Mycopathologia 82, 153–158 (1983).

Studies on thermophilic fungi associated with the spoilage of flue-cured tobacco leaves during storage

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Abstract

The ability of the thermophilic fungi *Humicola insolens* Cooney and Emerson, *Mucor pusillus* Lindt and *Thermoascus aurantiacus* Miehe which were obtained from stored tobacco leaves in Nigeria to cause biochemical changes in this product *in vitro* and studies of their nutritional physiology were determined. All the fungi were able to cause considerable losses in the various components of the blended leaves with consequent losses in dry weight on incubation at 45 °C for 14 days. Only *Humicola insolens* attacked the cellulose fraction while none of them was able to degrade the lignin component. The highest losses were recorded for *H. insolens* (29.18% of original dry weight) and *Thermoascus aurantiacus* (21.5% of original dry weight). When grown on various sources of carbon and nitrogen, the best results were obtained respectively on starch-dextrin and on casein hydrolysate. A C/N ratio range of 10.0 –15.0:5 –7.5 g per liter supported the best growth of these fungi. Because of the zoopathogenic nature of *Mucor pusillus* and *Thermoascus aurantiacus*, proper handling of cured tobacco leaves before and after processing becomes mandatory.

Introduction

The industrial processing of the dried leaves of the tobacco plant, *Nicotiana tabacum* Linn., involves some periods of storage after curing. Because of the high relative humidities obtaining in the tropics and the hygroscopic nature of dry tobacco leaves, the cured leaves are usually stored in heated barns. This keeps the moisture levels at 12% or below to prevent 'moulding'. In a previous survey however (11), moisture levels of between 36–42% were consistently recorded from the various samples of cured tobacco leaves analysed. Consequently, 'moulding' of the cured leaves was widespread. When properly handled, fermentation of cured tobacco leaves is due largely to bacterial activity rather than mould infestation. A search through the available literature shows that similar problems have been documented from other parts of the world (4, 12, 13, 16, 18, 19).

The presence of moulds on tobacco products also poses great health risks to the consumers for some of these moulds produce potent mycotoxins. In an earlier study (11), such moulds were consistently encountered on tobacco products in Nigeria. This report is on their ability to degrade blended tobacco leaves and studies of their nutritional physiology.

Materials and methods

Degradation of blended tobacco leaves and biochemical studies

The fungi used were *Mucor pusillus* (IMI 196724), *Humicola insolens* (IMI 196654), and *Thermoascus aurantiacus* (IMI 196731). The methods of their isolation and identification have been previously published (11).

Flue-cured tobacco leaves were shredded in a waring blendor, oven-dried (80 °C for 24 h) and 15 g portions placed in each of 250 ml conical

flasks. A basal medium containing $MgSO_4 \cdot 7H_2O$, 0.75 g; $NaNO_3$, 3.5 g; K_2HPO_4, 1.75 g; distilled water, 1000 ml was prepared and 30 ml portions added to each of the flasks. All salts are 'Analar' grade, BDH chemicals. The flasks were sterilized by autoclaving at 121 °C for 15 min. The inoculum was prepared by growing the fungi on the basal medium (supplemented with agar, 15 g; D-glucose, 5.0 g) for 3 days at 45 °C after which a 5 mm diam cork borer was flamed and used to cut agar and young mycelia, 5 mm diam. Each of the flasks was then inoculated with one disc before incubation as still cultures at 45 °C. At 7-day intervals, the contents of each flask were removed, oven-dried and weighed. They were then milled through a 0.55 mm sieve and a biochemical analysis of the powder obtained carried out to determine mould-induced changes in the ethanol soluble fractions, hemicellulose, cellulose, lignin and the nitrogen components. The ethanol soluble fraction was determined by continuous extraction in a Soxhlet apparatus until sugar free on testing with anthrone reagent. The diastase soluble fraction was determined by the enzymic hydrolysis of the ethanol-extracted material (17). Hemicellulose was assayed by extracting the diastase extracted product with 24% KOH for 4 h. Cellulose was assayed by hydrolysis of the alkali-extracted material with 72 % sulphuric acid for 2 h, followed by refluxing for 3 h on dilution of the acid to 5% (7). The residual fraction after acid hydrolysis was taken to be lignin.

Soluble nitrogen (ammonium and nitrate) was determined by the Conway process (1) while total nitrogen was by the micro-Kjeldahl method with modification to include the nitrate value (6). The values obtained were related to the original dry weight (%) before inoculation. Values obtained for day 0, also served as control. The results are means of three replicates with standard deviations.

Carbon source requirements for growth

The method described by Garrett (5) was used to determine the ability of these fungi to degrade native cellulose. Wads of filter papers (Whatman no. 3, 5.5 cm diam) were oven-dried and weighed. They were placed in 250 ml conical flasks and 30 ml portions of the basal medium added to each flask before autoclaving at 121 °C for 15 min. Each flask was inoculated with the test fungi as previously described and incubated as still cultures for 21 days at 45 °C.

At 7-day intervals, the filter papers were carefully removed, oven-dried and weighed. Loss in weight was taken to be due to the activities of the organisms. The control flasks were inoculated with blank agar discs. The ability of the fungi to utilize modified forms of cellulose was determined by growth on carboxymethyl cellulose (CMC).
A 1% (W/vol) solution of this was made with the basal medium and the fungi grown on 30 ml portions for 14 days at 45 °C. At the end of the incubation period, the mycelia produced were suction-filtered, oven-dried and weighed. The control flasks lacked the carbon source.

Utilization of hemicellulose, starch, dextrin and simple sugars by the test fungi was also determined. Five percent xylan (a hemicellulose) was weighed out and made to a liter with the basal medium. The fungi were grown on 30 ml portions of this in each of 250 ml conical flasks for 14 days at 45 °C after which the mycelia produced were harvested, oven-dried and weighed.

To determine the ability of the fungi to utilize other forms of carbon, the appropriate weights of each of the simple sugars lactose, glucose, maltose, fructose and the carbon source glycerol to yield 0.8 g C per liter of the basal medium was made and dispensed into 250 ml conical flasks, 30 ml per flask. For dextrin and starch, a 2% (w/vol) solution was used. All the flasks (3 for each carbon source) were inoculated with the test fungi and incubated as still cultures at 45 °C for 5 days. The mycelia produced were then harvested, oven-dried and weighed.

Utilization of nitrogen sources and the effects of C/N ration on growth

The basal medium was supplemented with D-glucose, 20 g per liter and the appropriate weights of each of L-alanine, sodium nitrate, ammonium sulphate, L-glutamic acid, L-asparagine, L-leucine + isoleucine, glycine, DL-serine and DL-tryptophan to yield 0.485 g N per liter. For caseine hydrolysate, 3.5 g per liter was used. The various media were dispensed into each of 250 ml conical flasks, sterilized by autoclaving and inoculated with the test fungi. All the flasks were incubated as still cultures at 45 °C for 5 days after which the mycelia

produced were harvested, oven-dried and weighed. The control flasks lacked the nitrogen sources.

The test fungi were also grown on different concentrations of glucose (2.5–15 g/l) and sodium nitrate (1.5–10.5 g/l) to determine their effects on mycelia production at 45 °C and 5 days of incubation. The results are means of three readings.

Results and discussion

All the fungi used in this study were able to grow on the blended tobacco leaves at 45 °C leading to considerable losses in their various components and consequently in their dry weights (Table 1).

These results show that *Humicola insolens* caused the highest losses. This was due to the rapid depletion of the cellulose and hemicellulose fractions of the tobacco leaves. By the end of the 14-day incubation period, losses of up to 11.39% were recorded for cellulose and 11.29% for the hemicellulose fraction. This was accompanied by a general rise in the total nitrogen content of the substrate probably due to protein synthesis with period of growth. Like the other fungi however, *Humicola insolens* was unable to degrade the lignin component of the leaves. These results are really not surprising for *H. insolens* is a known cellulose decomposer and has been variously implicated in the degradation and consequent spoilage of stored agricultural products (2, 15). In a previous work (10), the production and activities of the extracellular cellulases of this fungus were examined. The cellulases were found to be produced within 24 h of incubation on a cellulose medium at 45 °C with peak activity occurring within the 10-day incubation period.

From the results on Table 1, *Mucor pusillus* and *Thermoascus aurantiacus* were apparently unable

Table 1. Mould-induced changes in blended tobacco leaves on incubation at 45 °C. Figures (% original dry weight) are means of 3 readings with standard deviation.

Species	Fractions	Days of incubation			Total loss
		0	7	14	
Mucor pusillus	Cellulose	43.99 ± 0.22	43.93 ± 0.41	43.99 ± 0.48	
	Hemicellulose	35.79 ± 0.14	35.68 ± 0.24	36.68 ± 0.26	
	Lignin	9.59 ± 0.16	9.53 ± 0.16	9.59 ± 0.14	
	Diastase soluble	6.96 ± 0.24	2.19 ± 0.21	1.99 ± 0.06	
	Ethanol soluble	2.06 ± 0.15	0.79 ± 0.03	0.19 ± 0.01	
	Total N	0.83 ± 0.03	1.59 ± 0.05	0.42 ± 0.11	
	Ammonium N	0.76 ± 0.04	0.13 ± 0.002	0.55 ± 0.01	
	Total	99.98	93.84	93.56	6.45
Thermoascus aurantiacus	Cellulose	43.99 ± 0.22	43.96 ± 0.23	42.98 ± 0.21	
	Hemicellulose	35.79 ± 0.14	26.09 ± 0.18	23.2 ± 0.15	
	Lignin	9.59 ± 0.16	9.56 ± 0.04	9.58 ± 0.03	
	Diastase soluble	6.96 ± 0.24	2.48 ± 0.02	0.26 ± 0.01	
	Ethanol soluble	2.06 ± 0.15	0.32 ± 0.05	0.25 ± 0.02	
	Total N	0.83 ± 0.03	1.66 ± 0.01	1.64 ± 0.011	
	Ammonium N	0.76 ± 0.04	0.45 ± 0.003	0.48 ± 0.02	
	Total	99.98	84.52	78.39	21.59
Humicola insolens	Cellulose	43.99 ± 0.22	36.4 ± 0.24	32.6 ± 0.4	
	Hemicellulose	35.79 ± 0.14	26.2 ± 0.06	21.5 ± 0.18	
	Lignin	9.59 ± 0.16	9.58 ± 0.01	9.59 ± 0.14	
	Diastase soluble	6.96 ± 0.24	5.44 ± 0.05	4.49 ± 0.06	
	Ethanol soluble	2.06 ± 0.15	1.02 ± 0.04	0.98 ± 0.02	
	Total N	0.83 ± 0.03	0.80 ± 0.002	0.89 ± 0.03	
	Ammonium N	0.76 ± 0.04	0.72 ± 0.06	0.75 ± 0.11	
	Total	99.98	80.16	70.8	29.18

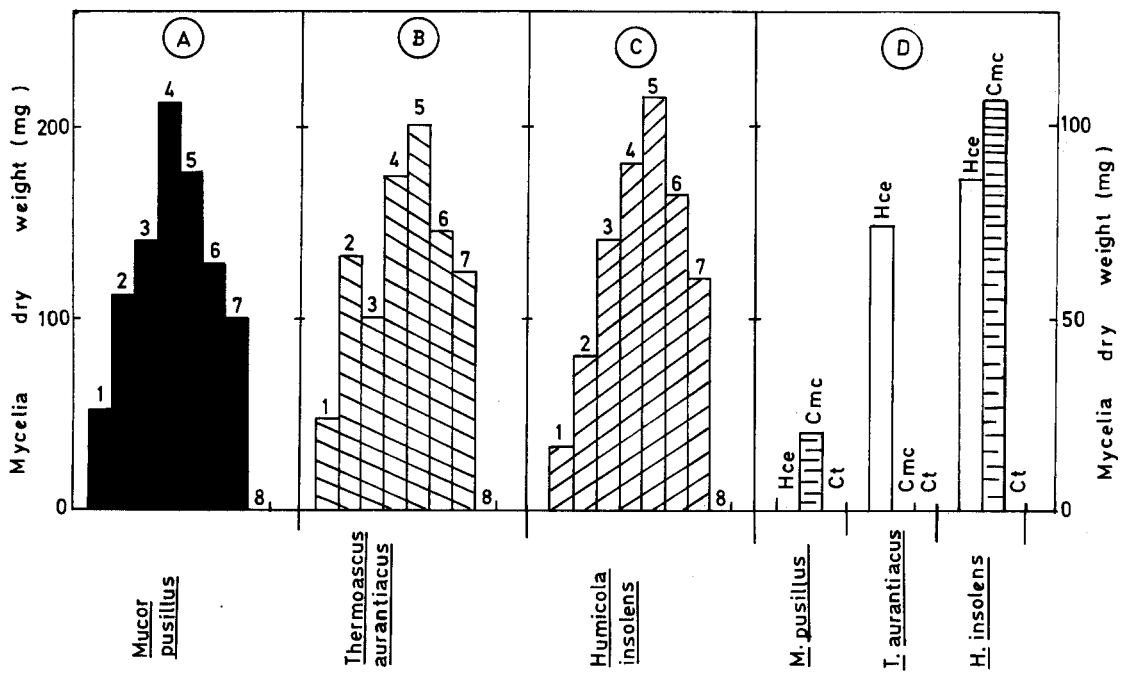

Fig. 1. (A–C), degradation of starch, dextrin and simple sugars (1 = lactose, 2 = glycerol, 3 = maltose, 4 = dextrin, 5 = starch, 6 = glucose, 7 = fructose, 8 = control) and (D), hemicellulose (Hce), carboxymethyl cellulose (Cmc). Ct = control.

to degree the cellulose component of the tobacco leaves. Whereas *T. aurantiacus* caused considerable losses (up to 12.6%) in the hemicellulose fractions at the end of the incubation period, no such results were obtained for *Mucor pusillus* which was unable to degrade the hemicellulose components. These results were confirmed when the fungi were grown on pure forms of cellulose and on hemicellulose (Fig. 1). While both *Thermoascus aurantiacus* and *Humicola insolens* had good growth on the hemicellulose (xylan), only *Mucor pusillus* showed some activity on CMC. When all the fungi were grown on filter papers however, only *H. insolens* was able to degrade the filter paper with losses of up to 112 mg/21 g of the papers in 14 days of incubation at 45 °C. The ability of some fungi to degrade modified forms of cellulose such as CMC while they fail to hydrolyse native cellulose such as filter papers has been the subject of investigation and controversy in the past (3, 8, 14). The current thinking as regards cellulose hydrolysis by microfungi is that degradation depends on the ability of the organism to produce two types of cellulases designated C_1 (exo-β-1,4,glucanases) and C_x (endo-β-1,4,glucanases). Whereas both the C_1 and C_x enzymes are needed to degrade native cellulose, only the C_x cellulase is required to hydrolyse modified cellulose such as CMC. Consequently, the ability of the isolate of *Mucor pusillus* used here to hydrolyse CMC and not native cellulose shows that it can produce only the C_x enzyme. This observation is in agreement with that of Somkuti *et al.* (8) working with *Mucor pusillus.*

When the fungi were grown on sources of carbon other than cellulose and hemicellulose (Fig. 1), all degraded to varying degrees the carbon sources supplied. Their best activity was recorded on the polysaccachride dextrin and on starch. This is understandable for on hydrolysis, these carbon sources yield simpler forms which themselves are good substrates for microbial growth. For all the fungi, no growth occurred in the control flasks.

When the test fungi were grown on both organic and inorganic nitrogen sources (Table 2), all had the best mycelial growth (>200 mg) on casein hydrolysate and on sodium nitrate. This result shows that the carbon in the molecules of the organic nitrogen sources did not contribute much to mycelial growth. The good activity recorded on casein hydrolysate is to be expected for this nitrogen

Table 2. Utilization of organic and inorganic nitrogen sources for growth. Cultures were incubated at 45 °C for 5 days.

Species	Mycelia dry weight, mg/30 ml medium		
	>200 mg	100–200 mg	<100 mg
Thermoascus aurantiacus	Caseine hydrolysate L-alanine Sodium nitrate	Ammonium sulphate L-glutamic acid L-asparagine L-leucine + isoleucine Glycine, DL-serine	DL-tryptophan
Mucor pusillus	L-asparagine Caseine hydrolysate L-glutamic acid Sodium nitrate	L-alanine Ammonium sulphate Glycine, DL-serine L-leucine + isoleucine	DL-tryptophan
Humicola insolens	Caseine hydrolysate Sodium nitrate Glycine DL-serine	Ammonium sulphate L-alanine L-asparagine L-glutamic acid L-leucine + isoleucine	DL-tryptophan

source is known to contain most of the common amino acids (9). Appreciable growth also occurred on the other forms of nitrogen sources except for DL-tryptophan which did not support their good growth. When the effects of the C:N ratio on the growth of these fungi were determined (Table 3), the best activities were recorded for *Humicola insolens* at a C:N ratio of 10.0:5.0 g per liter while for *Mucor pusillus*, 10.0:7.5 g per liter was best. With *Thermoascus aurantiacus* however, a C:N ratio of 15.0:5.0 g per liter supported the best mycelia production.

A glance through the available literature on microbial deterioration of stored tobacco shows that most of these reports deal with the isolations and descriptions of the associated organisms with little attention devoted to the specific roles these fungi play in the spoilage proper. The results obtained in this study and in earlier reports (10, 11) show conclusively that these organisms in association with other microbes are responsible for the deterioration of cured tobacco leaves during storage.

Table 3. Effects of C:N ratio on growth. Cultures were incubated at 45 °C for 5 days. Figures (mycelia dry weight, mg) are means of 3 readings with standard deviations.

Species	Glucose (g/l)	Sodium nitrate concentrations (g/l)				
		1.5	2.5	5.0	7.5	10.5
Humicola insolens	2.5	71.4 ± 0.62	87 ± 1.04	104 ± 0.65	76.4 ± 0.14	72 ± 0.46
	5.0	87 ± 0.51	99 ± 0.96	116 ± 0.49	98.2 ± 0.42	96 ± 0.39
	10.0	140 ± 0.48	143 ± 0.48	162 ± 0.16	155 ± 0.16	106 ± 0.62
	15.0	110 ± 0.16	113 ± 0.56	129 ± 2.01	102 ± 0.28	86 ± 0.56
Mucor pusillus	2.5	83 ± 0.36	96 ± 0.64	116 ± 1.13	188 ± 0.36	127 ± 0.19
	5.0	94.2 ± 1.2	152 ± 1.02	184 ± 1.04	221 ± 0.49	142 ± 1.02
	10.0	118 ± 0.98	184 ± 0.26	235 ± 0.25	246 ± 1.06	228 ± 0.6
	15.0	113 ± 1.1	182 ± 0.44	196 ± 0.48	226 ± 1.08	186 ± 0.99
Thermoascus aurantiacus	2.5	64 ± 0.13	70 ± 0.8	95.4 ± 0.42	78 ± 0.4	66 ± 0.23
	5.0	81 ± 0.02	96 ± 0.64	102 ± 0.46	91 ± 0.5	70 ± 1.02
	10.0	94 ± 0.16	114 ± 0.38	132 ± 0.52	102 ± 0.52	84 ± 0.32
	15.0	116 ± 0.21	128 ± 1.02	141 ± 0.64	106 ± 0.59	96 ± 0.54

Acknowledgements

This study was supported in part by a grant No. 2/SRG. 2/34 from the senate research funds of the University of Ibadan for which I am grateful. I am also indebted to Dr S. O. Osunlaja of the National Cereals Research Institute, Ibadan for technical assistance.

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