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ROLE OF PHENOLASES IN THE FORMATION OF QUINONOID FUNGAL METABOLIC PRODUCTS OF *ASPERGILLUS FUMIGATUS* FRES. AND *PENICILLIUM SPINULOSUM* THOM.

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SUMMARY

The relationship between the presence and activity of phenolases and the formation of fumigatin and spinulosin, quinonoid metabolic products of the fungi, *Aspergillus fumigatus* and *Penicillium spinulosum* was investigated.

It was found from colorimetric tests with a range of phenolic substrates that endo-cellular laccases with the same specificity are responsible for the formation of fumigatin and spinulosin. A *para*-phenolase mechanism is proposed for the formation of these substances.

Exo-cellular laccases were found only in strains producing fumigatin and these enzymes are responsible for the Bavendamm reaction. The intensity of this reaction is not quantitatively related to the gross phenolase activity or to the formation of fumigatin. The function of the exo-cellular phenolases has not yet been established.

INTRODUCTION

The fungi *Aspergillus fumigatus* and *Penicillium spinulosum* produce toluquinone derivatives, fumigatin (I) and spinulosin (II) respectively with related structures[1].

Fumigatin is somewhat unstable because its hydroxyl group may tautomerise with the oxygen atom in the *para* position to give the *o*-quinone[2]. Spinulosin, with hydroxyl groups in the 2,5-positions cannot undergo tautomerism and has a stable structure.

In the formation of fumigatin and spinulosin, it is presumed that a phenolase system is responsible for the oxidation of phenolic substances within the cells or in the substrate outside the cells. It has been shown[3] that *A. fumigatus*, from which humic acids were obtained, gives a positive BAVENDAMM reaction[4] whereas *P. spinulosum*

is negative. Tests on catechol and guaiacol indicated that the phenolase activity of *A. fumigatus* was of the laccase type, thus confirming the experiments of HIGUCHI[5] that laccases are mainly responsible for the BAVENDAMM reaction.

We compared the phenolase activity of various strains of *A. fumigatus* and *P. spinulosum* and investigated the relationship between this activity and the formation of quinonoid metabolic products.

MATERIALS AND METHODS

The following fungal strains were principally used:

A. fumigatus: strain AF 1 (Centraalbureau Schimmelcultures Baarn)
strain A 46 (London School Hygiene and Tropical Medicine)
strain A 49 (Imperial Mycological Inst. Kew, No. 89354)
P. spinulosum. strain PS 1 (FAL Braunschweig No. 1200/3)
strain PS 2 (Botany Dept. Univ. College Dublin, isolated from peat)

Culture media

Malt agar (Difco), Czapek-Dox solution, Raulin-Thom solution, modified for fumigatin production[1].

For the measurement of phenolase activity a colorimetric method was employed. The fungal strains were grown in liquid surface culture for fixed incubation periods. The contents of the flasks were then filtered, the mycelium washed and weighed and the filtrate volume measured. Three weighed samples of the mycelium were dried overnight at 105° for determination of dry weight. The mycelium was homogenised with a volume of phosphate buffer (pH 6.8) calculated to give the same concentration equivalent for the dried mycelium of each strain. The activity of these extracts was measured by incubating 3 ml of the solution in a waterbath overnight at 27° with 5 ml of a 0.5% solution of the following phenolic substrates: Catechol p.a. Merck, resorcinol p.a. Merck, hydroquinone B.D.H., pyrogallol p.a. Merck, guaiacol cryst. synth. Merck, *p*-cresol cryst. Merck, L-tyrosine Merck (aqueous suspension).

Since the filtrates obtained correspond to different mycelial dry weights, they also were standardised. The dry weight of the total mycelium harvested was calculated and the filtrate diluted to give a volume corresponding to the same weight of dry mycelium per 100 ml of the culture solution. The phenolase activity of this solution was estimated in the same way as the mycelial extracts.

The coloured products formed were estimated and compared using an "Eel Spectra" colorimeter at 500 mμ wavelength.

The methods for the isolation of fumigatin and spinulosin from their respective strains have been described[1,6]. The isolation of spinulosin from a strain of *A. fumigatus* (A 49) was also undertaken[7].

For the BAVENDAMM test tannic acid at a concentration of 0.5 g/100 ml was added to the various culture media, with agar and tests carried out by placing discs of agar from grown cultures onto the tannin-containing agar in petri dishes. The zones of darkening were then measured and compared. The technique described by

RÖSCH[8] whereby a test tube containing 10 ml of tannic acid agar is inoculated with the fungal strain, was also used. This method has the advantage that the extent of darkening of tannic acid can be more readily noted.

RESULTS

BAVENDAMM *test*

In our experiments we found that *A. fumigatus*, except the strain A 49 which produces spinulosin, gives a positive BAVENDAMM reaction, while *P. spinulosum* is negative (Table I).

TABLE I

BAVENDAMM REACTION OF *A. fumigatus* and *P. spinulosum*

Reaction determined by the depth of darkening in a test tube[8] containing Czapek-Dox agar + 0.5% tannic acid inoculated with the fungal strains and incubated at 27° for 8 days. F, fumigatin; S, spinulosin.

Fungus strain	*A. fumigatus*			*P. spinulosum*		
	AF 1	*AF 6*	*A 46*	*A 49*	*PS 1*	*PS 2*
Extent of darkening	+ +	+	+ +	−	−	−
Metabolic product	F	?	F	S	S	S

The intensity of the reaction varies with the strains of *A. fumigatus* but does not appear to be related to fumigatin production since strain A 46 which gives a high yield of this substance[1] has only moderate activity.

It has been shown[6,9,10] that the BAVENDAMM reaction is due mainly to exo-cellular laccases, though LYR[11] who reviewed the work on the enzymes concerned in this test, showed that peroxidases may also be responsible for the darkening of tannic acid. In order to investigate the occurrence and nature of the phenolases in the fungi under study, and to find what relationship, if any, exists between the phenolase activity, the BAVENDAMM reaction and the formation of metabolic products with quinonoid structure, colorimetric experiments were carried out on a variety of phenolic substrates with endo- and exo-cellular enzyme preparations.

The phenolases from various sources in nature can be readily classified as laccases or tyrosinases[12] and each of these types has a wide range of specificity[13,14]. On the basis of the substrate range we can show the precise activity of these phenolases[10,15] (Table II).

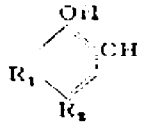
A. fumigatus and *P. spinulosum*, grown in liquid surface culture for 16 days at 24° were tested colorimetrically for phenolase activity with phenolic substrates. The mycelial extracts of all strains oxidised catechol, hydroquinone and pyrogallol but not *p*-cresol or tyrosine. From the key given in Table II it is clear therefore that the phenolases present in both *A. fumigatus* and *P. spinulosum* are laccases.

It may be noted that *p*-cresol is oxidised by laccases to a white precipitate[13] and not to a coloured quinonoid compound as with tyrosinase.

The development of endo- and exo-cellular phenolase activity by these strains

TABLE II

SUBSTRATE SPECIFICITY OF PHENOLASES

Enzyme	Activity	Substrate	Determining structure
Tyrosinase	monophenolase	Monophenols	
	o-diphenolase	o-Diphenol and derivatives	
	o-diphenolase		
Laccase	p-diphenolase	p-Diphenol and derivatives	

was then examined. Small Fernbach flasks, each containing 100 ml of modified Raulin-Thom solution[1] with the addition of 0.01% $FeSO_4 \cdot 7H_2O$ and a trace of $CuSO_4 \cdot 5\ H_2O$ were inoculated with liquid roller cultures of *A. fumigatus* (A 46 and A 49) and *P. spinulosum* (PS 2), six flasks of each strain. The conditions of inoculation and culture were maintained as uniform as possible. After 8 days incubation at 24° and every 4 days subsequently, to 28 days, a flask of each strain was taken and the phenolase activity of the filtrates and mycelial extracts measured.

The endo- and exo-cellular activities, expressed in terms of the extinction values for 15 mg dry mycelium with catechol, hydroquinone and pyrogallol are given in Fig. 1. There was no colour reaction with *p*-cresol or tyrosine and guaiacol gave slight activity with the strain A 46 only.

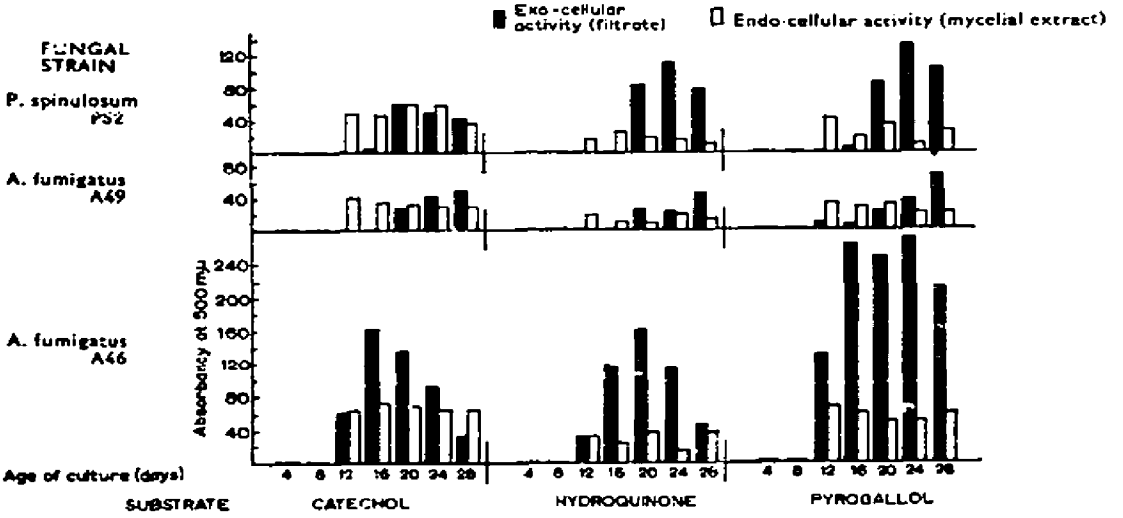


Fig. 1. Endo- and exo-cellular activities, expressed in terms of the absorbancy values for 15 mg dry mycelium, with catechol, hydroquinone and pyrogallol.

It can be seen (Fig. 1) that in the case of *A. fumigatus* strain A 49 there is no activity in the filtrate until after 20 days growth, when autolysis has begun. The endo-cellular phenolase activity remains relatively constant. Likewise, with *P. spinulosum* PS 2 there is no evidence of activity in the filtrate before autolysis has been observed. With *A. fumigatus* A 46 however, the filtrate has high phenolase activity as soon as the culture has completed its growth phase (about 12 days) and before autolysis, which occurs after about 20 days incubation. The activity of the mycelial extracts of the strains shows little variation.

From the data obtained in our experiments about these strains (Table III) we

TABLE III

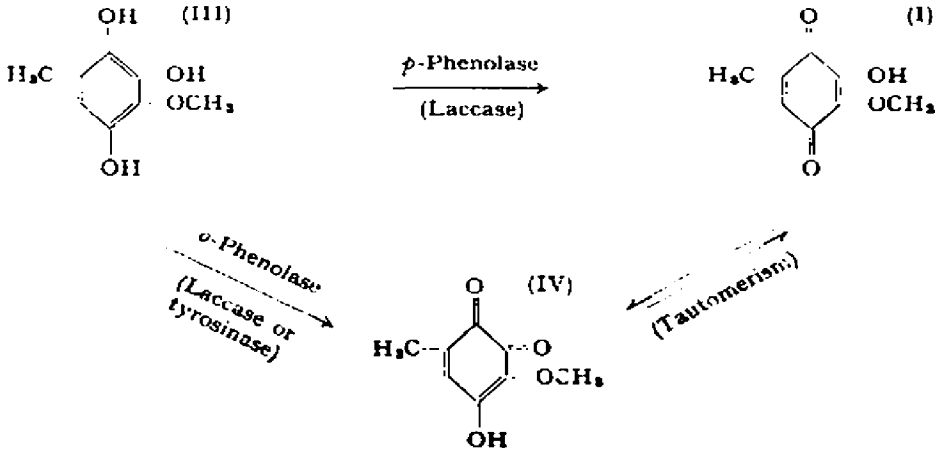
BAVENDAMM REACTION, PHENOLASES AND METABOLIC PRODUCTS OF STRAINS OF *A. fumigatus* AND *P. spinulosum*

Fungus strain	*A. fumigatus*			*P. spinulosum*	
	AF 1	*A 46*	*A 49*	*PS 1*	*PS 2*
BAVENDAMM reaction	+ + +	+ +	·	−	−
Phenolase activity exo- (laccase)	+	+	·	−	−
endo-	+	+	+	+	+
Metabolic product	fumigatin	fumigatin	spinulosin	spinulosin	spinulosin

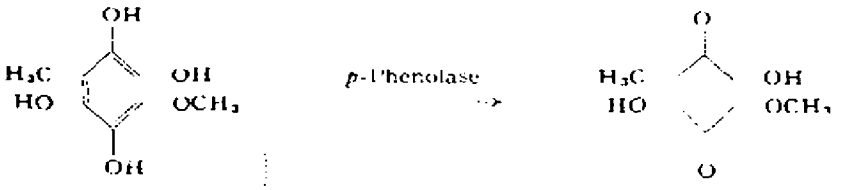
can conclude that laccases occur endo- and exo-cellularly in strains of *A. fumigatus* which produce fumigatin, but only endo-cellularly in strains of *A. fumigatus* or *P. spinulosum* which produce spinulosin. Thus we can confirm for these fungi that the BAVENDAMM reaction gives an indication only of the exo-cellular phenolases present.

DISCUSSION

In the formation of fumigatin it is possible that laccase or tyrosinase could be responsible for the oxidation of a polyphenolic precursor.



A. fumigatus was found to contain only laccase and it alone has *p*-phenolase activity (Table II). This suggests that fumigatin (I) must be formed directly from a *p*-phenol such as compound III above. In fact, this compound (1,2,4-trihydroxy-3-methoxytoluene) was found to occur in culture media with fumigatin[1]. Likewise, *P. spinulosum* was found to have laccase activity with the same substrate range as *A. fumigatus*, this leads to the conclusion that similar enzymes can be responsible for the formation of fumigatin and spinulosin (II) and, from theoretical considerations this is quite possible.



Since the *o*-quinone (IV) also occurs, by tautomerism, fumigatin can be formed by the *o*-phenolase activity of laccases. The formation of spinulosin, however, cannot be due to *o*-phenolase activity because the hydroxyls in the 2,5-position form a stable structure and tautomerism is not possible.

We have shown that endo-cellular phenolases only are associated with spinulosin formation while endo- and exo-cellular are present when fumigatin is formed. Spinulosin is presumably formed from the endo-enzymes whose activity we have measured. Similarly, fumigatin can be formed by the endo-cellular phenolases of *A. fumigatus*. We cannot say at present whether these enzymic oxidations take place in the cell or in the culture medium after autolysis. Further experiments are in preparation to establish the location of the phenolic and quinonoid metabolites with respect to the living fungal cell which should clarify this problem.

Previous observations[3] that *A. fumigatus*, which gave a positive BAVENDAMM reaction, formed humic acids led to the conclusion that there was a relationship between the type of phenolase and the formation of particular quinonoid metabolites (fumigatin). Our experiments have shown, however, that the BAVENDAMM reaction is due to exo-cellular phenolases only and these are not related quantitatively to fumigatin formation. Spinulosin, which does not form humic acids, is produced by the same phenolases as fumigatin. Therefore humus formation would depend on the structure of the precursors rather than on the type of enzymes forming them.

It is possible that the exo-cellular phenolases may have a function in humic acid formation by catalysing further polymerising dehydrogenation reactions of quinones, as is shown in the case of lignin formation[16], but there is, at present, no evidence of such a function.

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