasoning by the development of a fungi flora which can affect the structure and composition of the wood. In this study, we have emphasised the existence of antagonising phenomena between the different coexisting micro organisms. These relationships of a competitive nature explain, on the one hand, the succession of different types over the course of time and on the other, the progressive evolution of the wood in a cellulo-ligneous substrate in the decomposition process. The consideration of a fungi (*A. pullulans*), well adapted to the conditions of the environment (nature of the matter, humidity, temperature), as a source of selected fungi is conceivable for the future (Vivas, 1993a). A patent guarantees industrial protection.

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