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Antifungal Activities of Griseofulvin and Associated Bacteria of Cassava (*Manihot esculenta* Crantz)

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Abstract: The antifungal activity of bacterial isolates of cassava products origin as well as a known antifungal agent Griseofulvin was determined. The fungi used were *Aspergillus flavus*, *A. niger* and *Rhizoctonia solani*. The antagonists were: *Pseudomonas flourescens*, *Escherichia coli*, *Bacillus subtilis* and *B. pumilus*. The results obtained showed that the degree of antagonistic activity against the fungal isolates varied. *In vitro* bioassay using the disc diffusion technique showed that *Bacillus pumilus* had considerable degree of antagonistic activity against *Aspergillus flavus* with MIC value of 2.0 while *Escherichia coli* had significant activity against *Rhizoctonia solani* (2.0) and *Aspergillus flavus* (4.0). The antagonistic activity of *Pseudomonas flourescens* against *Aspergillus niger* was observed within 24-48 h of growth. Varying degrees of activity were observed after incubation period using the known antifungal agent Griseofulvin against fungal isolates and its activity against *Aspergillus niger* far exceeded 48 h. The minimal inhibitory concentration obtained using the agar well diffusion technique and measured in percentage varied considerably. The least concentration for inhibition was 2.0 using bacterial metabolites while the antifungal agent was able to inhibit the growth of a fungus at concentration of 0.5. The metabolites exhibited stronger antifungal effect at higher concentration and the preliminary results of the investigation appears to indicate the suitability of bacteria isolates in the antagonism of fungal pathogens of cassava.

Key words: Bacterial isolates, antifungal activities, Griseofulvin, cassava

INTRODUCTION

The wide and indiscriminate use of chemical preservatives has been the cause of the appearance of resistant microorganisms over and over, leading to the occurrence of emerging food borne diseases (Gibbons, 1992; Kaur and Arora, 1999; Akinpelu, 2001). As a result of this, the interest to obtain alternative antimicrobial agents for use against food pathogens has increased. The possibility of controlling pathogenic fungi by antagonistic microorganisms has been explored by various workers (Blakeman, 1985; Mercier and Reelender, 1987; Pandey *et al.*, 1993; Agarry, 2005). The development of antifungal drugs (agents) has lagged behind than that of antibacterial agents. This is a predictable consequence of the cellular structure of the organism.

Antagonism is the interference with or inhibition of the growth of a living organism due to the production of a specified antibiotic substance. An antagonist is an organism that adversely affects the growth of other microorganism growing in association with it. Antagonist is sometimes introduced to the surface of products with the aim of controlling a pathogenic organism of that product. This is referred to as biocontrol. Numerous antagonistic microorganisms with the potential for biocontrol of plant diseases has been reported and identified over the past few years (Fravel, 1988; Ferreira *et al.*, 1991).

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Effective antifungal agents frequently either extract membrane sterols or prevent their synthesis (Lansing *et al.*, 2005). Griseofulvin has been available since 1958 in treating diseases caused by fungi. Griseofulvin comes from the mould, *Penicillium griseofulvum*. It stops fungal cell's division (i.e., fungistatic) but does not kill them outrightly (Gull and Triner, 1973).

This study was carried out to compare and contrast the antagonistic activity of bacterial isolates of cassava product origin and griseofulvin against the pathogenic fungi.

MATERIALS AND METHODS

Test Organisms

Stock cultures of previously identified bacteria and fungi strains isolated from cassava products viz., grated cassava, starch and flour were used (Olowoyo *et al.*, 2001).

Determination of Spore Count

The fungi namely *Aspergillus flavus*, *A. niger* and *Rhizoctonia solani* were cultured on potato dextrose agar plates for 7 days. The cells were separately harvested by washing with sterile 0.85% physiological saline into suitable receptacles. The cell suspension was then diluted with the same liquid and the spores counted using Hawksley haemocytometer with Neubauer ruling (Evandro *et al.*, 2005).

Determination of Bacteria Population

The bacterial isolates were inoculated in broth and incubated at 37°C for 24 h. The broth cultures were then diluted to a dilution factor of 10⁻⁸. One milliliter of the broth culture was taken from the test tube with dilution factor of 10⁻⁸ and pour plated. The plates were incubated and after 24 h the colony forming unit per mL was counted.

Extraction of Metabolite

The 24 h broth culture was decanted aseptically into sterile centrifuge tubes at equal volumes. The tubes were then centrifuged for 20 min at 3000/rev/min. The supernatant containing the bacteria metabolite was prepared by adding 0.4 mL of the metabolite to 0.04 mL of Tween 80. The mixture was then made up to 5 mL by adding sterile distilled water and shaken vigorously for 5 min (Evandro *et al.*, 2005). The stock of the antifungal agent griseofulvin was prepared by dissolving 500 g of griseofulvin in 0.4 mL of sterile distilled water and then completing the mixture to 5 mL by adding sterile distilled water. The percentage final concentration of both the metabolite and griseofulvin were achieved.

Screening of Antifungal Activity

In vitro test of the antagonistic activities of bacterial isolates on fungi was performed using a modified disc diffusion tests described by Lima *et al.* (1993). One milliliter of the fungal suspension prepared with sterile physiological saline solution was uniformly spread on acidified potato dextrose agar plates. After inoculum's absorption, sterile filter paper discs (Whatmann No. 1 diameter 6 mm) were soaked with the bacterial metabolites and placed at different points on the potato dextrose agar plates inoculated with the fungal suspension. The plates were then incubated at 25-28°C for 5-7 days. At the end of the incubation period, the inhibition halo diameters were measured and expressed in millimeters. When the inhibition halo diameter observed was equal or higher than 10 mm diameter, it was considered a positive antifungal activity. The same procedure was followed while screening for the antifungal activity of griseofulvin.

Determination of the Minimum Inhibitory Concentration

Bacterial isolates that presented antifungal activity in the screening assay were evaluated for their minimum inhibitory concentration using the plate diffusion procedure involving wells in dishes (Hadeck and Gregor, 2000). This was achieved by uniformly spreading 1 mL of fungal suspension

prepared with sterile 0.85% physiological saline solution on acidified Potato Dextrose Agar (PDA) plates. After inoculum absorption by acidified PDA, wells were made using sterile cork borers, which were then filled with 0.1 mL of the different percentage final concentration of the bacteria metabolite. The control was carried out by filling the wells with 0.1 mL of sterile distilled water. The same procedure was followed for the determination of the minimum inhibitory concentration (MIC) of the antifungal agent griseofulvin.

RESULTS

In vitro Bioassay of Bacterial Antagonists

Not all bacteria isolates were antagonistic. Three bacteria namely *Bacillus pumilus*, *Pseudomonas fluorescens* and *Escherichia coli* were able to inhibit the growth of three fungi namely, *Aspergillus flavus*, *A. niger* and *Rhizoctonia solani*. The minimum inhibitory concentrations of the bacterial metabolites were obtained using the agar well diffusion technique at different time intervals (Fig. 1-3). *Bacillus pumilus* had MIC value of 2.0 on *Aspergillus flavus* while *Escherichia coli* had MIC values of 2.0 and 4.0 on *Aspergillus flavus* and *Rhizoctonia solani*, respectively.

In vitro Bioassay of Griseofulvin

The minimum inhibitory concentrations obtained using the agar well diffusion bioassay technique at different time interval for the fungi are presented in Fig. 4-6. The MIC values of griseofulvin on *Aspergillus flavus* was 0.5, *Aspergillus niger* (2.0) and *Rhizoctonia solani* (1.0). Griseofulvin inhibited the growth of the fungal isolates at reduced concentration than that of the bacterial metabolites.

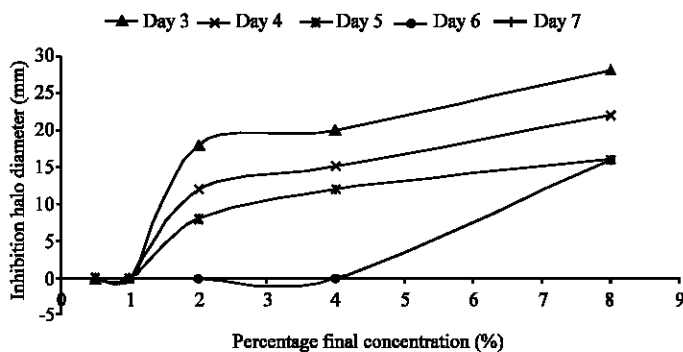


Fig. 1: Antagonistic activity of *Bacillus pumilus* on *Aspergillus flavus* at different time intervals

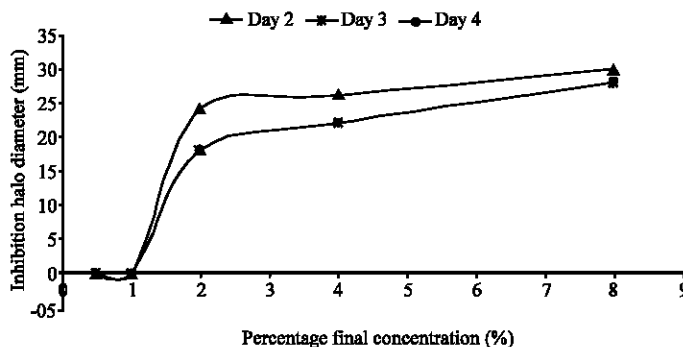


Fig. 2: Antagonistic activity of *Escherichia coli* on *Rhizoctonia solani* at different time intervals

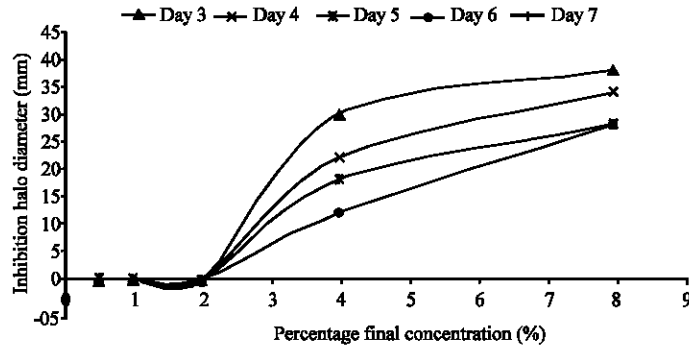


Fig. 3: Antagonistic activity of *Escherichia coli* on *Aspergillus flavus* at different time intervals

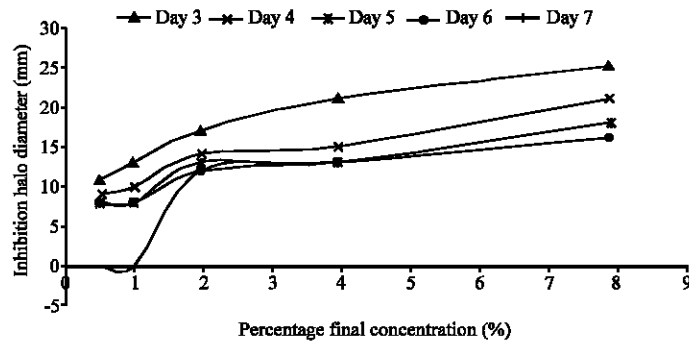


Fig. 4: Antagonistic effect of *Griseofulvin* on *Aspergillus niger* at different time intervals

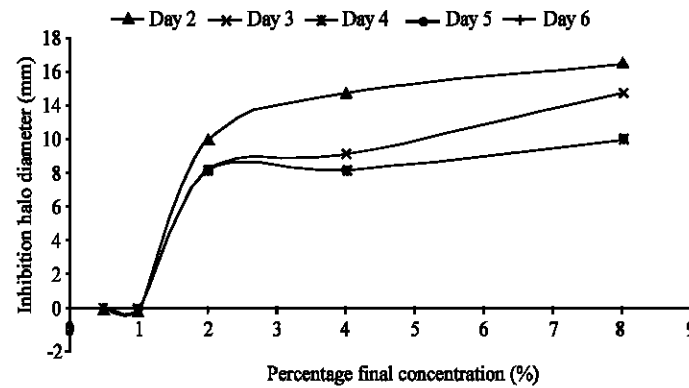


Fig. 5: Antagonistic effect of *Griseofulvin* on *Rhizoctonia solani* at different time intervals

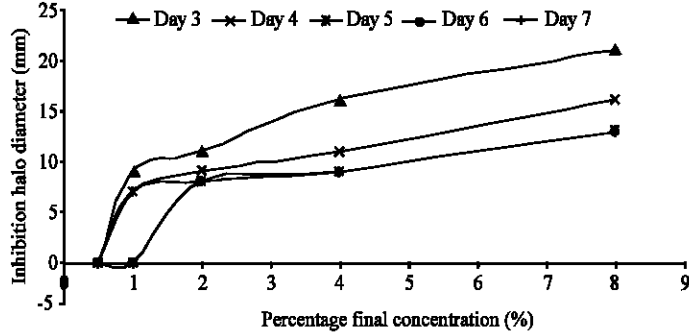


Fig. 6: Antagonistic effect of *Grisofulvin* on *Aspergillus flavus* at different time intervals

DISCUSSION

The growth of the mould was slow and resulted in abnormal misshapen colony development, which was difficult to quantify. Studies have shown that *Bacillus* sp are able to inhibit the growth of some species of *Aspergillus* while *Escherichia coli* has been shown to inhibit the growth of *A. flavus* (Agarry *et al.*, 2005). Also, susceptibility patterns were observed between *Escherichia coli* and *Rhizoctonia solani* while the antifungal activity of *Pseudomonas fluorescens* on *Aspergillus niger* was observed within 24-48 h of growth after which the inhibition halo diameter could not be observed. Previous research in which *Pseudomonas fluorescens* has been used as an antagonist against *Aspergillus* sp. revealed weak inhibition (Agarry and Osho, 2005).

The MIC values determined using the agar well diffusion technique were recorded at different time intervals. The MIC values for *Bacillus pumilus* on *Aspergillus flavus* were 2.0 while the values of *Escherichia coli* on *Rhizoctonia solani* and *A. flavus* was 2.0 and 4.0, respectively. For the fast growing fungal isolates namely *Aspergillus niger* and *Rhizoctonia solani*, the values were recorded after 48 h of growth while for the slow growing fungus *Aspergillus flavus*, the inhibition halo diameter was measured and recorded after 72 h of growth. The inhibition halo diameter at varying percentage final concentration gradually reduced and the values consistently decreased until a point was reached where the inhibition halo diameter became static. This may be attributed to the fact that the rate of killing slows down and subsequently stops due to the survival of more resistant strains of the fungal isolates (Lansing *et al.*, 2005). The significance of the values showing the extent of antifungal activity of the bacterial isolates was the function of the concentration of the bacterial metabolites.

When the test bacterial isolates were replaced with a known antifungal agent griseofulvin, the susceptibility of *Aspergillus niger* to the agent far exceeded 24 - 48 h, However, when the MIC was determined for the agent, the least value was 0.5 against *Aspergillus niger* while the highest was 2.0 for *Rhizoctonia solani*. *Aspergillus flavus* had a reduced MIC value of 1.0 as compared to when the bacterial metabolite was used. It was observed that the antifungal agent was more effective than the bacterial metabolites.

CONCLUSION

The bacterial isolates possess some degree of antifungal activity against fungal pathogens of cassava products. Further studies will be carried out to extract and purify the bioactive ingredients of the bacterial metabolites.

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