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Ecotoxicological aspects of Aspergilli present in the phylloplane of stored leaves of chewing tobacco (*Nicotiana tobaccum*)

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Abstract

Nine different species of *Aspergillus* were isolated from the phylloplane of stored chewing tobacco (*Nicotiana tobaccum*) of different ages. The maximum number of species were isolated from 12 and 18 month old leaves. *A. ruber, A. ochraceus, A. flavus* and *A. nidulans* were usually associated with older leaves while *A. niger, A. fumigatus* and *A. flavus* were isolated from 6 month old leaves. Approximately 18% of Aspergilli were found to be mycotoxigenic. Sterigmatocystin was produced by three different species. *A. ochraceus* produced patulin and ochratoxin. All aflatoxigenic strains of *A. flavus* produced aflatoxin B1 but none of the isolates of *A. flavus* produced aflatoxin G2. The percentage of toxigenic isolates of different species varied considerably.

Introduction

Different aspects of phylloplane fungi have been extensively reported [17, 4, 2] but little attention has been given to their toxigenic potential. Aspergilli, which often dominate the population of storage mycoflora, are known for their mycotoxigenicity [10, 9, 14]. Some of mycotoxins produced by species of *Aspergillus* are carcinogenic [22], teratogenic and mutagenic [15] and extremely detrimental to human and animal health [3]. Such toxigenic *Aspergillus* species associated with the phylloplane of chewing tobacco leaves warrant attention, in view of its raw consumption.

The agroclimatic conditions of tobacco growing areas of North Bihar, traditional post-harvest practices, poor storage facilities and prolonged storage, are inviting factors for the invasion of storage fungi in general and Aspergilli in particular. The presence of toxigenic fungi on the tobacco leaves is always associated with the risk of mycotoxin 'exposure' to the people addicted to chewing tobacco. Whether these mycotoxigenic fungi play a role in the development of oral cancer prevalent in tobacco chewing people needs further investigation. In the present communication the Aspergilli associated with the tobacco leaves of different storage ages and their toxigenic potential are reported.

Materials and methods

The sampels of tobacco leaves (saraisa variety) purchased from wholesalers and retailer shops were brought to the laboratory in separate polyethylene bags. Isolation of fungi was done by leaf

imprint method [16] on solid sterile potato dextrose agar (PDA) medium in 9 cm petri-dishes. Twelve leaf squares (10 × 10 mm) were cut at random from each leaf sample after stretching the leaves in aseptic conditions. Adaxial surface of 5 leaf squares were gently pressed against the medium and 5 leaf squares were used similarly for the abaxial surface. The petri-dishes were incubated for seven days at 25 ± 2 °C. Identification of *Aspergillus* species was done according to the key of Raper and Fennel [18]. Pure cultures were transferred to and maintained on PDA slants.

Screening for mycotoxins was done by growing the fungus on 50 g of sterilized polished rice (Basmati variety) and 50 ml tap water in 500 ml Erleynmayer flasks. The medium was inoculated with 1 ml of spore suspension in distilled water of 8–10 day old cultures. Flasks were incubated for 10 days at 24 ± 2 °C in static condition. After incubation the flask contents were submerged under 250 ml chloroform (BDH) and the caked rice was broken up with a glass rod. The mixture was boiled on a steam bath for five minutes and the molded rice was filtered off and discarded.

After evaporation of the chloroform, residues were stored at 0 ± 2 °C prior to qualitative estimation of mycotoxin. Each residue was dissolved in 5 ml chloroform and 50 µl aliquots were spotted on activated thin layer chromatography (TLC) plates coated with silica gel. The plates were developed to toluene : ethyl acetate : formic acid (50 : 40 : 10 v/v) in unequilibriated rectangular glass tanks. For the tentative identification of different mycotoxins the developed plates were examined under a longwave (360–365 nm) UV lamp. Aflatoxins were identified on the basis of their RF value and characteristic blue and green fluorescent spots. Detection of ochratoxin A were done by exposing the TLC plates in ammonia fumes for 5 minutes [20], which gives a greenish blue fluorescent spot. Treatment with ammonia fumes was also helpful in detection of palutin which gives yellow spots under visible light. For identification of sterigmatocystin, plates were dried and examined under longwave UV (360 nm) light. Sterigmatocystin fluoresces dull brick-red under longwave UV light. For confirmation by derivative formation, developed plates were sprayed with saturated solution of $AlCl_3$ in 95% ethanol and heated at 105 °C for 10 minutes in a hot air oven. When viewed under longwave UV light the sterigmatocystin appeared as a bright yellow spot [1]. Confirmation of all the mycotoxins were done by comparing RF values with reference standards from Rizk Institute, The Netherlands and Makor Chemicals, Israel.

Results

As shown in Table 1 the most *Aspergillus* spp. were isolated from 12 and 18 month old non-disinfected leaf surfaces. From 18 month old leaves the maximum number of colonies were recorded for *A. ruber* followed in decreasing number by *A. ochraceus, A. flavus, A. versicolor, A. nidulans* and *A. tamarii*. The spectrum of *Aspergillus* spp. associated with 12 month old leaves was slightly different and in order of decreasing number isolated: *A. flavus, A. ochraceus, A. niger, A. terreus, A. fumigatus* and *A. tamarii*. Comaratively fewer species were found to be associated with six month old leaves. Among all, *A. niger* was the most common species.

From disinfected surfaces of 18 month old leaves, the maximum number of colonies was recorded for *A. ruber* followed by *A. flavus, A. ochraceus, A. nidulans* and *A. terreus*. From 12 month old disinfected surface of leaves only three species, viz. *A. ruber, A. flavus* and *A. niger* were isolated, while only *A. niger* was isolated from disinfected 6 month old leaves.

Aspergillus flavus and *A. niger* were found to be the most common species present in the phyllophane of the leaves of all the samples. *A. fumigatus, A. tamarii* and *A. terreus* showed lesser frequency of appearance in comparison to other Aspergilli.

Regarding mycotoxigenic potential (Table 2), several isolates of *A. ochraceus, A. niger, A. flavus, A. versicolor* and *A. nidulans* were found to produce ochratoxins, patulin, sterigmatocystin and aflatoxin, respectively. The percentage of toxi-

Table 1. Aspergillus spp. isolated from the phylloplane of stored chewing tobacco leaves.

Species (according to Raper and Fennel, 1965 [18]	Approx. age of the leaf[a] 6 month		12 month		18 month	
	Number of colonies SND[b]	SD[c]	Number of colonies SND[b]	SD[c]	Number of colonies SND[b]	SD[c]
1. *A. ruber* Spick Brem	–	–	–	7	12	17
2. *A. fumigatus* Fres.	5	–	2	–	–	–
3. *A. ochraceus*	–	–	6	–	10	8
4. *A. niger* Van. Tiegh	6	9	5	4	–	–
5. *A. flavus* Link	3	–	7	5	9	11
6. *A. tamarii* Kita	–	–	2	–	2	–
7. *A. versicolor* Tirab.	–	–	–	–	4	–
8. *A. nidulans* Wint	1	–	–	–	3	2
9. *A. terreus* Thom.	–	–	3	–	–	2

[a] Age was determined from the harvest month.
[b] Surface non-disinfected.
[c] Surface disinfected.

genic isolates varied considerably in different species.

Table 2. Mycotoxin production by toxigenic *Aspergillus* species isolated from the phylloplane of chewing tobacco leaves.

Sl No.	Species	Total number of isolates screened	Total number of toxigenic isolates[a]	Types of toxin[b]
1.	*A. ruber*	36	None	Nil
2.	*A. fumigatus*	7	None	Nil
3.	*A. ochraceus*	24	3	Ochratoxin A
			1	Patulin and Ochratoxin A
4.	*A. niger*	24	5	Sterigmatocystin
5.	*A. flavus*	35	8	Aflatoxin B1
			3	Aflatoxin B1 & B2
			1	Aflatoxin B1, B2 & G1
6.	*A. tamarii*	4	None	Nil
7.	*A. versicolor*	4	3	Sterigmatocystin
8.	*A. nidulans*	6	2	Sterigmatocystin
9.	*A. terreus*	5	None	Nil

[a] Other isolates were not producing detectable amount of the toxin tested.
[b] Identification of mycotoxins based on Rf and colour of fluorescence after tlc in one solvent system against authentic standards (See Methods).

Sterigmatocystin was found to be produced by three species, *A. niger, A. versicolor* and *A. nidulans;* one isolate of *A. ochraceus* produced both patulin and ochratoxin A. One isolate of *A. versicolor* formed a blue fluorescence compound with a different RF value from aflatoxin but similar in color. Aflatoxin B1 was elaborated by all the toxigenic isolates of *A. flavus* followed in frequency of appearance by aflatoxin B2 and G1. None of the isolates produced aflatoxin G2, as ascertained by the screening method. The isolates of *A. ruber, A. fumigatus, A. tamarii* and *A. terreus* tested were non-toxigenic.

Discussion

Aspergilli, which frequently dominate the mycoflora of stored grains, were found to be predominant also in the phylloplane of stored tobacco leaves. Apparently, factors such as low moisture content of the stored leaves and minimum relative humidity levels of 65–88% favored *Aspergillus* species to become established during microecological succession. Favorable modifications of the substrate by the earlier invading fungi as seen in other stored commodities [21] may also con-

ribute in the advent and prevalence of Aspergilli on stored tobacco leaves. In addition, the multiple sources of contamination, viz. the wall, floor, ceiling of storage room and the jute sacs [6] provided a high load of inoculum.

The reason for predominance of *A. ruber* and *A. ochraceus* on the older leaves may be attributed to their ability to grow on the substrates of relatively low moisture content. Similar observations have been made by Mislivec and Tuite [12] and Mislivec *et al.* [13] while working with dried leaves.

Approximately 18% of Aspergilli isolated from the phylloplane of stored tobacco leaves showed toxigenic potentialities. Aspergilli of tropical origin show greater frequency of toxigenic strains than those of temperate regions [11].

Sterigmatocystin was the mycotoxin most commonly isolated and was produced by *A. niger, A. versicolor* and *A. nidulans*. Uraguchi and Yamazaki [19] have also recognised the wide range of fungal species producing sterigmatocystin. *Aspergillus versicolor*, the main producer of sterigmatocystin [19] was found to have 3 out of 4 isolates capable of producing sterigmatocystin (Table 2). Such a high percentage of toxigenic strains of *A. versicolor* has also been reported by other workers [13, 7]. The capacity to produce different types of aflatoxins by toxigenic isolates of *Aspergillus flavus* also varied considerably, however, aflatoxin B1 was produced by all of the aflatoxigenic isolates, which is a usual observation [5, 20].

Production of ochratoxin A by isolates of *A. ochraceus* was low compared with the levels reported in the literature [8]. The reason for this may be attributed not only to the poor toxigenic potential of the isolates, but also to the choice of substrate. The polished rice used for production of mycotoxins in these investigations has been reported to be a poor substrates for the laboration of ochratoxin [8]. The substrate factor may also be responsible for the identification of a lower percentage of toxigenic strain of *A. ochraceus* from the tobacco leaves (Table 2).

Investigations conducted with the chewing tobacco leaves showed that Aspergilli are the dominating mycoflora in storage. The various species of *Aspergillus* show a definite pattern of ecological succession in the course of storage. An appreciable percentage of toxigenic species among various Aspergilli indicates the possibility of mycotoxin contamination of tobacco leaves and, consequently, human exposure.

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