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Efficient degradation of tannic acid by black *Aspergillus* species

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A set of aspergillus strains from culture collections and wild-type black aspergilli isolated on non-selective media were used to validate the use of media with 20% tannic acid for exclusive and complete selection of the black aspergilli. The 20% tannic acid medium proved useful for both quantitative and qualitative selection of all different black aspergilli, including all recognized species: *A. carbonarius*, *A. japonicus*, *A. aculeatus*, *A foetidus*, *A. heteromorphus*, *A. niger*, *A. tubingensis* and *A. brasiliensis* haplotypes. Even higher concentrations of tannic acid can be utilized by the black aspergilli suggesting a very efficient tannic acid-degrading system. Colour mutants show that the characteristic ability to grow on high tannic acid concentrations is not causally linked to the other typical feature of these aspergilli, i.e. the formation of brown-black pigments. Sequence analysis of the *A. niger* genome using the *A. oryzae* tannase gene yielded eleven tannase-like genes, far more than in related species. Therefore, a unique ecological niche in the degradation of tannic acid and connected nitrogen release seems to be reserved for these black-spored cosmopolitans.

INTRODUCTION

Micheli (1729) introduced the designation *Aspergillus* for fungi with a characteristic aspersory, or mop-like, organisation of the conidiophore and spores. The name *Aspergillus niger* was introduced by van Tieghem (1867) when he described a black-spored *Aspergillus* capable of using the plant polymer tannic acid as carbon source. Rippel (1939) described the selective isolation of *A. niger* on high concentrations of this very acidic tannin, but this growth characteristic seems to have been largely forgotten ever since.

Nowadays, we recognize that the black aspergilli form a complex of closely related species. *Aspergillus* sect. *Nigri* consists of at least eight species based on recent morphological, RAPD, mitochondrial and RFLP studies (*A. carbonarius*, *A. japonicus*, *A. ellipticus*, *A. heteromorphus*, *A. aculeatus*, *A. niger*, *A. tubingensis*, and *A. brasiliensis*) (Kusters-van Someren, Samson & Visser 1991, Mégnégneau, Debets & Hoekstra 1993, Varga *et al.* 1993, 1994). All members of the section have characteristic dark brown to black conidia.

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Presumably all black aspergilli are asexual, and vegetative compatibility between natural isolates is very rare (van Diepeningen, Debets & Hoekstra 1997). In nature the black aspergilli are mainly saprophytic and occasionally pathogenic. The spores are distributed by air and the fungus has been detected from a large variety of substrates. They form a substantial part of fungal populations, and occur worldwide with a preference for tropical and subtropical areas (Rippel 1939, Raper & Fennel 1965, Domsch, Gams & Anderson 1980, Ploetz *et al.* 1985).

The black aspergilli are very versatile in their metabolism and *A. niger* was awarded with the 'generally recognised as safe' (GRAS) status by the US Food and Drug Administration. The GRAS status and their metabolic versatility mean that the black aspergilli are widely used in industry and biotechnology for the production of organic acids, enzymes and food fermentations (Finkelstein & Ball 1992). Though *A. niger* is known to grow on high concentrations of tannic acid, it was the non-black *A. oryzae* that was for a long time known for its tannase production (Hatomoto *et al.* 1996). Recently, however, the black *A. niger* and its close relative *A. foetidus* have been used in tannase

studies as well (Aguilar *et al*. 2001, Bhardwaj, Singh & Bhat 2003, Ramirez-Coronel *et al*. 2003, Mukherjee & Banerjee 2004).

The ability of *A. niger* to grow on tannic acid may be linked to its saprophytic life-style. Plants and plant parts can contain 5–20 % tannin by weight, and some plants that are used for tannin production may contain even up to 40 % (Clarke *et al*. 1949, Bollen & Lu 1969). In nature two types of tannins are recognised: the hydrolysable tannins such as tannic acid which have pentagalloylglucose as a core component (Mueller-Harvey 2001) and the condensed tannins or proanthocyanidins (Schofield *et al*. 2001). Both types of tannins occur in plant materials, both consist of a large range of molecules with different complexity, and both are capable of forming complexes with proteins, 'the tannin-effect' (Goldstein & Swain 1965). Tannin-protein complexes are difficult to mineralise, and thus are determinants of the proportion of nitrogen released in dissolved organic forms relative to the NH_4^+ and NO_3^- mineral forms (Northup *et al*. 1995).

In this study we show that: (1) the ability to grow on high tannic acid concentrations is unique to black aspergilli and can be used for qualitative and quantitative isolation; (2) there is no causal connection between the characteristic black pigment production and tannin degradation; and (3) the genome of *A. niger* contains many homologues to the *A. oryzae* tannase gene, more than in the related species examined. The possible unique ecological niche of the black aspergilli connected with their efficient tannic acid degradation is discussed.

MATERIALS AND METHODS

Strains and culture conditions

We used 50 Dutch, English and Indonesian wild-type black aspergillus strains, isolated on non-selective media, and 47 culture collection strains of black aspergilli and related aspergilli (Table 1). Regular incubation of strains was on minimal (MM) or complete medium (CM) (Pontecorvo, Roper & Forbes 1953) supplemented with $1\ mg \cdot l^{-1}$ $ZnSO_4$, $FeSO_4$, $MnCl_2$ and $CuSO_4$. Growth on tannic acid was tested on liquid complete medium supplemented with different amounts of tannic acid (Merck; tannic acid powder pure). The high acidity of the tannic acid prevents agar from solidifying. Medium with 5 % (w/v) tannic acid has a pH of 3.3, medium with 20 % tannic acid a pH of 2.65. The standard isolation of natural black aspergillus strains was done on CM with 20 % (w/v) tannic acid (CM + tan). All incubations took place at 30 °C.

Isolation of black Aspergillus *spp. on tannin medium*

Samples of the undisturbed top layer of soil and humus (5–50 g) were collected worldwide and used as inoculum on CM + tan. Samples were stored at 5 ° upon arrival in the lab and used for isolation within two weeks. Depending on the propagule density in the samples aliquots of the fresh soils or dilutions thereof were used. After 5 d all floating *Aspergillus* colonies were isolated and purified on MM. Estimates were made for local spore densities (quantitative assessment of the population density) and all isolates were classified according to mitochondrial haplotype (qualitative assessment of the population).

Mitochondrial DNA RFLP characterisation

All black *Aspergillus* strains, both culture collection and natural isolates were classified on the basis of their mitochondrial RFLP haplotypes. Total nucleic acids were isolated using a phenol/chloroform extraction adapted after Maniatis, Fritsch & Sambrook (1982): an overnight ± 0.1 g mycelial mat, grown on liquid CM, was frozen with liquid nitrogen in a 1.5 ml Eppendorf tube and disrupted with a pestle the size of a 0.5 ml Eppendorf tube, fitting in the bigger vial. Preheated extraction buffer (0.5 M NaCl, 10 mM Tris-HCl, 10 mM Na_2EDTA, 1 % SDS, pH 7.5) was added and the suspension was incubated at 65 °C for 30 min. Nucleic acids were then extracted once with 1:1 phenol: chloroform and once with 24:1 chloroform:isoamyl alcohol. Isopropanol was used to precipitate the nucleic acids, they were washed with 70 % ethanol and finally resupended in demi water or TE buffer (10 mM Tris/ 10 mM EDTA, pH 7.5). Restriction enzyme analyses were performed with both *Bgl*II and *Hae*III for *A. niger*, *A. tubingensis* and *A.brasiliensis* haplotypes (Varga *et al*. 1993, 1994) and with *Hae*III or *Eco*RI for *A. japonicus* and relatives (Hamari *et al*. 1997) and *A. carbonarius* and relatives (Kevei *et al*. 1996).

Isolation of colour mutants

Mutants with non-black conidiospore colours were obtained by plating conidiospores on MM after UV irradiation ($120\ J \cdot m^{-2}$) followed by visual inspection after two days of growth. Pure colour mutants were classified as fawn (*fwn*), brown (*brn*), white (*whi*), grey (*gry*) or olive green (*olv*).

Tannase gene(s)

The protein sequence of the *Aspergillus oryzae* tannase BAA09656.1 (D63338) was used to search (tblastn/ tfastx) the Dutch State Mines (DSM) database of the *A. niger* N400 genome sequence for putative genes with at least 60 % homology. The *A. oryzae* tannase and homologous tannase-like *A. niger* genes were subsequently used to further search Genbank for homologous published sequences. Preliminary sequence data of *A. fumigatus* Af293 was obtained from The Institute for Genomic Research website at http://www.tigr.org. Preleminary sequence data of *A. nidulans* FGSC-4a

Table 1. List of wild-type and culture collection strains used to validate the selectivity of medium containing 5 or 20% tannic acid for black *Aspergillus* spp.

Strain	Culture collection code[a]	Species	Haplotype	Growth on tannic acid[c] 5%	20%	Strain	Culture collection code[a]	Species	Haplotype	Growth on tannic acid[c] 5%	20%
Black *Aspergillus* spp.											
N050	CBS111.26	*A. carbonarius*	C	+	+	N068	CBS139.52	*A. usami*	1c	+	+
N051	CBS112.80	*A. carbonarius*	C	+	+	N069	CBS553.65	*A. usami*	1c	+	+
N1	–	*A. carbonarius*	C	+	+	N070	CBS117.32	*A. intermedius*	2a	+	+
N2	–	*A. carbonarius*	C	+	+	N071	CBS118.35	*A. hennebergii*	1b	+	+
N052	CBS707.79	*A. ellipticus*	n.d.	+	+	N072	CBS125.52	*A. hennebergii*	2d	+	+
N053	CBS677.79	*A. helicotrix*	1[a]	+	+	N073	CBS558.65	*A. pulverulentus*	2a	+	+
N054	CBS117.55	*A. heteromorphus*	n.d.	+	+	N074	CBS425.65	*A. pulverulentus*	2a	+	+
N055	CBS114.51	*A. japonicus*	J	+	+	N075	CBS121.28	*A. foetidus*	1a	+	+
N056	CBS621.78	*A. japonicus*	J	+	+	N076	CBS681.78	*A. foetidus*	1a	+	+
N057	CBS172.66	*A. jap. aculeatus*	J	+	+	N226	ATCC1015	*A. niger*	2c	+	+
N058	CBS115.80	*A. jap. aculeatus*	J	+	+	N400	ATCC9029	*A. niger*	1b	+	+
N059	CBS554.65	*A. niger*	1a	+	+	JHC601	–	*A. brasiliensis*	3	+	+
N061	CBS134.48	*A. niger*	2a	+	+	JHC603	–	*A. brasiliensis*	3	+	+
N062	CBS557.65	*A. awamori*	1c	+	+	JHC605	–	*A. brasiliensis*	3	+	+
N063	CBS563.65	*A. awamori*	n.d.	+	+	JHC606	–	*A. brasiliensis*	3	+	+
N064	CBS126.49	*A. phoenicis*	1a	+	+	Z1.1-2.25	–	26 black aspergilli[b]		+	+
N065	CBS135.48	*A. phoenicis*	n.d.	+	+	814-828	–	14 black aspergilli[b]		+	+
N066	CBS136.52	*A. nanus*	2a	+	+	Ind 1-6	–	6 black aspergilli[b]		+	+
N067	CBS131.52	*A. nanus*	1c	+	+						
Non-black *Aspergillus* spp.											
A003	–	*A. candidus*		+	–	704	–	*A. nidulans*		+	–
A004	CBS567.65	*A. candidus*		+	–	A002	–	*A. ochraceus*		+	–
A005	CBS225.8	*A. candidus*		+	–		NRRL 3174	*A. ochraceus*		+	–
	SZMC 918	*A. clavatus*		+	–		SZMC Z1	*A. ochraceus*		+	–
	IMI 015949	*A. clavatus*		+	–		SZMC O4	*A. ochraceus*		+	–
	NRRL 181	*A. fischeri* (*Neosartorya fischeri*)		+	–		CBS102.07	*A. oryzae*		+	–
	NRRL A-7223	*A. fischeri* (*Neosartorya fischeri*)		+	–		CBS818.72	*A. oryzae*		+	–
A001	–	*A. flavus*		+	–		CBS819.72	*A. oryzae*		+	–
	ATCC 16907	*A. fumigatus*		+	–	Ater1	–	*A. terreus*		+	–
	NRRL 163	*A. fumigatus*		+	–	Ater2	–	*A. terreus*		+	–
	IMI 156966	*A. longivesica*		+	–	Atam1	–	*A. tamarii*		+	–
701	–	*A. nidulans*		+	–		NRRL 5024	*Neosartorya spinosa*		+	–
702	–	*A. nidulans*		+	–						

[a] ATCC, American Type Culture Collection; CBS, Centraal bureau voor Schimmelcultures, Utrecht; SZMC, Szeged Microbiological Collection, University of Szeged.
[b] Different haplotypes: 1a/b and 2a/b/c/e.
[c] Growth (+) or no growth (–) on 5% and 20% tannic acid respectively.

Table 2. List of *Aspergillus* colour mutants tested on 20% tannic acid medium.

Strain	Name	Colour mutants[a]	20% tannin	Strain	Name	Colour mutants[a]	20% tannin
N050	*A. carbonarius*	*fwn*, *brn*	+	N067	*A. nanus*	*fwn*, *brn*, *gry*	+
N055	*A. japonicus*	*fwn*, *brn*, *whi*, *gry*	+	N068	*A. usami*	*fwn*, *brn*	+
N057	*A. jap. aculeatus*	*fwn*, *brn*, *whi*	+	N070	*A. intermedius*	*fwn*, *brn*	+
N059	*A. niger*	*fwn*	+	N076	*A. foetidus*	*fwn*, *brn*	+
N062	*A. awamori*	*fwn*, *brn*	+	N400	*A. niger*	*fwn*, *brn*, *gry*, *olv*	+
N064	*A. phoenicis*	*fwn*, *brn*	+				

[a] Conidium colours: *fwn*, fawn; *brn*, brown; *whi*, white; *gry*, grey; and *olv*, olive green.

(version 3.1) was obtained from the Center for Genome Research at http://www.broad.mit.edu. Genomic sequence data of *Neurospora crassa* (version 3) was obtained from the *Neurospora crassa* database of the Center for Genome Research (Galagan *et al*. 2003). Preleminary sequence data of *Podospora anserina* (release 1) was obtained from the *P. anserina* genome project website (http://podospora.igmors.u-psud.fr). Genscan (Burge & Karlin 1997) was used to predict the complete protein sequence of the *P. anserina* tannase homologues. ClustalW (Thompson *et al*. 1994) was used to align all sequences and TreeView (Page 1996) was used to further visualise the relationships between them, using *A. niger* and *A. tubingensis fea*A, ferulic acid esterase A (de Vries *et al*. 1997) to root the tree.

RESULTS

Tannin utilisation

Black *Aspergillus* strains that were not reported to be originally isolated on tannic acid medium were tested for the ability to grow on tannic acid. In total, 34 black *Aspergillus* strains from culture collections and 46 wild isolates, from air, soil, surfaces and other substrates, were individually tested on different concentrations of tannic acid. These strains were characterised by their mitochondrial haplotype and included strains belonging to the subgroups: *A. niger*, *A. aculeatus*, *A. ellipticus*, *A. heteromorphus*, *A. tubingensis*, *A. brasiliensis*, *A. carbonarius* and *A. japonicus*. All could grow on medium containing 20% tannic acid (Table 1). Some of the strains were tested on higher amounts and growth on 80% tannic acid polymers in the medium was not exceptional. 25 non-black *Aspergillus* strains of twelve different species were also tested, but none could grow on media containing more than 5% tannin. Therefore, growth on 20% tannin seems to be an exclusive trait for all the black *Aspergillus* species.

Eleven different black *Aspergillus* species were subjected to UV irradiation and visually checked for conidiospore colour mutants. Fawn (*fwn*) coloured mutants were most often found. Seven *fwn* mutations independently isolated from our laboratory strain *A. niger* N400 were found to be allelic in a complementation test (*fwnA*). Brown (*brn*), white (*whi*), grey (*gry*) and olive green (*olv*) mutants were found less frequently. The white colour mutations were restricted to the *A. japonicus* background. Fig. 1 shows the wild-type colour of *A. japonicus* N055 and four isolated different coloured spore mutants as an example for the colour mutants used in this study.

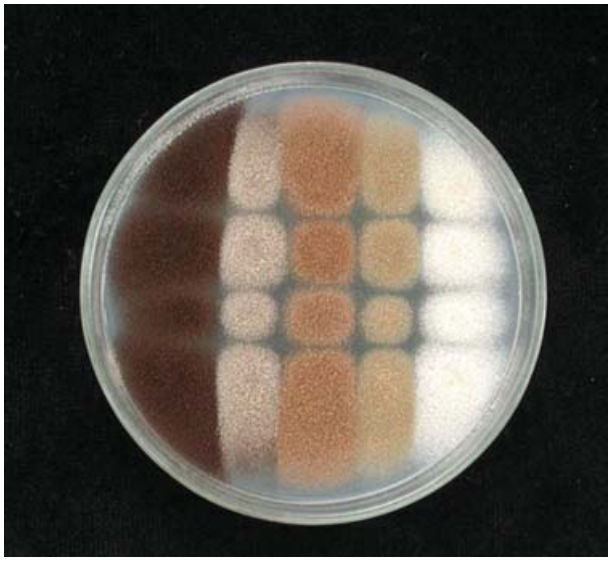


Fig. 1. Photograph of different colour mutants of *Aspergillus japonicus* N055. From left to right: wildtype black, grey (*gry*), fawn (*fwn*), brown (*brn*) and white (*whi*) coloured conidia.

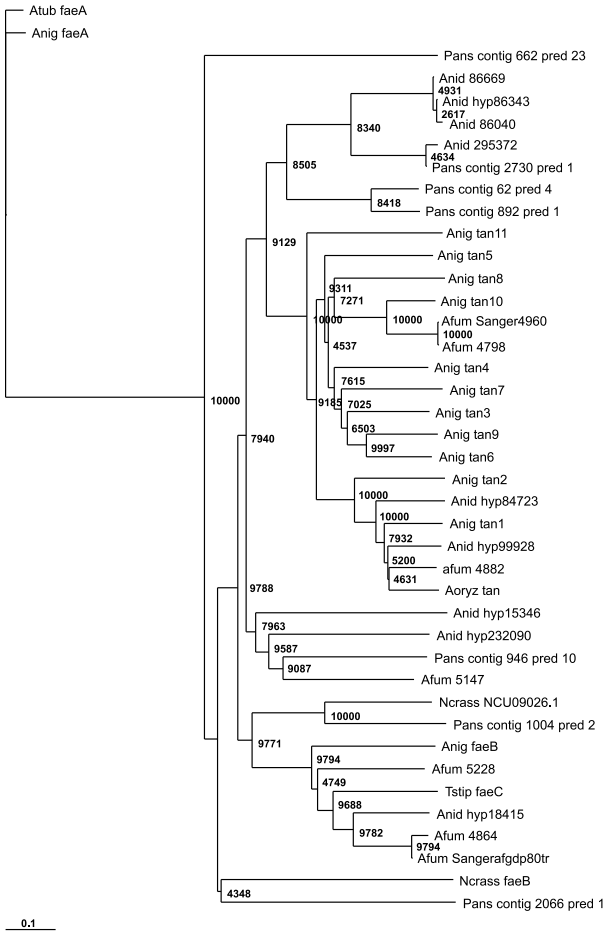
The spore colour mutants of the different black *Aspergillus* species were tested on CM + tan (Table 2). All colour mutants were able to grow on high tannic acid so the formation of black pigment in the conidia is not directly related to the ability to grow on high tannic acid concentrations.

After this validation we used medium with 20% tannin to select aspergilli from soil samples from vegetated areas, collected world-wide between 1990 and 1995. All colonies floating on CM + tan plates were isolated and purified and each was considered as a different strain. Each of the 642 isolates belonged to the black aspergilli and all were classified based on their mitochondrial RFLP haplotypes as *A. niger*, *A. tubingensis*, *A. carbonarius* or *A. japonicus* (Table 3). *A. niger* and *A. tubingensis* – each with different recognisable mitochondrial haplotyes – were the two most abundant species world-wide, whereas *A. brasiliensis* was not found in this study. No fungi other than black aspergilli were ever found growing on 20% tannic acid.

Table 3. Black *Aspergillus* spp. isolated on media containing 20% tannic acid from world-wide collected soil samples.

	Mitochondial haplotype													
	A. niger-aggregate													
	'*A. niger*'[b]				'*A. tubingensis*'[b]						*A. jap*[c]	*A. car*[d]		
Country[a]	1a	1b	1c	1d	2a	2b	2c	2d	2e	2f			No. of isolates	Density (No. of spores/g soil)
Canada (3)	–	–	4	6	–	–	–	–	–	–	–	–	10	0–2
France (4)	3	–	1	–	–	2	–	–	–	–	3	–	9	0–2
Netherlands (27)	1	6	5	–	40	6	–	–	–	–	–	–	58	0–8
Switzerland (2)	–	–	–	–	2	1	1	–	–	–	–	–	4	0–3
Morocco (2)	–	–	–	–	6	2	–	–	–	–	1	1	10	25–85
Egypt (2)	–	7	7	–	10	–	–	–	–	–	–	–	24	8–10
Israel (4)	–	1	–	–	4	–	–	–	–	–	–	1	6	2–3
Guinea (1)	–	6	3	–	–	–	–	–	–	–	–	–	9	65
Gabon (5)	3	24	5	–	4	7	1	–	1	–	40	–	85	40–60
Kameroon (6)	1	16	6	–	1	2	6	–	–	–	9	1	42	25–150
Brazil (4)	3	8	20	–	5	5	–	–	–	–	–	–	41	50–75
Indonesia (12)	27	77	74	1	50	33	4	9	14	1	23	7	320	50–250
Malaysia (1)	–	5	–	–	1	–	–	–	–	–	–	–	6	n.d.
Nepal (1)	–	6	–	–	–	–	–	–	–	–	–	–	6	4–6
Australia (1)	–	–	–	–	–	–	–	–	–	4	–	–	4	8–15
New Zealand (2)	–	7	–	–	1	–	–	–	–	–	–	–	8	6–12

[a] Per country the number of samples investigated is given between brackets.
[b] *A. niger* mitochondrial RFLP haplotypes 1a–1d and *A. tubingensis* haplotypes 2a–2f as recognised by Varga *et al.* (1993, 1994).
[c] jap, *A. japonicus* and direct relatives (Hamari *et al.* 1997).
[d] car, *A. carbonarius* and direct relatives (Kevei *et al.* 1996).



Spore densities in soil were highest in tropical (e.g. up to 250 spores g^{-1} soil in Indonesia) and subtropical regions and were lower in colder climate zones. Qualitative assessment of the population showed that the diversity in any given sample was generally very high. Thus, the different members of the group of black aspergilli can be found world-wide and well-mixed.

Tannases

A search in the *Aspergillus niger* DSM genome database using the tannase gene sequence from *A. oryzae* resulted in eleven sequences putatively coding for proteins of 570 amino acids and longer with a minimum amino acid identity of 46%. The two closest hits *anigtan1* and *anigtan2* hits were 581 and 580 amino acids long with an identity of 76 and 64% respectively to the *A. oryzae* tannase (86 or 80% positives and 1 or 3% gaps).

The *A. oryzae* tannase gene and its *A. niger* homologues were then used to search Genbank, two unfinished *Aspergillus* genome databases and two other

Fig. 2. Dendrogram showing the relationships between tannase homologs in different fungal species. 10 000 × bootstrap values are added. Anid, *Aspergillus nidulans* (hyp = hypothetical protein); Anig, *A. niger*; Atub, *A. tubingensis*; Afum, *A. fumigatus*; Aoryz, *A. oryzae*; Pans, *Podospora anserina* (pred = Genscan predicted gene); Ncrass, *Neurospora crassa*; and Tstip, *Talaromyces stipitatus*.

ascomycete genome fungal databases for other fungal tannase homologues. GenBank gave relatively few hits, whereas the *A. fumigatus* genome contained three potential homologues, *A. nidulans* four, *P. anserina* three and *N. crassa* none. The relationships between the putative tannase genes is shown in Fig. 2, where the *A. niger fae*A and its homologues are used as outgroup. Thus, *A. niger* contains a whole group of homologues not present in the other species.

DISCUSSION

All black aspergilli tested shared the efficient tannic acid utilisation trait, whereas non-black aspergilli all failed the 20% tannic acid test. However, the trait was not linked to the black conidiospore colour. The dark brown to black conidiospore colour itself seems to be a separate trait and this must have an additional selective advantage.

A preference of black aspergilli for warm and wet tropical climate zones has been noted previously (Rippel 1939, Raper & Fennel 1965; Domsch, Gams & Anderson 1980). But, the diversity and density of the black aspergilli we observed in the spore bank of the relatively dry top layers of soils found was enormous, suggesting that the dispersal must be very efficient to reach such a mixed world-wide distribution.

Aspergillus oryzae is capable of growing on up to 5% of tannic acids and a tannase gene has been isolated from *A. oryzae* by Hamamoto *et al.* (1996). Tannase homologues can also be found in different *Aspergillus* species capable of growing on up to 5% tannic acid as well as in other ascomycete fungi that have not been tested on tannic acid. However, in none of the other species examined have so many homologues been found as in *A. niger* N400 (where 11 homologous sequences were identified). One of these putative *A. niger* tannase homologues may be responsible for the efficient tannic acid degrading activities of black aspergilli or this ability may be produced by the joint effect of so many homologues in the genome enabling the degradation of many different tannin molecules. Other enzymes may also participate in the degradation of tannic acid. Ramirez-Coronel *et al.* (2003) have also found a β-glucosidase in *A. niger* with tannase activity which has no homology to the tannase genes described in this study.

Locally high concentrations of tannin may occur in nature where desiccated tannin-rich plant materials are accumulating. Northup *et al.* (1995) suggested that polyphenols such as tannins may play a role in the control of nitrogen dynamics in soils. The unique ablility of black aspergilli to utilize high concentrations of tannic acid sets them apart from all related fungi. The world-wide occurrence of black aspergilli may be attributed to their unique role in the degradation of tannin-rich plant compounds and related nitrogen release.

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