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***In-vitro* and *In-vivo* Inhibition of *Aspergillus fumigatus* by *Pseudomonas fluorescens* Used as a Microbial Antagonist**

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Abstract: The potential inhibitory effect of *Pseudomonas fluorescens* from cassava starch and flour was studied by using: (i) *in-vitro* inhibition assays with *Aspergillus fumigatus* and (ii) the *in-vivo* *A. fumigatus* rats model of infection. *In-vitro* activity against *A. fumigatus* revealed a moderate capacity of the bacterium in inhibiting the growth of the fungus. *In-vivo* experiments were performed by oral administration of *P. fluorescens* to 12 -14 week-old rats previously infected with *A. fumigatus*. Toxicological data of rat plasma showed that the bacterium had liver improvement functions. Alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities of the rats dosed with *P. fluorescens* alone were lower (2.65 and 20.67 IU/L) than the control. Moreover, no significant ($P>0.05$) rise was observed with the plasma albumin, globulin and total protein. There was a reduction in the count of *A. fumigatus* in rats dosed with both organisms after 7 days of feeding trials. The weight gain by rats in the treated group compared favourably with the control. Histopathological data confirmed protection of the liver, spleen, and kidney while the stomach and small intestine had partial erosion of the mucus membranes.

Key words: Cassava starch, flour, *pseudomonas fluorescens*, *aspergillus fumigatus*, inhibition, toxicology

Introduction

Aspergillus fumigatus is the most commonly isolated species out of the genus *Aspergillus*. It is one of the most potent fungal pathogens reported as causative agents of opportunistic infections in man. It is a thermotolerant fungus and can be isolated from air, plants, foodstuffs, soil, sand beaches, caves and mines and cotton fabrics in the tropics. It is one of the most common causes of systemic fungal disease in humans and animals causing acute or chronic respiratory tract infections. It is a well documented allergen. It is known to cause fungal balls/masses which grows in air spaces without invasion of tissue. *Aspergillus* species are well-known to play a role in three different clinical settings in man: (i) opportunistic infection; (ii) allergic states; and toxicoses. These infections may present in a wide spectrum, varying from local involvement to dissemination and as a whole called aspergillosis. Owing to the ubiquitous, disease causing ability and toxicity of *Aspergillus fumigatus*, humans have discovered and synthesized numerous antifungal compounds (Singh and Singh, 2000). The repeated use of such chemicals has encouraged the development of chemical resistance in target organisms (Goldman *et al.*, 1994). Moreover, these chemicals being non-degradable pollute the atmosphere and can be environmentally harmful, as the chemicals spread out in the air and accumulate in the soil (Nannipieri, 1994). A common wish for the reduction of the environmental impact has caused an increasing interest in the development of alternative control. Microbiological

control methods include bacteria, fungi and vira. One of the methods to reduce the resistance to antibiotics and other adverse effects on host is by using antibiotic resistance inhibitors from plant-origin (Linume *et al.*, 1994; Kim *et al.*, 1995). The possibility of controlling the pathogenic fungi by antagonist microorganisms has been explored by various workers (Blakeman, 1985; Mercier and Reelender, 1987, Pandey *et al.*, 1993).

Pseudomonas fluorescens has the ability to grow at 4°C and hydrolyse gelatin. These characteristics help explain its frequent involvement in spoilage of refrigerated food. The main property that conspires against its becoming important opportunistic pathogen is the inability to grow at body temperature. It is rarely pathogenic for humans, even though they have been found associated with empyema, urinary tract infections and septicemia. Some *Pseudomonads* have been recognized as antagonists of plant fungal pathogens and antibiotic producers (O' Sullivan and O' Gara, 1992). This is probably due to the abundance of this diverse group of bacteria and their obvious importance in the soils. *Pseudomonas* plasmids confer resistance to many antibiotics and antibacterial agents. The purpose of this study was to examine the activity of *Pseudomonas fluorescens* from cassava starch and flour against *Aspergillus fumigatus* *in vitro* and *in vivo*.

Materials and Methods

Cassava tubers collection: Sweet variety of fresh cassava tubers were collected within Akure metropolis of Ondo State, Nigeria and processed into starch and

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flour using the methods of Onabolu *et al.* (1998). The tubers were between 7-12 months old.

Test organisms: The microorganisms used in this investigation (*Aspergillus fumigatus* and *Pseudomonas fluorescens*) were isolated from cassava starch and flour (Olowoyo *et al.*, 2001). The growing fungus was identified based on macro and microscopic characteristics. The physiological and biochemical characteristics of the bacterial strain were examined using the methods of Holt *et al.*, 1994.

Detection of *in-vitro* antifungal activity

Conventional streak method: Antagonism on agar plates, was studied using a modified method of Fokkema (1973). A mycelium disc (7mm diameter) of the fungus was aseptically transferred onto the centre of a fresh and dry Malt Extract Agar (MEA) plate. Then a streak 40mm long of the bacterium was made 23mm from the fungus plug. Control plates consist of only fungal plugs without bacterial streak. Plates were incubated at 25°C and monitored for 7 days. Antagonism was assessed by measuring zones of inhibition between the fungus plug and the bacterium streak. Measurement of the percentage inhibition and intercolony distance were taken daily for 7 days.

Novel ring method: For the novel ring method of Adetuyi and Cartwright (1985), bacterium was cultured on agar plate into confluent growth. Prepared MEA plates were inoculated separately with concentric ring culture of the organism. The fungal plug (7mm) was transferred aseptically into the centre of the plate already inoculated with the test antagonist. Plates were incubated at 25°C and monitored for 7 days. The measurement of the percentage of inhibition and intercolony distance were taken daily.

***In vivo* evaluation of antifungal activity:** The inhibitory efficacy of *Pseudomonas fluorescens* against *Aspergillus fumigatus* infection in rats was verified *in-vivo* by some plasma biochemical markers and histopathological data. Clinically, healthy adult albino rats (12-14 week old) obtained from the Department of Physiology, University of Ibadan were held under specific pathogen-free conditions and given a standard basal diet broiler starter (Amo-Byng Feeds and Concentrates, Oyo State, Nigeria) and demineralized water ad libitum. Animals were subjected to four treatments using the modified method of Henriksson and Conway, 2001; appropriate control group of uninfected, untreated rats, fed with basal diet only; *Aspergillus fumigatus* - infected but untreated animals (A) + basal diet; fungus - infected and bacterium-treated rats (B) + basal diet; uninfected animals given *Pseudomonas fluorescens* (C) + basal diet. Animals were orogastrically dosed with 0.3ml each

of 108cfu/ spores of the fungus and 106cfu/g of the bacterium respectively. The treatments were repeated the second day. A post-ingestion period of 14 days was observed after the administration of the cultures. The rats were killed by cervical dislocation and blood samples collected into EDTA bottles for analysis of some plasma biochemical indices. The liver, kidneys, stomach, spleen and small intestines were removed and grossly observed for any pathological effects.

Monitoring the progress of infection and faecal levels of *A. fumigatus*:

The body weights of animals were recorded daily up to 14 days post-pathogen challenge. The data gathered were used to calculate the following parameters: (i) weight gain = final weight-initial weight (ii) percentage weight gain = weight gain/final weight X100.

For enumeration of viable faecal *Aspergillus fumigatus*, freshly voided faecal pellets were collected and pooled from each rat (0.3-0.4g per rat) at 1,2,7 and 14 days post-dosing (Chang *et al.*, 2001). Faeces were weighed and homogenized. Faecal homogenates were serially diluted in sterile water, and 0.1ml aliquot was added in duplicate onto MEA plates. Plates were incubated in aerobic condition for 4 days at 25°C. Colonies were characterized on the basis of morphology and pigmentation. The population levels were converted to log values before plotting out in graphs.

Histopathological tests: At autopsy the internal organs were inspected for morphological lesions. Samples of the liver, kidney, stomach, spleen and small intestine from each animal were fixed in 10% formalin, dehydrated in different percentages of alcohol, cleared in xylene for 2h and impregnated in liquid wax for 2h for embedding. The embedded organs were sectioned using micro tone and stained with haematoxylin eosin (Silva *et al.*, 1999).

Statistical analysis: Results are expressed as means + standard error of the mean. For statistical comparison, the data gathered were processed by one way analysis of variance (ANOVA), SPSS 10.0. Means were compared by Duncan Multiple Range Test.

Results and Discussion

A study of the morphological and physiological characteristics of the bacterium revealed that the bacterium is non-spore forming gram-negative rod. Cells from 24h culture on nutrient agar are motile, colonies are raised, shiny, slightly greenish-yellow and filiform or slightly spreading. Their consistency is butyrous. Broth is turbid in 12h with a green fluorescent pigment produced in the surface layer. Gelatin is liquefied and nitrates are reduced to nitrites. Indole and hydrogen sulfide are not formed. The bacterium is

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Table 1: Effect of the administration of *Aspergillus fumigatus* and *Pseudomonas fluorescens* on biochemical indices of albino rat plasma

Treatment	AST (IU/l)	ALP (IU/l)	ALT (IU/l)	Albumin (g/dl)	Globulin (g/dl)	Protein (g/dl)
Control	9.42 ^a ±2.28	21.09 ^a ±3.89	3.38±0.96	11.70 ^a ±0.82	29.93 ^a ±0.75	47.41 ^a ±9.04
A	9.54 ^{ab} ±3.80	19.60 ^{ab} ±6.49	2.77 ^{ab} ±0.99	11.77 ^a ±0.52	22.51 ^a ±0.75	32.98 ^{ab} ±4.07
B	8.09 ^{ab} ±3.72	25.14 ^c ±6.00	3.98 ^{ab} ±0.99	12.34 ^{bc} ±0.12	24.96 ^{ac} ±0.56	35.56 ^a ±6.13
C	9.63 ^a ±3.22	20.67 ^c ±2.45	2.65 ^a ±0.48	12.25 ^b ±0.39	45.65 ^b ±0.15	63.52 ^c ±3.59

AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; ALT, alanine aminotransferase. Values are mean ± S.E. (n=4). Means with the same superscript letters along same column are not significantly different (P<0.05).

Treatment: Control: Basal diet alone, B: Basal diet + fungus and bacterium, A: Basal diet + fungus alone, C: Basal diet + bacterium alone

catalase - positive. When grown in a synthetic medium, the bacterium utilize glucose, fructose, arabinose and mannitol with acid production. There is no growth with sucrose and maltose and very slight growth with lactose. Starch is not hydrolyzed. Optimum temperature is about 27°C. Growth occurs at 35°C, but not at 37°C. The isolate is non-pathogenic.

The strain of *Pseudomonas fluorescens* reported in this article inhibited the growth of *A. fumigatus*. Physical contact between the bacterium and mycelium was never observed during the incubation period, though inhibition was relatively weak (Fig. 1). An uncolonised zone was maintained throughout suggesting that a diffusible toxin of bacterial origin was responsible for the inhibition of the mycelium growth. There was considerable variation in inhibitory action, more apparent in the novel ring method. Subsequent increase in inhibition was observed and complemented by a small but progressive decrease in the distance between bacterium and fungus. However, total cessation of growth was achieved after 48h. Fluorescent *Pseudomonas* sp. have been suggested to be important natural antagonist of plant pathogens particularly in relation to the microbial suppression of the take-all fungus (Kloepper *et al.*, 1980).

Table 1 shows the plasma levels of Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein, albumin and globulin of albino rats infected with *Aspergillus fumigatus* and inhibited with *P. fluorescens*. There was no significant difference (P<0.05) between the AST of the control and that of groups A and C. The slight increase in AST of rats fed with basal diet and fungus only could be attributed to possible secretion of mycotoxins; aflatoxin B1 has been implicated in liver damage (Oboh *et al.*, 2000). AST is widely distributed in body tissues; hence many other diseases involving cellular injury may be accompanied by increases in AST activity e.g. pulmonary infections and tumor (Cheesehrough, 1991). Increase in blood AST values may be attributed to both liver damage and possible damage to the heart (American Liver Foundation, 1995; David and Johnson, 1999). Moreover, there was a mild elevation of the ALT

levels in the blood of albino rats in group B. Mild or moderate elevations of ALT and AST are non-specific and may be caused by a wide range of liver diseases. ALT and AST are enzymes that are located in the liver cells and leak out and make their way into the general circulation when liver cells are injured (American Liver Foundation, 1995, 1997). The ALP activity of rats in group B was significantly higher (P<0.05) that of others. ALP is regarded to be a more specific indicator of liver inflammation. The ALP values indicate obstruction to the biliary system; either within the liver or in the larger bile channels outside the liver. The ALP are elevated in a large number of disorders that affect the drainage of bile, such as gallstone or tumor blocking the common bile duct. In affected liver, both the ALT and ALP levels are increased (Strove, 1989). The ALP serves as an indicator of liver damage when there is cholestasis (lack of bile flow) (Johnson, 1999).

The plasma albumin and globulin levels for group B showed significant difference (P<0.05) from that of control. This probably indicates the ability of the bacterium to affect the biosynthesis of albumin from the liver (David and Johnson, 1999) and to stimulate the immune system of the rats. Immunoglobulins are often sought in children with recurrent infections or a combination of infections with injury (Baron *et al.*, 1994). Changes in plasma proteins occur in chronic hepatitis and cirrhosis, which are results of prolonged or extensive liver cell impairment. The obtained plasma protein was not significantly different from that of control. This implies that the administration of *P. fluorescens* to rats will prevent liver damage (Fig. 2).

Weight was monitored as an index of the general well-being of the animals for over 2 weeks. Animals dosed with both fungus and bacterium (Group B) had no significant different (P<0.05). The weight gain in the control was higher than the treated group on the fourteen day after challenge (Fig. 3) but compared favourably with those of the control. This implies that the treatment enhances the growth of the animals. Animals dosed with fungus alone lost less weight than animals treated with the bacterium at post-pathogen test.

Seven day post-challenge with *Pseudomonas*

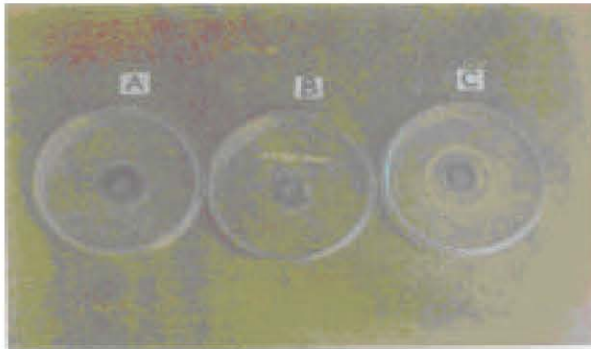


Fig. 1: Antagonistic zone between *Aspergillus fumigatus* and *Pseudomonas fluorescens*. Photograph taken 5 days after inoculation

- A. control
- B. fokkema/conventional streak method
- C. concentric ring bioassay

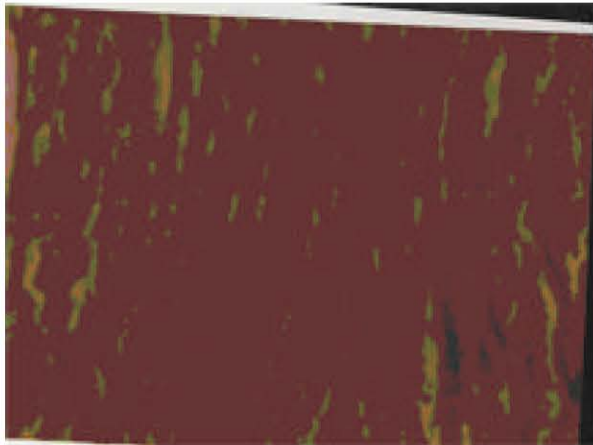


Fig. 2: Section of the liver of rat fed with *Aspergillus fumigatus* and challenged with *Pseudomonas fluorescens* showing normal hepatic features (X 200)

fluorescens, animals of group A generally contained low faecal numbers of the pathogen (Fig. 4). The faecal levels of the bacterium increased in animals of groups B & C. Although animals were dosed with isolate for only 2 days after pathogen challenge, they were protected beyond that time. These observations are in line with observations made by Henriksson and Conway (2001), who demonstrated that a range of new bifidobacteria may provide protection against infection by *Salmonella typhimurium* in mice resulting in both initial reduction of *S. typhimurium* levels in faeces and reduced weight loss of animals challenged with the pathogen. Bernet - Carmard *et al.* (1997) also demonstrated that the protective effect on mice against *Salmonella typhimurium* remained for at least 3 days post-termination of the supply of a *Lactobacillus acidophilus* strain. There is

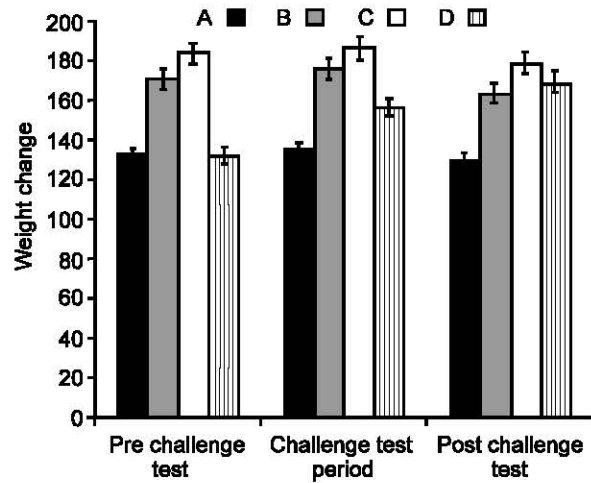


Fig. 3: Average weight changes of rats dosed with *Aspergillus fumigatus* and *Pseudomonas fluorescens*

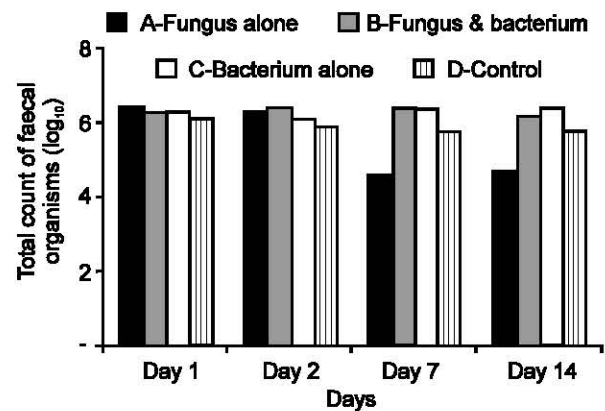


Fig. 4: Total count of faecal organisms in rats dosed with *Aspergillus fumigatus* and *Pseudomonas fluorescens*

evidence (Fig. 4) to suggest that inhibition of *A. fumigatus* may remain up to 2 weeks after termination of supply of *P. fluorescens*.

The result of the histopathological examination of the kidney, spleen and liver of the rats revealed that *Pseudomonas fluorescens* protected the organs from any damage. However, it causes the mucosa surface of the small intestine to be detached from the submucosa layer and partial erosion of the mucus membrane of the stomach.

As reported in this paper, an attempt has been made to investigate whether the phenomenon of 'microbial antagonism' could be utilized to control the infection of *A. fumigatus*. Our experiments have shown that the bacterial strain referred to has some protective action against this opportunistic fungus.

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