Biological Sciences Section

Pak. j.sci. ind. res., vol. 35, no. 9, September 1992

STUDIES ON *ASPERGILLUS* AND *CLADOSPORIUM* SPECIES INFECTING STORED CEREALS

S.O. FAPOHUNDA

Department of Biology, Adeyemi College of Education, Ondo, Nigeria

(Received July 7, 1991; revised September 3, 1992)

Some aspects of the cultural behaviour of *Aspergillus terreus* and *Cladosporium cladosporioides* were studied. Glucose (4 and 8%) and potassium nitrate (0.05 and 1.0%) were best suited for germ tube elongation. In an agar plate assay, secretion of pectate lyase and RNase was much greater than other polysaccharases. NaCl was deterimental to growth and sporulation of *A .terreus and C. cladosporioides*; both the species exhibited better growth in a mineral salts medium (MSM) over an Aspergillus medium (AM).

Key words: *Aspergillus terrues, Cladosporium cladosporioides*, Cereals enzyme.

Introduction

The presence of fungi as contaminants of cereal grains is adequately reported [1-4]. *Aspergillus terreus* and *Cladosporium cladosporioides* are known to elaborate certain metabolites that aid biodeterioration. However, more information is required about the growth, substrate utilization and elaboration of extracellular enzymes by these microfungi so that better control measures can be developed. It would be recalled that *A. terreus* has been reported as toxigenic [3] in contrast to an earlier result [5].

The aim of this work was to investigate the environmental factors that promote the synthesis of some hydrolysing and degrading enzymes and to determine the cultural conditions that inhabit fungal growth with the aim of controlling them on stored cereal grains.

Materials and Methods

The strains of *Aspergillus terreus* IMI 301001 and *Cladosporium cladosporioides* IMI 301004 were originally isolated from *Zea mays* L. grains in store and stock cultures were maintained on malt extract agar medium (pH 4.5).

Induction of conidiation. A modified method of Zuber and Turian [6] was employed. 10 ml malt extract medium contained in 50 ml Erlenmeyer flasks were inoculated with 0.5 ml of a conidial suspension (10^5 conidia/ml) and incubated on a reciprocating shaker (200 rpm) at 25° for 8hr. Germinated conidia were rapidly washed by centrifugation in the basal medium containing per litre of distilled water. KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.25g; NaCl, 0.15g; $CaCl_2$, 0.1g; $FeCl_3.6H_2O$, 5 mg; biotin 4 µg; trace elements i.e. 1 ml of a solution containing per 100 ml: H_3PO_3, 6 mg; $CuSO_4.5H_2O$, 40 mg; $MnSO_4.H_2O$, 5mg; $(NH_4)_6(MO_7)_{24}.4H_2O$, 3.5mg; $ZnCl_2$, 400 mg. Desired concentrations of carbon and nitrogen sources were added and the flasks incubated, on the shaker under continuous light. Biomass was removed by pre-dried and pre-weighed Whatman No. 1 filter paper. Germ tube elongation and percentage range of microcycle conidiation was determined as described earlier [6]. Microscopic observations were made 5 hr. after the experiment.

Determination of extracellular enzyme profile. Xylanase, amylase, DNase, RNase, Pectate lyase and lipase were determined for *A.terreus* and *C. cladosporioides* the fungi by the method of Hankin and Anagnostakis [7].

Determination of minimum inhabitory concentration (MIC) of sodium chloride. The basal medium contained D-glucose, 5.0g; $MgSO_4$, 0.75g; KNO_3, 3.5g; KH_2PO_4; 1.75g; distilled water 1 litre (pH 5.9). Inoculation was by means of 3mm agar discs of mycelium, and growth and sporulation were measured after 5 days of inoculation at 30°. The sodium chloride concentrations (%) were: 0,2, 5,10,15 (w/v). Mycelial weight was determined on oven-dried (80°) and weighed sintered crucible.

Degradation of hemicellulose was observed on a 2% (w/v) birch xylan per litre of basal medium.

Effect of cyclic 3'5'-adenosine monophosphate (AMP) on mycelial growth. Growth was determined on two media, mineral salts 329 and Aspergillus 627 [9] containing 0.5% glycerol as carbon source; AMP was added at a final concentration of 1.6g/litre [10]. Growth was monitored for 7 days at 30° control cultures had neither AMP nor 0.5% glycerol.

Results and Discussion

Glucose, (48%) and potassium nitrate (0.05, 1%) as carbon and nitrogen sources respectively were effective in the induction of conidiation in the two fungi, *A. terreus* and *C. cladosporioides*. The percentage range of microcycle conidiation also fell in these ranges. However, at glucose concentration higher than 10, vegetative elongation results in *C. cladosporioides* which is in contrast with the observations earlier obtained in *Trichoderma* [6] (Table 1). It has been suggested that a regulatory mechanism operates that coordi-

TABLE 1. INDUCTION OF CONIDIATIONS TO *ASPERGILLUS TERREUS* AND *CLADOSPORIOIDES* IN A BASAL MEDIUM CONTAINING VARIED CONCENTRATION OF GLUCOSE AND POTASSIUM NITRATE.

Glucose (% w/v)	KNO (% w/v)	Germ tube elongation *A. terreus*	Germ tube elongation *C. cladosporioides*	% microcycle conidiation *A.terreus*	% microcycle conidiation *C. cladosporioides*
Control					
0	0.0	0	0	0	0
	0.05	0	0	0	0
	0.1	0	0	0	0
0.5	0.0	0	0	0	0
	0.05	+	++	5-10	5-15
	0.1	+	++	10-20	10-20
1	0.0	++	+	0	0
	0.05	++	+	5	5
	0.1	++	+	5-20	5-20
2	0.0	++	+	10-25	10-15
	0.05	++	+	0	10-15
	0.1	+++	++	0	10-25
4	0.0	++	++	10-30	5-15
	0.05	++	++++	95	0
	0.1	+++	+++	20	15-20
8	0.0	+++	+	10-25	25-35
	0.05	++	+++	90	0
	0.1	+++	+++	5	20
10	0.0	+	+++	5	30-40
	0.05	++	+++	0	15-20
	0.1	+	+	0	15-20

Control: 15th after transfers

0= no elongation; + short i.e. less than 10 times the germinated conidium diameter; ++ intermediate i.e. 10-20 times, the germinated conidium diameter; +++ long i.e. more than 20 times the germinated conidium diameter.

TABLE 2. DETERMINATION OF EXTRACELLUAR ENZYME PROFILE IN AGAR MEDIUM.

Enzyme	Substrate in agar medium	Assay reagent	Species	Activity
Xylanase	25% xylan in YNB agar pH 6.5***	96% (v/v)* ethanol	*A. terreus*	7.8±0.10
			C. cladosporioides	5.1±0.08
Amylases	0.2%(w/v) starch in NA medium pH 6.0	Iodine solution*	*A. terreus*	7.4±0.15
			C. cladosporioides	6.0±0.05
DNase	Oxoid	In HCl*	*A. terreus*	15.6±0.03
			C. cladosporioides	15.0±0.05
RNase	RNase	In HCl*	*A. terreus*	10.8±0.06
			C. cladosporioides	14.4±0.16
Pectate lyase	2% apple pectin in NA pH 7.0	1% (w/v) hexadecyltrimethyl ammonium bromide*	*A. terreus*	14.0±0.18
			C. cladosporioides	17.7±0.36
Lipases	Glyceryltributyrate in PDA pH 6.2	-	*A. terreus*	6.6±0.08
			C. cladosporioides	6.1±0.12

* Flood plates with reagent., ** Diameter (cm) of cleared zone., Result are means of 3 reading and incubation was at 30° for 4 days.

nates glucose utilisation, fungi metabolism and growth rate of fungi [6].

As qualitatively observed, all the enzymes assayed for were produced in sufficient quantities (Table 2), after 4 days the production and activity of pectate lyase and RNase were highly significant in the organisms. The high production rate of exocellular enzymes explains the ease with which the organisms are known to degrade agricultural products particularly grains [1,3,12,13]. Little or no growth and sporulation occurred in both the fungi at 10 and 15% NaCl (Table 3). At about the third day of incubation, the cultures were observed and there was no appreciable growth compared to those under 2% NaCl. The growth of up to 67.8mg in *A. terreus* and 45.6mg in *C. cladosporioides* started after the third day which suggest a possible re-adjustment in physiology. It is known that at inhibitor concentrations not leading to the death of cells, there occurs a change in biochemical composition of the microbial biomass, the kinetic characteristic of the culture and the appearance of physiological types resistant to the action of the inhibitor [15].

On the 14th day of incubation, *C. cladosporioides* showed a sharp rise in spore count; at this stage, there was significant

TABLE 3. DETERMINATION OF MIC OF SODIUM CHLORIDE.

Salt concentration (% w/v)	*Aspergillus terreus* Mycelial weight (mg/00ml^{-1})	*Aspergillus terreus* Sporulation (x10^5 ml^{-1})	*Cladosporium cladosporioides* Mycelial weight (mg/00 ml^{-1})	*Cladosporium cladosporioides* Sporulation (x10^5 ml^{-1})
0	86.1 ± 0.07	48	100.1 ± 0.12	56
2	118.9 ± 0.05	59	156.8 ± 0.11	58
5	67.8 ± 0.08	8	45.6 ± 0.02	3
10	3.4 ± 0.01	a	3.0 ± 0.00	1
15	—	–	—	–

Incubation was at 30° for 5 days. Result are mean of 3 replicates with standard deviations. [a]Dashes indicate no growth, no spore.

TABLE 4. DEGRADATION OF HEMICELLULOSE (XYLAN) BY *A. TERREUS* AND *C. CLADOSPORIOIDES*.

Isolate	Days of incubation 7 MN	7 S	14 MN	14 S	21 MN	21 S
A.terreus	48.1±0.04	29+.06	52.7±011	60±0.01*	57.5±0.07	78±0.06
C.cladosporioides	35.6±0.01	34±0.03	86.5±0.06	142±0.03*	90.0±0.11	156±0.08
Control	–	–	–	–	–	–

Flasks were still culture at 35° for 21 days. Result at 7days intervals represent spore count (x10^6 /ml medium) and dry weight of mycelium (mg/30ml), and are means of 3 replicates with standard deviations.

* Significant at 5% level of probability.

difference at 5% level of probability with spore production in *A. terreus*. Degradation of hemicellulose therefore, appeared to enhance the growth of the fungi (Table 4). Since it is a substantial component of cereals. Minerals salts medium (MSM) resulted in better growth and sporulation of the two fungi (Table 5) than Aspergillus medium (AM). Adenosine mono phosphate (AMP) inhibited their growth with more impact on *C. cladosporioides*. Addition of 0.5% glycerol, however, led to an appreciable enhancement in growth and sporulation. It is perhaps possible to link effect of glycerol or the growth with a descrease in AMP in the fungal cell and reduced permeability of cell wall to the nucleotide. Cyclic

TABLE 5. EFFECT OF CYCLIC 3.5 AMP ON THE GROWTH OF *ASPERGLLUS TERREUS* AND *CLADOSPORIUM CLADOSPORIOIDES*.

Media	MW*	Sporulation	MW	Sporulation
MSM (Control)	68.6±0.02	46±0.01	61.8±0.11	58±0.01
MAM+AMP	40.6±0.01	24±0.03	26.8±0.07	19±0.05
MAM+AMP+glycerol	140.1±0.17	100±0.05	116.8±0.11	86±0.14
AM(control)	41.1±0.10	29±0.02	40.0±0.03	35±0.02
AM = AMP	28.0±0.03	18±0.01	21.7±0.06	16±0.04
AM+AMP+Glycerol	98.8±0.11	64±0.02	84.6±0.05	60±0.07

MW = Mycelial dry weight mg/30ml; sporulation x10^5/ml, MSM = Mineral salts medium 32g., AM = Aspergillus medium 627.

Growth, determined on MSM and AM, with or without glycerol was observed at 30° for 7 days. Results are means of 3 replicates with standard deviations.

nucleotides have been reported to penetrate poorly into microbial cells [16]. Although stimilating effect of AMP has been reported [17], these results are in agreement with the earlier reports wherein it was shown to exert an inhibitory effect [18-20].

References

1. C. M. Christensen and H. H. Kaufman, C.M. Christensen (ed.), *Storage of Cereal Grains and Their Products*, (Am. Assoc., Cereal Chem., St. Paul Minnesota, USA, 1974), pp. 158.
2. B. J. Blaney and R. L. Dodman, Aust. J. Agric. Res., **39**, 21 (1988).
3. S. O. Fapohunda, Biochemical and Physiological Studies of Toxigenic Fungi from Malted Grains of *Zea mays* Linn, Ph.D. Thesis, University of Ibadan (1987).
4. R. G. Rotter, A. A. Frohlich, R. R. Marquardt and P. A. Mills, Can. J. Anim. Sci., **69** (1), 235 (1989).
5. G. Odamtten and E. Kampelmacher, Int. J. Fd. Microbiol., **3**, 57 (1986).
6. J. Zuber and G. Turian, Trans. Brit. Mycol. Sci., **76**, 433 (1981).
7. L. Hankin and S. L. Anagnostakis, Mycologia, **67**, 597 (1975).
8. S. O. Fapohunda and V. W. Ogundero, Int. Biodeter, **26**, 23 (1990).
9. S. C. Jong and M. J. Edwards (ed.), ATDC Update Fungi/Yeasts, 39 (1989).
10. G. A. Mideeva, Microbiology, **54** (6), 719 (1986).
11. L. M. Baryshnikov, R. I. Aminov, L. A. Okoroko and E. L. Golovier, Microbiology, **54** (6), 719 (1986).
12. R. Waldner, M. S. A. Leisola and A. Fiechter, Appl. Microbiol. Biotechnol., **29** (4), 400 (1988).
13. H. C. Dube and K. Prabakaran, Perspectives in Phytopathology, Today and Tomorrow's Publ., 181 (1989).
14. A. V. Furyaova, G. I. Tushkova, T. I. Pisman and N. S. Pechurkin, Microbiology, **54** (6), 708 (1986).
15. T. I. Pisman, Nauka Moscow, **1**, 67 (1980).
16. Y. Pastan and R. Perlman, Science, **169**, 3943 (1970).
17. J. Patrick and W. Dobrogosz, Biocheme. Biophys. Res., Comuns., **54** (2), 555 (1974).
18. M. M. Dedyukina and S. Ya Barikhin, in Proceedings of the Institute of Ecology of Plants and Animals, UNTS Academy of Science of the USSR, **104**, 70 (1977).
19. G. A. Menav, Microbiol. Shurn, **39** (5), 665 (1977).
20. N. Judewicz, E.De Roberts and A. Torres, Biochem. Biophys. Res. Comuns., **52** (4), 1257 (1973).