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Full Length Research Paper

Control of *Botryodiplodia theobromae* causing Tissue Rot of White Yam (*Dioscorea rotundata* Poir).

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Seven organisms (*Aspergillus niger, Penicillium expansum, Penicillium sclerotigenum, Fusarium solani, Botryodiplodia theobromae, Fusarium moniliformes* and *Rhizopus stolonifer*) were isolated as major rot-causing organisms of yam in Calabar, Cross River State, Nigeria. Of these, *Rhizopus stolonifer* was the most frequently isolated and *B. theobromae* the most virulent recording a percentage rot severity of 57.5 and was used as the test pathogen throughout the course of this study. Six fungicides (forcelet, ridomil, caocobre, nordox, hydrox and borax) were used in the management of the *Botryodiplodia* – incited rot of yam in storage. The effect of the test fungicides was concentration-dependent. There were significant differences in the level of inhibition of different fungal structures by a particular test fungicide. At 20g/l, inhibition of spore germination by forcelet and coacobre were high (81% and 70% respectively) while reduction in mycelial dry weight by same fungicides dropped to 56.3% and 30% respectively. However, borax was the least effective of the fungicides tested in both cases. Incubation period positively affected the inhibitory performance of the test fungicides. Except for borax, all other fungicides recorded increasing inhibitory values against the test pathogen with increasing incubation period. Within 2 days of incubation, coacobre, hydrox and mancozeb recorded mean radial growth values of 0.87cm, 0.95cm and 1.39cm respectively as compared with 3.05cm recorded in the control experiment. Eight days thereafter (10 days), coacobre improved in its effectiveness reducing the colony size of the test fungus from 1.30cm recorded on the 8th day to 1.16cm as against 4.50cm observed in the control experiment.

Key words: *Botryodiplodia theobromae, Dioscorea rotundata,* tissue rot, yam

INTRODUCTION

A great magnitude of losses of tuber crops resulting from many causes (pathological and non-pathological) in storage has been reported throughout the world (Noon, 1978, Ricci *et al*., 1979, Foua-Bi *et al*., 1979, Arinze, 2005). Of these two causes of food loss, pathological losses (losses resulting from the activities of parasitic organisms or pathogens) is the most severe. In yams, a record of about 50% reduction of the total stored tubers has been reported within the first 6 months of storage (Arinze, 2005). This is a major threat to food security. To avert this, various measures have been developed and directed at managing losses (Thompson *et al.,*1973). Some of these measures are preventive (indirect) and other (control) direct. The indirect measures include gentle handling of the yam tubers to minimize risk of injury during harvesting, transport and storage; modification of the storage environment of the yam tuber as to reduce the incidence of the disease and discouraging attacks by the causative agents, curing of the crop before storage at an optimum temperature of 30 - 40 ^{o}C (Arinze, 2005).

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Preventive measures are effective before incidence of the disease. However, when the organism(s) have already invaded the plant tissues, the best management approach is direct attack on the pathogenic organisms. One of the most effective methods of direct attacks on pathogenic organisms is the chemical method. Chemical method of disease control involves the use of chemical compounds (usually synthetic chemicals) in the management of diseases. Several chemicals have been reported to reduce the storage losses of yam. They include; sodium orthophenylphenate, borax, captan, thiabendazole and Benomyl (Ogali *et al*, 1991; Okigbo and Ikediugwu, 1999). High *in vitro* toxicity of some systemic fungicides has been reported against *Pythium aphanidermatum* (Ramachandran *et al*, 1989). They reported ED_{50} values as low as 0.25 and 0.74 for ethazole and metalaxyl fungicides respectively. Complete inhibition of spore germination of *Botryodiplodia theobromae*, *Fusarium moniliformes* and *Penicillium sclerogenum* has been recorded with captan, while benlate and thiabendazole were able to arrest the germination of the spores as well as stall the growth of these organisms (Plumbley *et al*, 1984). However, there have been reports of ineffectiveness of some fungicides on some stored crops. Singh and Singh (1989) reported the ineffectiveness of Benlate and Bavistin 50 WP at 500 and 1000ppm respectively on *Rhizopus* rot of stored jackfruits in India. According to Plumbley *et al.,* (1984), for effectiveness, chemical treatment requires the application of the appropriate compound, at the recommended dosage, by the most appropriate method and at the most suitable time. The method of application may be by dipping, spraying or dusting. The effective use of some fungicides for the control of tissue rot of white yam is here presented.

MATERIALS AND METHODS

Sample collection and pathogen identification

Symptomatic and asymptomatic tubers of white yam (*Dioscorea rotundata* Poir) were sampled from open market stalls in three markets in Calabar, Cross River State, Nigeria. The markets were Akim, Marian and Watt. Tissues about 5mm in diameter from the symptomatic and asymptomatic white yam tubers were removed following surface sterilization with 70 % ethanol for 10s, blotted dry with sterile paper towel, and plated onto chloramphenicol-amended Potato Dextrose Agar (PDA). After three days of incubation at 28^{o}C, microbial growth was assessed by microscopy. Cultures of the isolates were transferred to new PDA-containing plates, from where axenic cultures were generated (Gevens *et al*., 2008). Identification of the isolates were based on morphological characteristics, described in the 1987 illustrated genera of fungi by Barnett and Hunter and with literature on identification of pathogenic fungi by Rossman *et a*l., (1997). Confirmation was made by comparing with cultures identified by International Mycological Institute, Egham, UK.

Pathogenicity test

To confirm the pathogenicity of isolates from white yam, axenic cultures of the isolates were used to inoculate three white yam minisetts per pathogen with 5-mm-diameter mycelial agar plugs of a 4-day-old culture. On appearance of symptoms, the tissues at the margins of the healthy and diseased parts were surfaced-sterilized, excised and plated onto PDA for incubation at 28^{o}C for four days. At the end of this period, morphological characteristics and growth patterns observed in each case were compared with the ones of the original isolates.

Severity Index (SI)

Following appearance of symptoms, 15 to 21 days post inoculation (dpi), rot severity index (SI) was assessed on a scale of 0- 4, (0 = no disease, 1=1-25% rot, 2=25- 50% rot, 3=50-75% rot and 4=75-100% rot), using a modification of the formula given by Groth *et al.,* (1999);

$$SI = \frac{(X_0xY_0)+(X_1xY_1)+(X_2xY_2)+(X_3xY_3)+(X_4xY_4)}{(Y_0+Y_1+Y_2+Y_3+Y_4)}$$

Where X = Disease scale
Y = Volume of rot (cm^3)

Effect of fungicides on spore germination of *Botryodiplodia theobromae* in culture

The fungicides were evaluated following the methods of Vijay and Gupta (1990). Suspensions of the fungicides were prepared to give the following concentrations: 20g/l, 40g/l, 60g/l, 80g/l and 100g/l. One ml of each concentration of fungicide or sterile distilled water (0g/l, control) was sprayed with a syringe on the solidified surface of PDA in each of the series of Petri dishes and was allowed to stand for 4 hours. One drop of conidia suspension ($5.0x10^4$ conidia/ml of distilled water) was placed at the centre of each four sectors in each Petri dish. The plates were incubated at 30^{0}C for six hours and then fixed with Formalin-Acetic-Acid –Alcohol (FAA). The conidia germinating and those not germinating from each of the four inoculated sectors in each plate were carefully counted under the low power (x10) of the light microscope. The percentage inhibition of germination

Table 1: Fungal isolates of yams obtained from the study area, their frequency of occurrence and rot severity.

Organisms Isolated	Markets and frequency of occurrence_(%)__				Type of rot	Severity (%)
	Marian	Akim	Watt	Mean		
Aspergillus niger	13.00	31.00	0.00	14.67	Dry	34.5
Penicillium expansum	41.00	57.00	10.00	36.00	Dry	45.0
Penicillium sclerotigenum	3.00	23.00	5.00	10.30	Dry	12.5
Fusarium solani	0.00	3.00	25.00	9.30	Dry	11.25
Botryodiplodia theobromae	42.00	81.00	16.00	46.30	Dry	57.5
Fusarium moniliformes	21.00	47.00	10.00	26.00	Soft	32.5
Rhizopus stolonifer	70.00	87.00	42.00	66.30	Soft	52.5

was calculated from the data obtained.

Effect of fungicides on mycelial dry weight of *Botryodiplodia theobromae* in culture.

The effect of fungicides on the mycelial dry weight was carried out using nutrient broth. Fifty millilitres of nutrient broth were each dispensed into 100 ml conical flasks and a 5mm - diameter disc of a four – day old culture of the pathogen taken from the advancing margin of the culture and dropped in the flask and one ml of each concentration of fungicide or distilled water (g/l, control) was introduced into the nutrient broth – pathogen mixture in the flask. This set-up was incubated at 30^0C for ten (ten) days and then filtered using an already weighed sterile- 9cm diameter Whatman N0. 1 filter paper. The content of the filter paper was dried in an oven at 80^0C for 24 hrs to a constant weight (Markson *et al,* 2005).

Effect of fungicides on the growth of *Botryodiplodia theobromae* in culture

Seven fungicides were evaluated for inhibition of mycelial growth both in molten and solid media, and conidia germination of *Botryodiplodia theobromae.* The fungicides were: forcelet (carbendazim, 50% WP), Borax (Sodium tetra borate), Nordox (cuprous oxide, 86.2% WP, equivalent to 75% metallic copper.), Hydrox (50% metal copper in the form of copper hydroxide), Mancozeb (Mancozeb 75% WP), Coacobre (56% w/w of cuprous oxide, equivalent of 50% w/w of pure copper metal), and Ridomil (66% WP contains 60g of metalaxyl-M and 600g in the form of cuprous oxide). One ml of each concentration of fungicide or sterile distilled water was sprayed with syringe onto the solidified surface of PDA in each of the series of Petri dishes. Each plate was inoculated at the centre with a 5mm culture disc of the fungus and incubated at 30^0C (Amadioha and Uchenna, 2003). Measurements of the colony diameter were taken at two days interval for 10 days. The percentage inhibition was calculated from the data obtained as follows:

% growth inhibition = colony diameter of control – diameter of the treatment x 100

Diameter of control 1

RESULTS AND DISCUSSION

Sample collection, isolation and pathogen identification

Following the results obtained from isolations of rot-causing pathogens of yam, *Botryodiplodia theobromae* was the most frequently isolated after *Rhizopus stolonifer* and the most virulent (Table 1). Cultures of this fungus on PDA were initially white, fluffy and feathery, becoming grey and eventually black. The growth was radial in pattern from the centre of the plate outwards. Literature on identification of pathogenic fungi (Rossman *et al*., 1997) corroborates this observation and the appearance of this fungus fitted the description of *Botryodiplodia* Pat. (=*Lasiodiplodia theobromae* (Pat.) Griff and Maubl.) given by Marley (1998) and confirmation was made by comparing with a culture identified by International Mycological Institute, Egham, UK. (IMI 347961).

Pathogenicity test

The *B. theobromae* isolate was pathogenic on the three white yam minisetts used for the test. Symptoms of decay (rot) were seen on the re-inoculated yam minisetts as dry black rot. On re-isolation. Morphological characteristics and growth patterns similar to those earlier observed on axenic cultures were exhibited, a confirmation of pathogenicity.

Severity Index (SI)

The results obtained from assessment of rot severity index on the potato (Sweet and Irish) tissues revealed that *B. theobromae* recorded the highest rot severity index of 57.5%, closely followed by a 52.5% rot created by *R. stolonifer*. *P. expansum* was next in virulence with a 45.0% rot index. The other four pathogens tested recorded rot indexes within the range of 12.5% to 34.5% within the test period (Table 1).

Effect of fungicide on spore germination of *Botryodiplodia theobromae*

Conidia of the test fungus were treated to varying concentrations of the seven fungicides and the result is presented in Fig.1. The percentage inhibition of spore germination increased with increase in concentration of the fungicides. Forcelet recorded the highest inhibition of spore germination throughout the course of the experiment with 81% inhibition level at the least concentration level of 20g/l. This was followed by other fungicides (coacobre, mancozeb, ridomil Nordox, hydrox and borax). Borax at 20g/l did not make any impact on the spore germination of the test fungus. There was no difference between the performance of forcelet, mancozeb, coacobre and ridomil in the inhibition of spore germination of *B. theobromae* irrespective of the level of concentration. Enokpa, (1995) reported on the effect of lichen extracts and synthetic fungicides on spore germination of *Penicillium sp.* and recorded as high as 94% inhibition in spore germination of the test fungus by mancozeb at 10.00µg/g whereas Ramachandran *et al*, (1989) reported ED_{50} value as low as 0.25 and 0.74% for ethazole and metalaxyl fungicides respectively.

The poorest result was recorded with borax. The report given by Ogali *et al*, (1991) naming borax (Sodium tetraborate) as one of the effective fungicides against storage diseases of yam did not match the findings in this study, suggesting that borax may be effective in controlling other pathogens of yam but not *B. theobromae.* Plumbley *et al*, (1984) had documented a complete inhibition of *B. theobromae* with captan (a copper – based fungicide) which corroborate the current work where high level inhibition of spore germination was recorded for forcelet [carbendazim (50% WP), Mancozeb (75% WP), Ridomil (66% WP) metalaxyl-M and cuprous oxide] and Coacobre (50% pure metal copper), all copper – containing compounds, at 20g/l concentration.

Effect of fungicide on mycelial dry weight of *Botryodiplodia theobromae*

Two fungicides (Forcelet and mancozeb) gave appreciable growth reduction of the test fungus at a very low concentration (20g/l). The performance of forcelet (63.3%) was however significantly different ($P<0.05$) from that recorded by mancozeb (55.1%) at this level of concentration. Borax was the least effective as it recorded only a 17.0% inhibition of the test fungus even at 100g/l concentration (Fig.2). Forcelet was recorded as the best fungicide in reducing the mycelial dry weight of the test fungus, followed by mancozeb. This was followed by ridomil (36.7%). The effect of coacobre and nordox in reducing the growth of *B. theobromae* was not significantly different ($P<0.05$).

This was followed by hydrox and borax. At concentration levels above 40g/l, coacobre was superior to nordox which was comparable to hydrox. At 100g/l, forcelet was not significantly ($P<0.05$) different from mancozeb in inhibiting the growth of *B. theobromae*. From the result above, some fungicides that showed high inhibition of spore germination (Fig.1.) were not as effective in reducing the mycelial dry weight (Fig.2.) of this same fungus suggesting that the nature and composition of the fungal structures, spore coat and the mycelial cell wall respectively may be responsible for the difference in the activity of these fungicides or the difference in the chemical composition of the protective structures (Singh, 2007).

Effect of fungicides on the growth of *Botryodiplodia theobromae* in culture

The various synthetic fungicides showed variations in the inhibition of growth of the test fungus. The level of inhibition increased with the concentration of the fungicides used. Period of incubation also significantly influenced the efficacy of the fungicides indicating that the effect of the active compounds of the fungicides used were persistent and increased with the incubation period (Amadioha, 2003).

The effect of different concentrations of the synthetic fungicides on the radial growth of *B. theobromae* after two days of incubation showed that fungicides showed significant ($P<0.05$) growth inhibitions during this period. Hydrox, coacobre and mancozeb were the best fungicides recording mean radial growth values of 0.95cm, 0.87cm and 1.39cm respectively whereas borax (2.46cm) was the least in reducing the radial growth of *B. theobromae* after 2 days. This mean radial growth value recorded by borax was not significantly ($P<0.05$) different from the values obtained in the control experiment (3.05cm). On the fourth day of incubation all the fungicides indicated improvements on their performance compared with values obtained on the second day (Table 2). Coacobre gave the best results followed by mancozeb, forcelet, nordox and hydrox which were comparable in their growth inhibition effects. Borax gave

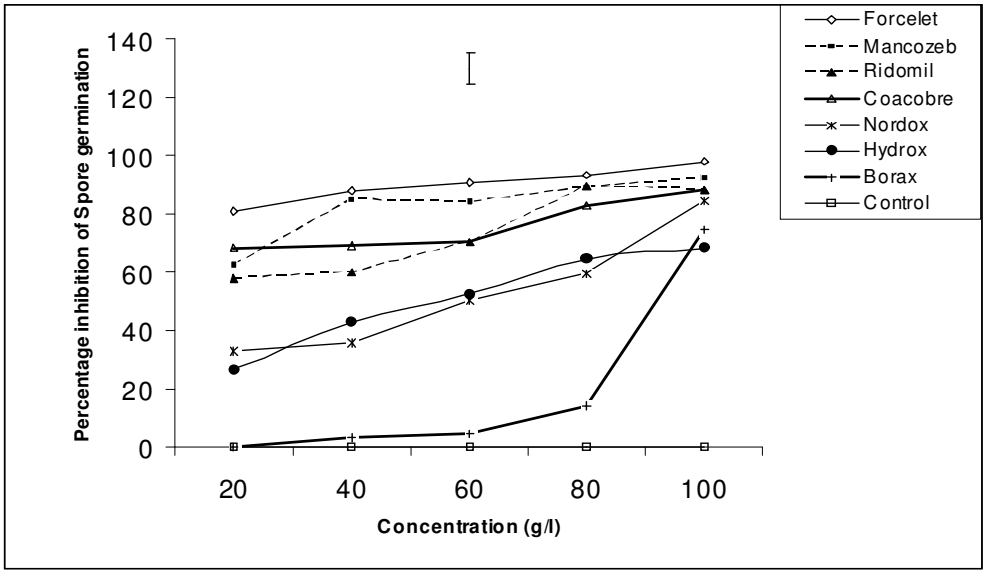

Figure. 1: Effect of fungicides on spore germination of *Botryodiplodia theobromae*. Bar represent LSD (P<0.05).

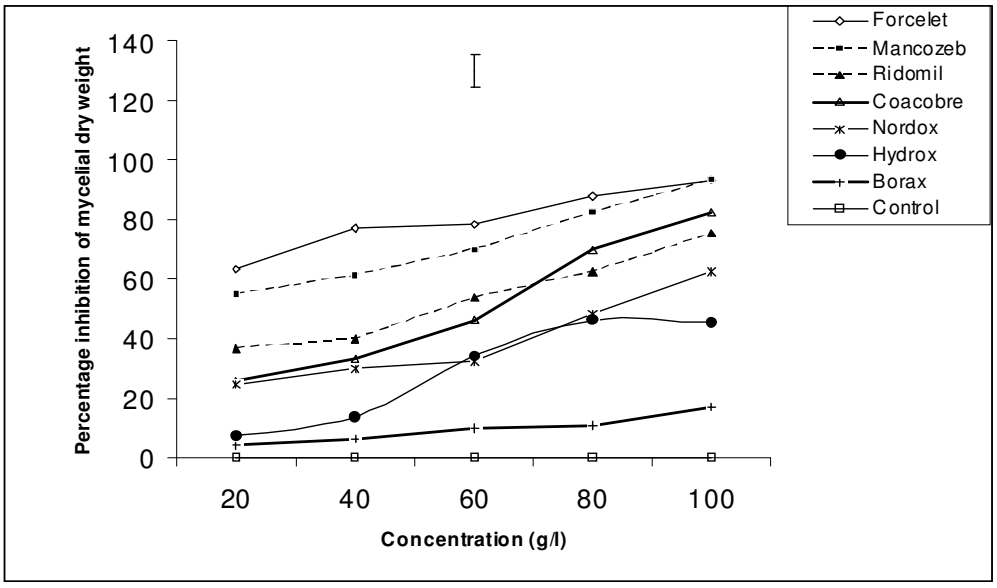

Figure 2: Effect of fungicides on mycelial dry weight of *Botryodiplodia theobromae*. Bar represent LSD (P<0.05).

(3.03cm) the least inhibition effects which was however significantly (P<0.05) different from a mean radial growth value of 4.40cm recorded in the control experiment. Six days following the commencement of the test, there were marginal increment in growth indicating a slight reduction in efficacy of the test fungicides except for coacobre which recorded marked reduction in the colony size of the test fungus from 1.08cm to 1.00cm.

Forcelet and mancozeb recorded a steady rise in activity. The effect of forcelet was not significantly (P<0.05) different from that of mancozeb. Nordox and ridomil were comparable in their activity. Borax was the

Table 2: Effect of incubation period on the performance of fungicides on the growth inhibition of *B. theobromae* in culture.

Fungicides	Period of incubation (days) and growth (cm)								
	2	4	6	8	10	2	4	6	8
Forcelet	1.43	1.57	1.72	1.77	1.82	1.43	1.57	1.72	1.77
Mancozeb	1.39	1.43	1.44	1.37[e]	1.59	1.39	1.43	1.44	1.37[e]
Ridomil	1.82	1.86	2.12	2.54	2.32	1.82	1.86	2.12	2.54
Coacobre	0.87	1.08	1.00	1.30	1.16	0.87	1.08	1.00	1.30
Nordox	1.49	1.48	2.15	2.54	2.62	1.49	1.48	2.15	2.54
Hydrox	0.95	1.09	1.37	1.60	1.96	0.95	1.09	1.37	1.60
Borax	2.46	3.03	3.31	3.66	3.83	2.46	3.03	3.31	3.66
Control	3.05	4.40	4.50	4.50	4.50	3.05	4.40	4.50	4.50
LSD	0.62	0.66	0.37	0.42	0.41	0.62	0.66	0.37	0.42

Table 3: Effect of different concentrations of fungicides on the growth inhibition of *B. theobromae* in culture.

Fungicides	Concentration (g/l) and growth (cm)				
	20	40	60	80	100
Forcelet	3.74	3.39	0.77	0.28	1.66
Mancozeb	3.30	2.01	1.16	0.69	1.44
Ridomil	3.30	3.38	2.39	1.63	2.35
Coacobre	1.61	1.08	1.01	0.96	1.08
Nordox	3.76	3.32	2.00	0.60	2.01
Hydrox	2.58	1.97	1.43	0.56	1.39
Borax	4.01	3.67	3.14	3.14	3.26
Control	4.19	4.19	4.19	4.19	4.19
LSD	0.06				

least effective of all. Results obtained eight days following the commencement of the experiment, showed that all the fungicides recorded reduced activity at various levels of concentration (Table 2). However, coacobre still led in the growth inhibition activity with 1.30cm growth inhibition value which was not significantly ($P<0.05$) different from 1.37 and 1.77cm recorded by mancozeb and forcelet respectively. On the tenth day of incubation, coacobre became more effective reducing the colony size of the test fungus from 1.30cm recorded on the 8th day to 1.16cm.

This performance was significantly different ($P<0.05$) from those of forcelet, mancozeb and hydrox which were comparable in their growth inhibition activity. Borax was the least effective recording below 50% (2.45cm mean growth value) radial growth inhibition of the test fungus even at 100g/l concentration. In all, means separation results indicated that coacobre gave the highest growth inhibition value compared with others, followed by hydrox and mancozeb.

All the tested fungicides were effective against the radial growth of *B. theobromae* in culture (Table 3). Coacobre was the best fungicide followed by hydrox, and borax was the least effective at 20g/l concentration. There was no significant ($P<0.05$) difference between mancozeb and ridomil in their growth inhibition effects at this level of concentration with a 3.30cm mean growth each. At 100g/l, forcelet and mancozeb gave the lowest mean growth values of 0.12cm and 0.06cm respectively which were comparable. Borax was still the least effective at this level of concentration giving 2.33cm mean radial growth which was however significantly ($P<0.05$) different from the values obtained in the control experiments. Irrespective of the level of concentration, coacobre gave the best growth inhibition results and borax still remained the least effective of all the fungicides tested.

Assessment of the effect of synthetic fungicides on the radial growth of the test fungus showed that increase in the concentration of the chemicals positively correlated with the growth inhibitions that resulted (Table 3). Similar observations have been made by Ekpe *et* al (1990), Amadioha and Markson (2007), Osai (1992), Madunagu *et al*, (2001), Wokocha and Okereke (2005), Madunagu *et al*, (2005) and Chiejina (2005) using plant extracts; Amadioha (2000), Agrawal and Mehrota (1988), Amiri (2008) on fungicides. The different levels of reductions of radial growth by the synthetic fungicides may probably be due to varying extent of interference of these chemicals with the metabolism of the fungi involved. Deacon (1980) reported that in addition to genetic requirement, the

metabolism of fungi depends, among other factors, on the substrate composition which could have been the active principles in the synthetic fungicides that affect the qualitative state of the fungus.

This influence according to Cooke (1980) is peculiar to each fungus. Agrawal and Mehrota (1988), studying the effect of synthetic fungicides on *Phytophthora colocasiae* observed a positive correlation between mycelial growth inhibition and inhibition in the rate of respiration. Rebollar-Aluiter (2007), in a similar study on the sensitivity of some fungicides to isolates of *Phytophthora cactorum,* came up with the findings that Salicylhydroxamine Acid (SHAM) inhibited the growth of the fungus through inhibition of the alternative pathway which was dependent on the AOX enzyme in mitochondrion respiration operated by plants and fungi. Going by the findings obtained from earlier researches in this area, it appears that the chemicals (whether synthetic or of plant origin) effect their actions through interfering with or inhibiting the normal metabolic activities (especially respiration) of the target pathogen. However, the gravity of the action primarily depends on the type and relative amounts of the active principles in the chemical (fungicide) tested.

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