Studies Of Heat Resistant Fungi In The Soil: *Talaromyces flavus* Isolated In Nigerian Soils

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Abstract Heat resistant fungi are a group of fungi which are able to withstand thermal processes employed in the preservation of most acid foods. A total of 85 soil samples collected from gardens/farmlands in Ohodo, Enugu-Ezike, Ogbede, Adani, Oturkpo, Umuahia/Orlu, Imilike, and Gboko were examined for the presence of heat resistant fungi using standard methods.HRF were isolated from 74 soils representing approximately 94%. Fungal counts ranged from non-detectable to 437 colony forming units per 2g of soil. *Neosartorya fischeri* was the most common isolate. Other isolates were *Talaromyces flavus, Eupenicillium cinnamopurpurem, Eupenicillium crustaceum,Emeriella rugulosa and Paecilomyces variotii/Byssochlamys fulva.* The occurrence of *T. flavus* in this study is important as it has previously not been reported in Nigerian soils. [New York Science Journal 2010;3(12):8-14]. (ISSN: 1554-0200).

Key words: Heat resistant fungi, Nigeria, *Talaromyces flavus*

Introduction

Fungi are eukaryotic organisms important to humans in both harmful and beneficial ways. They are among the most important microorganisms associated with food. For example in England, Olliver and Rendle (1934) observed instances in which canned fruit disintegrated with no gas production, no abnormal flavour and no evident colour change and found that such spoilage was due to the activity of an ascomycete which had previously been named by Olliver and Smith (1933) as *Byssochlamys fulva.*

This fungus is the cause of an important problem in fruit preservation since it was found to be capable of disintegrating processed fruit even under conditions of reduced oxygen tension. The ascospores were found to be viable after being heated at 86^{o}-88^{o}C and held at that temperature for 30min in fruit syrups (Williams, 1959). This organism probably comes into the cannery on fresh fruits since it is known to be present on fruit in fields and orchards. *Byssochlamys spp* are historically the most widely encountered mold causing spoilage of heat processed fruits and have therefore been most extensively researched. Recently however, spoilage due to *Talaromyces flavus* and *Neosartorya fischeri* has been frequently observed in North America, Europe and Australia (Beuchat, 1986). It has been reported that *Talaromyces* and *Neosartorya* ascospores can survive 100^{o}C for 5-12min (Kavanagh *et al*., 1963; McEvoy and Stuart, 1970; Van der Spuy, 1975). Besides spoilage, the heat resistant molds produce a number of toxic secondary metabolites such as byssochlamys A, byssochlamyic acid, carcinogenic patulin, the tremorgenic substances, fumiremorgin A and C, fischerin which caused fatal peritonitis in mice and eupenifeldin, a compound possessing cytotoxicity as well as in vivo antitumor activity.

In the developing countries of Africa, little is known about incidence of spoilage of processed fruits by heat resistant molds. Spoilage of heat-processed fruit juices was first reported in Nigeria in 1995 (Ugwuanyi and Obeta, 1995). There are also a few documented reports on the incidence of HRF in Nigerian soils (Okagbue, 1989; Ugwuanyi and Obeta, 1991). *Neosartorya* has been the most commonly isolated among other important HRF but there is yet no report on the occurrence of *Talaromyces. Byssochlamys* that was once reported (Ugwuanyi and Obeta, 1991) but was later renamed *Paecilomyces variotii* due to its inability to produce ascospores in Kew Gardens in London.

This work was therefore designed to

1) examine the distribution and types of heat resistant fungi in the soils of vegetable and fruit producing zones of South Eastern/Middle belt in Nigeria.
2) To check for the presence of *Talaromyces* in Nigerian soils.

Materials and Methods

Isolation of heat resistant fungi from the soils

Collection of soil samples

A total of 85 soil samples were collected between November 2000 and April 2001, using the method of Okagbue (1989). Soils were collected in sterile dry screw capped universal bottles (25ml capacity). Soils were collected from gardens, orchards and farmlands in the following towns: Ohebe- dim, Ohodo, Imilike, Nsukka, Gboko, Adani, Enugu Ezike, Umuahia and Orlu. Metallic spoons were used for the collection of soil and they were sterilized before and between collections of different samples by dipping in alcohol followed by flaming. Samples were generally analysed within 24-48 hours of collection. At each location, sampling was done at random but a distance of not less than 4m was kept between any two sampling sites. Samples were collected from soil surface and the maximum depth did not exceed 4cm.

Isolation of heat resistant fungi from soil samples.

Two grams of each soil sample was weighed out aseptically and transferred into 10ml sterile distilled water in a sterile 20ml capacity screw capped test tube, which was then mixed by shaking vigorously and by inverting it up to ten times. The content of the tube was then heated in a water bath at 70^{o}C for 1 hr to activate ascospores of any HRF that may be present and to kill mycelia and reproductive structure of non-HRF present in the soil (Splitstoesser *et al*., 1971; Okagbue, 1989). A thermometer was inserted into an identical flask to ensure that the temperature of 70^{o}C was maintained. The water level in the bath was not less than 3cm above the level of the content of the flasks. The flasks and contents were shaken intermittently to mix well.

After heating, flasks were allowed to cool to room temperature. A 2ml volume of content was aseptically pipetted into duplicate 9cm Petri dishes, followed by 18ml of molten (approx. 50^{o}C) single strength potato dextrose agar (PDA) containing chloramphenicol (40μmg / ml final concentration). Agar and sample were mixed gently in a circulatory motion and allowed to set. The plates were incubated at room temperature (approx 28^{o}C) for 3-5 days. After the incubation period, representative types of fungal colonies were counted.

Purification of isolates

Representative types of fungal colonies were subcultured on PDA plates for purification. Subculturing on PDA plates was carried out at least twice for each fungal type to ensure purity.

Confirmation of heat resistance of the isolates

Large amounts of fully developed and mature ascospores were obtained by inoculating selected isolates on PDA slants (16 x 1.6cm test tubes) and incubating for 30 days at room temperature (Splittstoesser *et al*., 1970, 1971; Splittstoesser and Splittstoesser, 1977). Ascospores in asci or cleistothecia were then dislodged by flooding each slant with 2-3ml of sterile 0.1M potassium phosphate buffer (PB), pH 6.0, followed by scrapping the culture surface with a sterile wire loop. The washing was poured into a sterile screw capped test tube. The slant was then rinsed and the rinsate pooled in the screw-capped test tube. Sterile PB was added by rinsing the sides of the tube to reach a volume of 10ml. The test tubes with their contents were heated in a water bath at 70^{o}C together with an identical tube containing 10ml of PB in which a thermometer was immersed to monitor the temperature. The tubes were heated for 1hour at 70^{o}C with the water level in the bath at least 3cm above the level of suspension in the tubes. After heating, the tubes were cooled to room temperature and 2ml volume of ascospore suspensions from each test tube was then plated out as described above with single strength PDA and incubated at room temperature (28-30^{o}C) for 3-5 days. Heating achieved inactivation of mycelia and conidia and activation of ascospores if any heat resistant ones were produced. Fungal growths, which occurred after incubation, were considered to be heat resistant fungi. The colonies were further purified on PDA plates and transferred to slide cultures for preliminary identification. Cultures were also put on slants of PDA for preservation.

Identification of isolates

Preliminary identification of isolates by slide culture technique

The preliminary colony characteristics and microscopic morphology of isolates were determined by cultivation on appropriate media and by the use of slide culture technique.

Sterile sabouraud dextrose agar medium (SDA) in a petri dish was cut into square blocks 1cm x 1cm with a sterile blade and placed on the centre of a sterile slide on a v-tube in a sterile petri dish (Haley and Callaway, 1978). Inoculum was taken from the advancing edge of growth of the isolate and inoculated onto the mid-point of each of the four edges of the agar block. With the aid of sterile forceps a sterile cover slip was placed to cover the surface of the inoculated agar block. Approximately 10ml of sterile distilled water was poured into the bottom of the petri dish to arrest dehydration of agar block and the dish was then covered. Duplicate set-up was made for each isolate. The slide culture plates were incubated at room temperature and the growth examined after 3-5 days or as soon as reproductive

(asexual) spores became visible. Two wet mounts were made for each set-up for microscopic examination at x10 and x40 magnification by placing the cover slip on a clean slide and by removing the agar block and covering the slide with a clean cover slip. The slides were stained with a drop of lactophenol blue.

After this, the isolates were grouped into the two genera *Aspergillus* and *Penicillium* and further identification was carried out by microscopic and macroscopic observations according to the keys of Pitt (1985), Onions *et al.* (1981) and Raper and Fennel (1965).

Identification of common *Penicillium* species

Plates of cpazek yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N) were used for the identification of *Penicillium spp* and their telemorphs according to the methods and keys of Pitt (1985). Each plate was inoculated at three points equidistant from the edge of the plate and each other. For each culture, five standard sized petri dishes (100mm) were used; three of CYA and one of each of the other media. Cultures on MEA, G25N and CYA were incubated for 7 days at 25^{o} and CYA also at 5^{o} and 37^{o}C. Incubation below room temperature was carried out in a cold incubator (Gallenkamp). At the end of the incubation period, macroscopic characteristics including colony diameters, mycelia colours, exudate, colony reverse or soluble pigment, colony depth, texture and sulcation were observed and recorded. Microscopic characteristics were observed at x10 and x40 magnifications on wet mounts. If cleistothecia (or sclerotia) were present, keys to identification of *Eupenicillium* were further used, if gymnothecia were present, keys to *Talaromyces were* used, when none of these were observed, keys to *Penicillium* subgenera were followed (Pitt, 1985). Cultures identified as *Paecilomyces* were incubated at 30^{o}C for 12 days as some cultures of *Byssochlamys* do not produce asci at 25^{o} or 37^{o}C except at 30^{o}C (Beuchat and Rice, 1979).

Identification of common *Aspergillus* species and their telemorphs

The identification system was based on growth and cultural characteristics on three media (CYA, MEA and czapek yeast extract agar with 20% sucrose (CY20S) at two temperatures (Klich and Pitt, 1988). For each culture, four standard sized petri dishes (100mm) were used; two of CYA and one of each the other media. Each plate was inoculated at same points as described in section above. One CYA plate of each culture was incubated at 37^{o}C and all of the others incubated at 25^{o}C in the dark. All plates were observed after 7 days incubation. After initial observation, isolates of telemorphic genera required a continued second incubation of 7days or more for completion of ascospore development. At the end of incubation period, macroscopic characteristics including colony diameters, mycelia colours, exudate, colony reverse or soluble pigment, colony depth, texture and sulcation were observed and recorded. Microscopic characteristics were also observed at x10 and x40 magnifications on wet mounts.

Identification was also aided by comparing the isolates with reference samples of *Neosartorya fischeri var spinosa* (IMI 333692*), Aspergillus flavus* (IMI 333693), *Aspergillus flavus* (IMI 333694), *Neosartorya fischeri* (IMI 333695) *Paecilomyces variotii* (IMI 333697), *Penicillium citrinum* (IMI 333698), *Neosartorya fischeri* (IMI 333699), *Neosartorya fischeri var spinosa* (IMI 333700), *Neosartorya fischeri* (IMI 333701), *Botryodiplodica theobromae* (IMI 3336966), *Neosartorya quadrcincta* (IMI 312830), *Neosartorya fischeri* (IMI 312820). These samples were supplied by Dr. J. O. Ugwuanyi of Microbiology Department, University of Nigeria Nsukka.

Results and Discussion

On Table 1 is shown the occurrence and distribution of HRF in soil samples from different sites as well as the predominant species in each site. In general the incidence of HRF was high. Out of a total of 85 soil samples examined in this study 78 representing approximately 94% bore HRF.

On Table 2 is shown the rate of occurrence of HRF propagules per 2g of soil. The number of propagule-forming units ranged from non-detectable to 437. *Neosartorya spp* were isolated most frequently and were present in most soil samples examined. The next most frequently occurring fungi were *Eupenicillium spp*, followed by *Talaromyces spp*, *Paecilomyces sp* while *Emericella sp* occurred least frequently.

Colonial and microscopic characteristics of *Aspergillus spp*

Table 3 shows the colonial and microscopic description of those isolates described as *Aspergillus*.

Most of the fungal colonies, which developed on PDA, appeared to be granular white to milk coloured. Some of them formed yellow pigments, which readily changed or disappeared entirely on subculturing. Microscopic examination showed that the granules were cleistothecia and the isolates were later identified as *Neosartorya fischeri* (Wehmer)

Malloch and Cain. The less frequently occurring member of the genus was identified as *Emericella rugulosa* (Thom and Rapper) C. R. Benjamin.

Table 1: Heat resistant fungi (HRF) in soils samples from South Eastern / Middle Belt of Nigeria

Site	Fraction of soil bearing HRF	Predorminant specis
Enugu-Ezike	6/6	N, Tal, Em
Ogbede	10/10	N,Tal, Eu,P
Ohodo	9/10	Tal, Eu, P
Imilike	9/10	N, Eu
Nsukka	10/10	N, Eu, Em
Gboko	8/9	N, Eu
Adani	10/10	N, Tal
0turkpo	7/10	N, Eu
Umuahia and Orlu	10/10	N, Eu

Tal = *Talaromyces;* N = *Neosartorya;* P = *Paecilomyces;* Eu =*Eupenicillium;* Em = *Emericella*

Colonial and microscopic characteristics of *Penicillium spp*

Table 4 shows the colonial and microscopic description of those isolates described as *Penicillium*.

Out of a total of 5 representative isolates identified to be *Eupenicillium spp*, 3 were later identified as *Eupenicillium cinnamopurpurem* Scott and Stolk while the rest were identified as *Eupenicillium crustaceum*. All the representative isolates of fungal colonies characterized by the presence of abundant yellow gymnothecia were identified as *Talaromyces flavus* (Wôcker) Stolk and Samson. The remaining isolates were identified as *Paecilomyces variotii* Bainer.

The incidence of HRF in the soils of Nigeria has been previously reported (Ugwuanyi and Obeta, 1991; Okagbue, 1989). In this work, the predominant organisms were *Neosartoya spp* that were isolated in all positive soil samples. This finding agrees with those reported in the Southern and Northern soils earlier mentioned. *Paecilomyces* for the second time was reported in the tropical soils of Nigeria. The occurrence of *T. flavus* in this study is important as it has previously not been reported in Nigerian soils. One of the isolates, *Eupenicillium*, has already been described as an important spoilage agent of fruit juices in Australia (Hocking and Pitt, 1984) and in South Africa (Anon, 1967). Considering the fact that *Neosartorya*, *Eupenicillium* and perhaps other heat resistant fungi not only cause spoilage of fruit products but also produce highly toxic and sometimes carcinogenic compounds; these findings are of utmost importance to fruit processing industries which are presently increasing in number in Nigeria and other developing countries.

Table 2: Rate of occurrence of propagules of HRF per 2g of soil

	Site								
Soil sample	**Enugu ezike**	**Ogbede**	**Ohodo**	**Imilike**	**Nsukka**	**Gboko**	**Adani**	**0turkpo**	**Umuahia/Orlu**
1	Tal = 1, N=7	N=35, T=25	N=4	-	N=7	N=35, Eu=18	N=129	N=4, Eu=3	N=70
2	N=4	N=4,Tal=4, Eu=4	N=4	Eu=7	N=4, Eu=7	Eu=39, N=11	N=10	Eu=4	N=46
3	N=4	N=4, Tal=7,Eu=4	N=7	N=1, Eu=4	N=51, Em=10	N=10, Eu=25	N=12	-	N=14
4	N=7, Tal=10	N=119, P=14	-	N=7	N=10	N=77, Tal=35	N=136,Tal=35		
5	Tal=10	N=126	N=4, P=4	N=14	N=196, Eu=10	N=46	N=7, Tal=10	-	N=32
6	N=4, Em=7	N=144	N=4, Eu=7	Eu=4, N=14	N=186	N=432,Eu=105	N=35, Tal=10	N=4	N=46
7		N=105,Tal=84	N=7	N=35, Eu=14	N=4, Eu=4	N=300,Eu=227	N=354	-	N=10
8		N=51,P=105	N=28	N=18	N=437, Eu=70	N=4, Eu=4	Tal=7, N=27	N=4	
9		N=81, Tal=7	N=4	N=14, Eu=4	N=5	N=25	N=3,Tal=32	N=4	
10		N=16		N=4	N=18		N=10		N=66,Eu=46

Table 3: Colonial and Microscopic Morphology and Identification of *Aspergillus spp (*media: CYA, MEA, CY205) Incubation for 7 days: CYA, MEA and CY20S at 25^{o} and CYA also at 37^{o}C

Isolate	Incubation temperature	Cultural characteristics on MEA	Cultural characteristics on CYA	Cultural characteristic on CY20S	Microscopic Morphology	Probable Identification
3c	25^{o}C	Colony diameter 70mm+, conidial sporulation sparse, mycelium white to cream, cleistothecia light cream; reverse dull yellow .	Colony diameter 70mm+ mycelium white to cream coloured cleistothecia, exudate clear texture velutinous, conidia sparse and grey green	Colony diameter 70 mm+, velutinous in texture, coloured as on CYA	Ascospores with two longtitudinal thin flanges, Aspergilla uniseriate, Ascospores are spherical, conidial heads spherical to ellipsoidal	*Neosartorya fischeri*
	37^{o}C		Colony diameter 70mm+, texture as described above			
2k	25^{o}C	Colony diameter 12-14mm conidia usually dark green, cleistothecia which are pale at first and become dull red with age, colony with an irregular margin	Colony diameter 10-15mm, conidial dull greyish green, mycelium white with dull yellow hulle cells embedded in the mycelium exudate reddish reverse orange brownsh, radially sulcate	Colony diameter 12mm, conidial colours similar to those on CYA except reverse brown	Conidial head radiate on CYA, columnar on MEA. Aspergilla biseriate metulae covering two third of vesicle, conidial globose, Ascopores walnut shape with two longitudinal crests	*Emericella rugulosa*
	37^{o}C		Colony diameter 54mm, texture as described above			
E7		Similar to 3C			*Neosartorya fischeri*	
4B		"			"	
4A		"			"	
5B		"			"	

Table 4: Colonial and microscopic morphology and identification of *Penicillium spp* (media:CYA,MEA, G25N) incubation for 7 days CYA, MEA and G25N at 25^{o}C and CYA also at 5^{o} and 37^{o}C

Isolate	Incubation temperature	Cultural characteristics on MEA	Cultural characteristics on CYA	Cultural characteristics on G25N	Microscopic morphology	Probable identification
9A	25^{o} C	Colonies 13-15mm, diameter, central area of brown cleistothecia surrounded by white mycelium	Colonies 20mm in diameter of closely textured white to brown mycelium enveloping cleistothecia, clear exudated and purple soluble pigment typically produced, reverse purple	Colonies 9mm diameter of dense white mycelium, conidial production moderate, reverse purple	Ascospores ellipsiodal with two longitudinal flanges, conidiophores monoverticillate, phialides gradually tapering, conidia subspherodial to ellipsoidal	*Eupenicillium cinnamopurpurem*
	5^{o}C		No germination			
	37^{o}C	Colonies 5-6mm diameter of white mycelium				
1A	25^{o}C	Colonies on MEA 40mm, centrally radially sulcate velutious or lightly floccose mycelium white at the margins, clear exudate and yellow soluble pigment produced, reverse pale brown	Colonies 40mm, radially sulcate, dense with a floccose overlay mycelium white at the margins, cleistothecia abundantly produced, clear exudates, reverse pale brown	Colonies 20mm, diameter, radially sulcate, floccose mycelium, white cleistothecia produced, reverse pale brown	Ascospores broadly ellipsoidal ornamented with two longitudinal flanges, stripes bearing terminal biverticillate penicillium, phialides tapering abruptly. Conidial spherical to ellipsoidal	*Eupenicillium crustaceum*
	5^{o}C		Colonies 3-5mm, diameter formed of white mycelium			
	37^{o}C		no growth			

2E	25°C	Colonies 45mm diam, moderately floccose, mycelium bright yellow concealing developing gymothecia, reverse reddish	Colonies 22mm, diameter, similar to those on MEA	Colonies 3mm diameter (micro colonies)	Ascopores ellipsoidal stripes bearing terminal biverticillate or less commonly monoverticillate penicillium. Conidia ellipsoidal	*Talaromyces flavus*
	5°C		No germination			
	37°C		Colonies 23mm similar to those at 25° but with white mycelium			
4G	25°C	Colonies grow well on all substrates (60mm); dull brown or yellowish brown, loosely floccose and mostly ropy mycelium that drops down and flatten with age, underside brownish pigments, colonies become patchy and granular with age, production of abundant macrospores	Phialides are flask shaped or tapering gradually to the apices, big asci containing eight prominent ascospores Asci nearly spherical	*Paecilomyces variotii*		
	5°		Microcolonies were formed			
	37°		no growth			
5A	Similar to 2E					*Talaromyces flavus*
4X	"					"
3B	"					"
X	"					"
9B	Similar to 9A					*Eupenicillium cinnamopurp-urem*
2A	"					
1B	Similar to 1A					*Eupenicillium crustaceum*
2B	Similar to 4G					*Paecilomyces variotii*

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