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EFFECT OF FUNGICIDES ON FUSARIUM GRAIN ROT AND ENZYME PRODUCTION IN MAIZE (ZEA MAYS L.)

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ABSTRACT

Two isolates of *Fusarium moniliforme* Sheld caused biochemical changes in maize grains on infection. Infection was achieved by dipping the grains in spore/mycelial suspension of the isolates. Biochemical components such as levels of starch, sugar, protein, fat, fibre and dry matter were reduced on infection. However other components such as ash and water content increased. The fungicides benlate, calixin, captan, demosan and vinclozolin variously reduced the incidence and severity of grain rot. However benlate, calixin and captan were the most effective. The trio also inhibited the activity of polygalacturonases and cellulases produced by the two isolates of *F. moniliforme.*

KEY WORDS: Fusarium, fungicides, enzymes.

INTRODUCTION

During pathogenesis, plant tissues are degraded by microbial enzymes. This degradation includes biochemical changes in the host tissue. The biochemical changes vary with organism type, part, age of plant, type of enzyme and the environmental conditions under which the pathogen operates (Adesiyan *et al* 1975). Owens and Specht (1966) analysed the components of the gall of tomato roots and found a decrease in carbohydrates when compared with healthy roots. They also found that *Scutellonema bradys* caused a significant reduction in the percentage of starch, amylopectin and amylose. However, total reducing sugars increased in dry rot but decreased in yam tubers affected by wet rot. Other reports of biochemical changes on infection of plant tissues by pathogens include Ogundana *et al* (1971) for F. *moniliforme* with yam tubers Hancock (1966) for *Sclerotinia sclerotiorum* with sunflower and tomato tissues and Bateman et al (1966) and Bateman (1972) for *Rhizoctonia solani* on bean hypocotyls.

Fungicides have been used to control rot of plant tissues caused by rot pathogens. These reports include works on benomyl (Ronald, 1975; Nielson, 1977) captan (Hampton, 1979; Mark *et al*, 1982) and calixin (Siegel, 1981; Jimenes and Ulrich, 1982).

Apart form preventing rot, mycelial growth and spore/conidial germination, some fungicides have been reported to inhibit extracellular enzymes. Arinze *et al* (1975) reported that cellulase and polygalactronase of *B. theobromae* were variously inhibited by captan, benomyl and kocide - 101. Earlier report of Mehlitz and Mass (1935) showed that formaldehyde was inhibitory to polygalacturonases of some plant pathogens. This work considers the biochemical changes occurring in maize grains on infection with isolates of *F. moniliforme.* The effect of pre and post inoculation chemical treatment on incidence and severity of grain rot and the activity of extracellular pectolytic and cellulolytic enzymes produced by the isolates, is also investigated.

MATERIALS AND METHODS

Fungal isolates: Two isolates of *F. moniliforme* used in this work were isolated from rotted maize gains and maintained on Potato Dextrose Agar. These isolates designated Fm-1 and Fm-2 had white cottony appearance on PDA. Both had two types of conidia, the micro and macro conidi a.

The micro conidia of Fm-1 were 2 - 3 celled, oval in shape arranged in chains and measured 8 - 12 x 2.5 - 3 µ . The macro conidia of Fm-1 were boatshaped, 5 septate and measured 25 - 60 x 2.5 - 4 µ. On the other hard the micro conidia of Fm-2 were also oval in shape but non septate, borne singly and measured 5 12 x 1.5 - 2.5µ. Also the macro conidia of Fm-2 were sickle shaped but 3 septate and measured 32 - 53 x 3 - 4.5 µ.

Maize grains: The maize grains of sweet corn were obtained from the Rivers State Institute of Agricultural Research and Training, Port Harcourt.
Fungicides. The following fungicides were obtained from the Rivers State Agricultural

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Development Programme (ADP) Headquarters in Port Harcourt:

Benomy (Benlate) (methyl-i-(butylcarbamyl)-2-benizmidazole carbamate) manufactured by E. I. dup Pont de Nemouvs & Co. (Inc).

Vinclozolin (3-(3, 5 - dichlorophenyl)-5-methyl-5-vinyl-1, 3 -oxazolidine - 2, 4 dione)

Calixin (tridemorph) (C_{11}-C_{14} -4-4 alkyl-2-6-dimethyl-morphon) manufactured by Basf Aktiengsell Schaft.

Captan (N-trichloromethylthio-4-cyclohexane-1,2-dicarboximide) manufactured by Standard Oil Co.

Demosan (1,4-dichloro-2, 5-dimethoxybenzene)

Effect of isolates of *F. moniliforme* on Biochemical components of Maize grains: Bacteria and fungi-free seeds were inoculated by dipping them in spore/mycelial suspensions of the isolates of the fungus and incubated in sterile flasks at 25°C for 14 days. To prepare the spore suspensions, ten ml sterile distilled water was used too flood the surfaces of sporulating mycelia of 12-day old cultures. The cultures were slightly agitated with a sterile glass rod to dislodge the spores. The suspension was filtered through a sterile muslin cloth. The concentration of the conidia was adjusted using aliquots of 0.01ml of the suspension in a haemocytometer to achieve a spore density of 600 conidia/ml. The biochemical components of the infested grains after 14 days were determined. Also determined were the components of the uninfected grains of days 0 and 14 respectively. The biochemical components were determined as follows:

Total sugars: These were determined according to the method of Dubois *et al* (1956). Twenty grams of grain were homogenised in 20ml of sterile distilled water in a sterile blender (Soryal Ominmixer 5). The resulting homogenate was centrifuged (EL 583-140/01) at 2,500g for 10 minutes. Two millilitre of freshly prepared 5% dinitrosalysilic acid reagent was rapidly added to 1ml of the supernatant fluid and heated for 5 minutes in a boiling water bath. When cool, the intensity of the resulting colour was read at 540mm in a spectrophotometer (Beckman model DB). The amount of total sugars in the aliquot was determined from a calibration curve obtained by plotting absorbance prepared for dextrose. In the control, uninfected seeds were analysed on the day the experiment was set up and 14 days thereafter.

Total starch: Sugars were extracted from 20g of grains with 100ml of hot 80% elthanol (analar). To ensure complete extraction of the sugars, the process was allowed to last for 15 minutes, during which qualitative tests with anthrone sulphuric and reagent showed no green colour. Starch was estimated using the method of McCready *et al* (1950). To the sugar-free residue, 20mls of 52% perchloric acid were added, cooled in an icebath, stirred for 15 minutes and centrifuged at 2500g for 10 minutes. The supermatant was poured off into a 100ml flask. 5mls of distilled water were added to the residue in an icebath, after which 10mls of perchloric acid were added. The mixure was stirred with a glass rod for 30 minutes, centrifuged at 2500g and the supernatant added to the contents of the 100ml flask. The flask content was finally made up to 100ml with distilled water. Ten millilitre of this solution was diluted to 500mls and 5mls of the diluted solution mixed thoroughly with 10ml of the anthrone sulphuric acid reagent in an icebath and then heated for 10 minutes in boiling water. It was rapidly cooled and the optical density read at 630mm using a spectrophotometer (Beckman DB). The amount of starch was then read off from a dextrose calibration curve prepared with the anthrone sulphuric acid reagent.

Total protein: The amount of protein was estimated using the Kjeldhal method as outlined by the Association of Official Agricultural Chemists methods of Analysis cited by Oyejola (1973). Five grams of the grain sample were put into a Kjeldhal digestion flask and 5g of a catalyst mixture (96% K_2SO_4, 0.5% $CuSO_4$, 0.5% selenium dioxide) and 25mls of concantrated sulphuric acid were added and placed on a hot plate until the samples were completely digested (samples became colourless). After digestion, the solution was allowed to cool and the digest transferred into a 100ml volumetric flask and made up to the mark with distilled water. Aliquots of 10ml of the digested sample were transferred into a micro Kjeldhal distillation apparatus and 10mls of 40% sodium hydroxide were added. This caused a colour change (from red to yellow). The content was steam distilled and the distillate taken up in 10mls of 2% boric containing two drops of bromophenol blue indicator. Distillation was continued until the solution of boric acid assumed a violet colour. 40mls of the distillate were titrated against 0.1NHCl until a change in colour from violet to yellow was obtained. The amount of total nitrogen in the sample was calculated on the basis of 1ml 0.1N HCL = 0.0014g nitrogen. The protein content was obtained by multiplying the nitrogen value by 6.25.

Total Fats: 10g of grains were cut into pieces. The cut pieces were put into a specimen bottle containing 50mls of n-hexane. After storage at 30 °C for 72 hours the n-hexane was poured into a previously weighed dry crucible and the n-hexane was allowed to evaporate under vacuum at 50°C. The crucible was placed in a Soxhlet connected to a distillation flask. The Soxhlet apparatus was heated using an electical mantle set at 50°C. This evaporated the n-hexane leaving the extracted fat inside the crucible. The crucible with the lipid extract was dried in an oven at 45°C for 15

minutes; cooled and weighed.

Fibre/ash: 2g of grain were put into a 500ml conical flask and 250mls of 5% sulphuric acid was added. The mixture was hydrolysed for 30 minutes at 50 using an electrical heater (model EL 582 - 310/01). Distilled water was added occasionally to avoid burning or drying of the mixture. The mixture was filtered after 30 minutes using filter paper. The residue was washed with hot water to remove traces of sulphuric acid and again transferred into a flask containing 200mls of 50% potassium hydroxide for second hydrolysis. After this second hydrolysis, the mixture was again filtered and the redidue rinsed with ethanol into a porcelain crucible dish, allowed to dry and first weight taken,. The crucible containing the dry residue was put into a muffle furnace and ashed at 500°C for 3 hours and the second weight taken.

Dry Matter/Moisture: 10g of the maize grains were dried in an oven at a temperature of 60°C for 2 hours in a porcelain crucible, cooled in a desiccator and weighed. This was repeated until a constant weight was obtained. The total residue represented the amount of dry matter while the loss in weight represented the moisture content.

Effect Of Fungicides on Disease Incidence and Severity

Grains were sprayed separately with a concetration of 10mg/ml of benlate, calixin, captan, vinclozolin and demosan. After 1 hour, the grains were sprayed with conidial/mycelial suspensions of the appropriate isolate of *F. moniliforme* and incubated at 25° C for 14 days. In the post inoculation cemical treatment, grains were sprayed with mycelial/conidial suspensions before chemical treatment. Disease incidence (incidence of rot) was determined after 14 days. Rot severity was expressed as rot index using the formula:

$$\text{R. I.} = \frac{\sum na}{N}$$

Where a = number of categories, each expressing degree of rot

n = number of rotted grains in each category

N = total number of treated grains.

Effect Of Fungicides on Activity of Pectolytic and Cellulolytic Enzymes Of Isolates of *F. moniliforme*

The effect of benlate, calixin, vinclozolin, captan and demosan on activity of Polygalacturonase(PG) and cellulase was determined using the method of Arinze et al (1975). Enzyme samples were obtain by growing the fungal isolate in Rease and Levinson's medium (1952) with Sodium Polypectate (Napp) as carbon source for the production of PG and Carboxymethyl Cellulose (CMC) for cellulase for 4 days. Partial purification of culture filtrates were done using the method of Spalding (1969). One millilitre sample of the appropriate fungicide at a concentration of 5mg/ml was added to 2mls of the culture filtrate containing appropriate enzyme, mixed thoroughly by shaking and the resultant solution was allowed to stand for 1 hour to ensure inactivation of the enzyme before the addition of the substrate. At intervals of 5 minutes, for 25 minutes 1ml fungicide/enzyme mixture was withdrawn and added to 9mls of the substrates which consisted of 1% Napp in phosphate buffer pH 5.0 or 1% CMC in phosphate buffer pH 5.0. The loss in viscosity of the reaction mixture was estimated viscometrically (using size 300 Ostward-Fenske viscometers) 1ml sterile distilled water was substituted for the fungicides in the control experiment.

RESULTS

Biochemical changes occurring in infected grains, are summarised in Table 1.

Fourteen days after inoculation, there was decline in the amount of starch, sugar, proteins, fat, fibre and dry matter while the ash and water contents rose sharply following inoculation. Isolate

Table 1. Effect of infection of maize grains with isolate of *F. moniliforme* on biochemical components of the grains

	Starch	Sugar	Protein	Fat	Ash	Fibre	Dry matter	Water content
Control (uninoculated)	76.6[a]	80.3[a]	34.7[a]	33.0[a]	3.0[a]	8.0[a]	74.0[a]	15.0[a]
F. moniliforme - 1	35.5[b]	53.0[b]	19.3[b]	16.1[b]	6.8[b]	12.0[b]	68.4[b]	33.0[b]
F. moniliforme - 2	55.0[c]	60.0[b]	23.4[b]	20.3[b]	5.6[b]	10.5[b]	65.7[b]	28.9[b]
Fm - 1/Fm - 2	27.8[d]	41.2[c]	15.3[c]	10.8[c]	12.1[c]	12.5[b]	58.7[c]	42.9[c]

Starch determined as mg/g fresh weight of grains; Total sugars as mg/g glucose equivalent; Total proteins as mg/g fresh weight of grains; Total fat as mg/g fresh weight of grains; Ash as mg/g fresh weight of grains; Fibre as mg/g fresh weight of grains; Dry matter as %; Water content as %; Values are means of triplicate determinations; means with different letters within the same column are significantly different ($P < 0.05$).

Table 2: Effect of Fungicide Treatment on Incidence and Severity of grain rot caused by F. moniliforme strains Fm 1 and Fm 2.

Fungicide	Fm - 1				Fm - 2			
	1. Pre-inoculation		Post-inoculation		Pre-inoculation		Post-inoculation	
	Incidence %	R. I.*	Incidence %	R. I.*	Incidence %	R. I.*	Incidence %	R. I.*
Control	71.5[a+]	2.15[a]	71.3[a]	2.09[a]	66.5[a]	1.92[a]	68.8[a]	2.06[a]
Benlate	15.5[b]	0.43[b]	17.2[b]	0.45[b]	14.5[b]	0.39[b]	13.5[b]	0.42[b]
Calixin	17.5[b]	0.46[b]	17.5[b]	0.56[b]	15.6[b]	0.40[b]	19.3[b]	0.51[b]
Captan	18.0[c]	0.45[b]	24.0[c]	0.66[c]	15.3[b]	0.46[b]	19.0[b]	0.52
Demosan	19.0[c]	0.48[b]	25.0[c]	0.69[c]	16.3[c]	0.47[b]	25.5[c]	0.65[c]
Vinclozolin	21.0[c]	0.60	25.5[c]	0.70[c]	17.5[c]	0.51[b]	28.5[c]	0.80[c]

+ Values are means of triplicates determination; means with different letters within the same column are significantly different ($P < 0.05$).

* R. I. = rot index.

Fm-1 caused more dramatic changes in biochemical composition than Fm-2. When the isolates were combined, the changes were even more pronounced than when each isolate acted separately (Table 1).

Effects of fungicide treatment on disease incidence and severity in the grains:

Table 2 shows that all the fungicides variously reduced disease incidence and severity in both pre and post inoculation chemical treatment of grains.

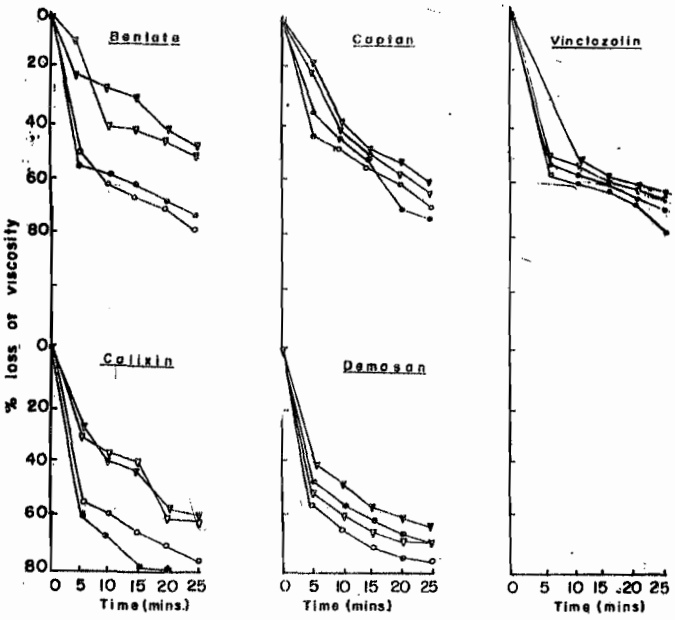

Fig. 2: Effect of Fungicides on activity of Celulase of F. moniliforme
▽—▽ FM.I.treated; o—o FM I.control; ▼—▼ FM 2 treated;
●—● FM 2.control

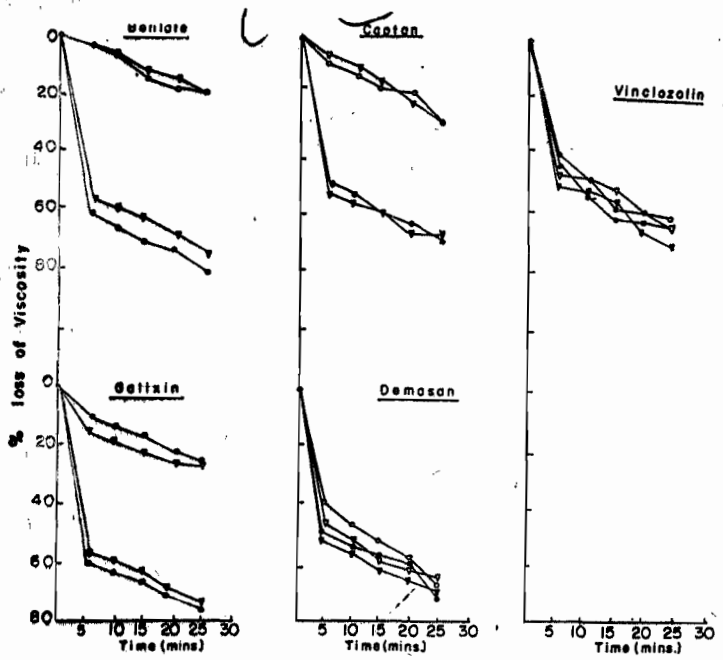

Fig. 1: Effect of Fungicides on Activity of PG of F. moniliforme.
o—o FM.I treated; ●—● FM. I control; ▽—▽ FM. 2 treated;
▼—▼ FM. 2 control

Effect of fungicides on activity of PG and cellulase produced by isolates of *F. moniliforme*

Figs. 1 and 2 show that although benlate, calixin and captan caused appreciable reduction in activity of PG and cellulase enzymes, benlate was the most inhibitory. Vinclozolin and demosan only caused slight inhibition of pectolytic and cellulolytic activities.

DISCUSSION

When plant materials are infected by pathogens, there is deterioration in quality and quantity of their biochemical components,(Onuegbu, (1999)). These changes were observed on infection of maize grains with *F.moniforme*. Studies on the constituents of the maize grains have been reported (Oyenuga 1978). When uninoculated after 14

days, there was a slight decrease in starch, sugar, protein, fat and water contents. This could be attributed to the metabolic activities of the respiring grains. Coursey (1967) working on yams in storage, attributed these changes to sprouting, respiration and evaporation. Isolates of *F. moniliforme* were found to cause a decrease in starch, sugar, protein, fibre and dry matter contents of the grains. However, water and ash content increased sharply. Arinze *et al* (1975) reported a drop in the carbohydrate, protein and lipid contents of sweet potato infected by *L. theobromae* after inter and intracelluler establishment. Earlier Ogundana *et al* (1971) showed that *F. moniliforme* penetrated the parenchyma cells of yam tubers, established itself within the cells and cleared most of the starch grains, so that the carbohydrate contents of the cell consequently decreased. The increase in water content observed in this work may be metabolic due to the breakdown of the pectic bonds by pectic enzymes into water. According to Stephens and Wood (1975) enzymes probably acting on pectic polymers of the cell wall allow water to be taken up from the ambient solution into the protoplast. In contrast to the finding of this work, Adesiyan *et al* (1975) reported an increase in sugar content of yam tubers infected by the nematode - *Scutellonema bradys.*

The fungicides, especially benlate and calixin, reduced the incidence and severity of grain rot due to *F. moniliforme.* This means that maize grains should be subjected to the appropriate chemical treatment before/during storage.

The fungicides Benlate, Calixin, Captan, Demosan and Vinclozolin inhibited the activities of pectolytic and cellulolytic enzymes to varying degrees and this may account for their differential ability in controling Fusarium seed rot. Demosan and Vinclozolin were less inhibitory on the activity of pectolytic and cellulolytic enzymes. The greater ability of Benlate, Calixin and Captan to inhibit the activity of these enzymes may account for their effectiveness in reducing disease incidence and severity. Arinze *et al* (1975) had earlier reported the inhibition of cellulolytic and pectolytic enzymes of *Botryodiplodia theobromae* by some fungicides. As suggested in that report, this fungicides could be general poisons rather than inhibitors of specific enzymes. It is recommended that the toxicity levels of these fungicides to man be first determined before practical application.

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