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Antagonistic Potential of Soil Bacteria against Plant Pathogenic Fungi: *Aspergillus niger*

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Authors' contributions

This work was carried out in collaboration between both authors. Author PTN designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Author APR managed the analyses of the study. Authors APR and PTN managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was carried out with the aim of assessing the antagonistic potentials of soil bacteria against the plant pathogenic fungi *Aspergillus niger.*

Study Design: The complete Randomized Design was the experimental design for this study.

Place and duration of study: This study took place in University of Calabar, Cross River State Nigeria within the pace of one month.

Methodology: Standard microbiological methods were used to identify bacteria and obtain pure culture of fungi

Results: Bacterial isolates were evaluated for their potential of antagonism against *Aspergillus niger* isolated from spoilt tomatoes. The percentage inhibition of mycelia growth by these isolates recorded values as 27%, 0%, 66%, 40%, 97%, 0% and 23% respectively. The isolates were analyzed and identified as *Bacillus* spp, *Enterobacter* spp., *Pseudomonas* spp., *Proteus* spp., *Escherichia* coli, *Streptococcus* spp. and *Staphylococcus* spp respectively.

Conclusion: In the present work high levels of in vitro control of *A. niger* have been verified by *E. coli* isolates. However, in future investigations molecular studies should be carried out confirming that the isolates obtained from *E. coli* are not pathogenic to humans.

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Keywords: Aspergillus niger; antagonisism; isolates; mycelia; dilution; botanical garden and biochemical.

1. INTRODUCTION

The soil is made up of billions of microorganisms. There is usually an interaction between biotic and abiotic factor within the soil. It is the most reliable and important reservoir of biodiversity within the planet earth. In recent years, species of gram positive to gram negative bacteria have been evaluated for their antagonistic effects on fungi [1,2]. Their ability to degrade complex organic compound and soil minerals to liberate nutrients that are essential for plant growth are some of their outstanding benefits [3].

Aspergillus niger is one of the major pathogens of plant. It has been identified as the etiological agent of plant diseases like black mold or black rot of onions, garlic and tomatoes, crown rot of peanuts [4] Reduction in plant production and commercial loses are some of the economic aftermath of some diseases caused by this fungi. Black mold caused by *Aspergillus* is the foremost post harvest diseases of tomatoes and oranges. Imazole and thiabendazole are synthetic fungicide currently used to control post harvest infection. However, it has often resulted in fungicide residue in the fruit which may cause harm to human health if consumed.

Antagonism in this case according to [5] is the reaction of any organisms that suppresses or interferes with the normal growth and activity of plant pathogens. These antagonist organisms can be used for pest control and are referred to as biological control agents. An understanding of the mode of actions of these microbes is important for developing protocols for choosing microbial agents. Commonly recognized mode of action of microbial agent include: antibiosis, competition for nutrient and space and induction of host resistance [6].

Enzymes like Lytic enzymes produced by microbes are capable of breaking down polymeric compounds including chitin, protein, cellulose, hemicelluloses and DNA [7].Volatile compounds such as Hydrogen cyanide is reported to be one of the antifungal secondary metabolites produced by bacterial antagonism [5]. Hydrogen cyanide effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic micro-organism at picomolar concentrations [8].

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were collected from the Botanical garden of University of Calabar. Using a hand auger the sample was taken from a depth of 10 to 20 cm below the soil surface. Soil sample was air dried at ambient temperature, mesh, sieved and preserved in polyethylene bags in the laboratory.

Spoiled tomato fruit was conveyed to the laboratory in sterile polythene bag from Watt market in Calabar south L.G.A.

2.2 Culture Media Preparation and Dilution

One gram of the soil sample was added to conical flask containing 100 ml of water. The soil and water were thereafter thoroughly shaken well to obtain a homogenous suspension. From the mixture, 1 ml of the sample was used and serially diluted into seven fold dilutions. The tomato sample was also diluted serially according to standard procedures. Nutrient Agar, Macconkey agar, Eosin methylene blue agar and Potato Dextrose Agar were prepared according to manufacturer's instruction for the isolation of heterotrophic bacteria, gram negative and enteric bacilli, *coliforms,* and fungi.

2.3 Isolation and Identification

2.3.1 Bacteria

After serial dilution of samples (1 ml of each soil dilution (10^{-3}, 10^{-5} and 10^{-7}) were inoculated into sterile petri dishes. Molten agar media were thereafter poured into it, swirled carefully and allowed to solidify. The plates were incubated at 37° for 24 hrs. At the end of the incubation period emerging colonies were enumerated using the colony counter.

Discrete colonies were subjected to gram staining and microscopy to differentiate gram negative (-ve) and gram positive bacteria. Various biochemical tests such as oxidase test, indole test, motility test, sugar fermentation, hydrogen sulphide production test, gas production test, methyl red test, catalase test,

citrate utilization test, urease test, were also carried out for proper identification of organisms.

2.3.2 Isolation of *Aspergillus niger*

With the aid of a sterile pipette, 1ml of tomato dilution was cultured 10^{-3}, 10^{-5} and 10^{-7} in PDA at ambient temperature for 72 hrs. *Aspergillus niger* was identified in mixed culture using colonial morphology and sub-cultured in a PDA plate that was supplemented with ampicillin in order to obtain a pure culture.

2.4 Evaluation of soil Bacteria for Antagonistic Potentials

2.4.1 Multiplication of isolates

An aliquot of 25 ml peptone broth was poured into test tubes which was covered with a plastic cap and sterilized in an autoclave for 15 min at 121℃. Bacteria isolated and fungal pathogen were then separately transferred from pure culture into test tubes containing peptone broth and kept to stand for 24 hrs at ambient temperature.

2.4.2 Screening for fugal antagonism

Agar well diffusion method was used to screen for potential antagonist. PDA plates were prepared by sterilizing the media at 121℃ for 15min. The media was then poured into plates and kept to solidify. With the aid of a sterile borer, three wells were made on 10 PDA plates. Using a sterilized wire loop, a loop full of the pathogen in peptone broth was streaked on the PDA surface in the plates. Using a sterile pipette, 0.2 ml of each of the nine bacteria isolates was transferred from the peptone broth into 9 plates inoculated with the pathogen. One plate served as a control.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Characterization of isolates

After serial dilution and culturing of microbes, nine different microbes were identified. These organisms presented different morphological characteristics and reacted to the series of biochemical tests. The biochemical characterization of isolates is represented in Table 1. Their colonial morphology and reaction to gram stain is represented in Table 2.

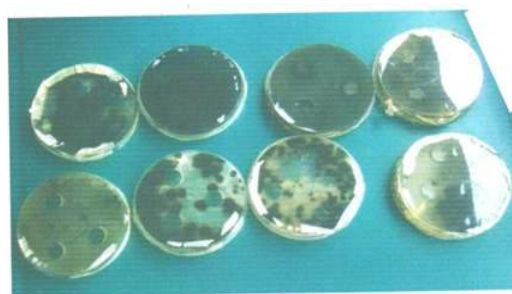

Plate 1. Showing antagonism activities of soil bacteria

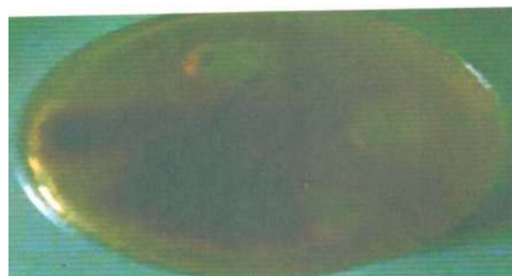

Plate 2. Mycelia reduction by *Bacillus* spp

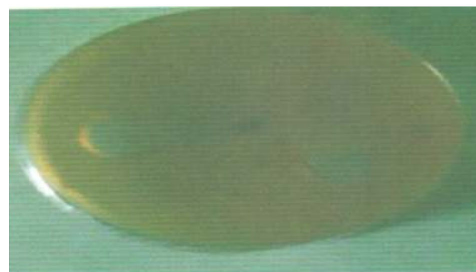

Plate 3. Mycelia reduction by *Escherichia coli*

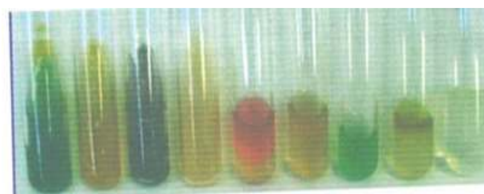

Plate 4. Biochemical reaction of *Escherichia coli*

3.1.2 Screening for fugal antagonism

Antagonist activity was measured with respect to the reduction in growth of fungal mycelia during the interaction with different bacterial isolates. Zones of inhibition (ZOI) were measured in millimeters. Bacterial isolated with ZOI of diameter 20 mm and above were considered

effective antagonist. Percentage of mycelia reduction was calculated using the formular.

$$\% \text{ reduction} = \frac{A\text{-}Dgp}{Dgc} \quad 00$$

Where:

A = 1
Dgp = Diameter of mycelium growth on plate
Dgc = Diameter of mycelial growth on control

Plate 5. Biochemical reaction of *Pseudomonas spp*

4. DISCUSSION

From this study it is clear that bacterial antagonism to fungal growth is a reality and different soil bacteria posses varying capacities of antagonism to fungi in the soil. While some bacteria posses high level of antagonism (eg *Pseudomon spp* and *Bacillus spp*), bacteria like *Escherichia coli* posses a very high level of antagonism against fungi growth. No doubt some bacteria as well posses little or no inhibition to fungal growth around them. *Escherichia coli* from the soil in the University garden in this research recorded the highest mycelia inhibition of up to 97% followed by *Pseudomonas spp* which recorded up to 67.7% inhibition in close relation to bacillus with 67.7% *Enterobacter spp and streptococcus* did not show any antagonistic potential against the test fungus. These findings are in agreement with [4], which stated that the mycelia growth of many species of *Aspergillus* and *Fusarium* was inhbited by antifungal potential of *bacillus* spp, *Pseudomonas* spp and *Streptococcus* spp.

Furthermore, the studies revealed that the degree of inhibition of mycelia growth varies between different strains of bacteria of the same species. For instance *Escherichia coli* with colony code (SB3) showed differed percentage of mycelia inhibition from *Escherichia coli* with colony code (SB7).It was also observed that *bacillus* spp with the colony code (SB1) with had higher mycelia inhibition percentage than B*acillus spp* colony code (SB5).The high rate of resistance of mycelia growth by species of Bacillus and Pseudomonas is in agreement with the findings of [9].

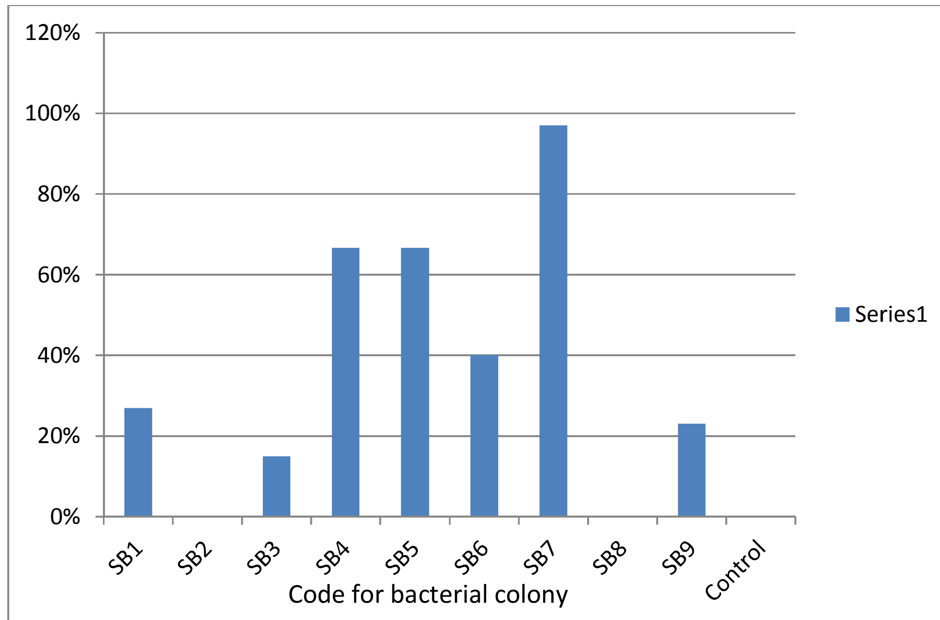

Fig. 1. Percentage of mycelia inhibition

Key: SB1- Bacillus, SB2- Enterobacter, SB3- E. coli, SB4- Pseudomonas, SB5 - Bacillus Spp SB6 - Proteus spp, SB7 - E. coli, SB8 – Staphylococcus, SB8 - Staphylococcus

Table 1. Biochemical characterization and identification of biochemical reaction

Colony code	Lac	Man	Glu	Cit	Ind	Urea	Oxid	Mr	Vp	H_2s	Gas	Mot	Slope	Butt	Cat	Probably organism
SB_1	-	-	+	+	NR	NR	NR	NR	NR	-	-	-	R	Y	-	*Bacillus*
SB_2	+	+	+	+	-	-	-	+	-	-	+	+	Y	Y	+	*Enterobacter*
SB_3	+	+	+	-	+	-	-	-	+	-	+	+	Y	Y	+	*E. coli*
SB_4																*Pseudomonas*
SB_5	-	-	+	+	+	NR	NR	NR	NR	NR	-	-	R	Y	-	*Bacillus Spp*
SB_6	-	-	+	+	+	+	-	+	-	+	+	+	R	Y	+	*Proteus spp*
SB_7	+	+	+	-	-	+	-	+	-	-	+	+	Y	Y	+	*E.coli*
SB_8	+	-	+	-	-	NR	NR	NR	NR	NR	-	-	Y	Y	-	*Staphylococcus*
SB_9	+	-	+	+	-	NR	NR	NR	NR	NR	-	-	Y	Y	+	*Staphylococcus*

H_2S -hydrogen sulphide test, MOT- Motility test, Y - Yellow, NR - Not Required, LAC - Lactose fermentation test, MAN - Mannitol Utilization, GLU -Glucose utilization, CIT - Citrate test IND - Indole test, OXID-Oxidase test, MR-Methyl Red test, VP-vogues proskaver test

Table 2. Colonial morphology and gram stain reaction of isolates from soil sample

Colony code	Colony description	Gram reaction
SB_1	Gray, Irregular, dry raised	G+Ve long rods
SB_2	Translucent, round, moist, flat	G-ve short rods
SB_3	Pink, circular, moist, raise	G-Ve short rods
SB_4	Cream circular moist round smooth and raise	Gram-Ve
SB_5	Irregular, dry and raised	G+Ve rods
SB_6	Gray circular convex moist	G-ve rods
SB_7	Gram circular convex dry	G+ve cocci inchains
SB_8	Cream circular convex dry	G+ve cocci incharns
SB_9	Cream irregular dry raised	G+ve cocci in cluster

SB ⟶ *Soil bacteria*

Table 3. Illustrates the diameter and mean diameter of fungal mycelia growth

Colony code	Mean diamete of fungal mycelia inhibition	Diameter of mycelia growth
SB_1	16.5 ± 1.53	43.7mm
SB_2	0.0 ± 0.0	60.0mm
SB_3	5.0 ± 2.0	50.7mm
SB_4	25.3 ± 1.15	20mm
SB_5	24.0 ± 2.0	20mm
SB_6	21.3 ± 1.53	36mm
SB_7	30.0 ± 1.0	2mm
SB_8	0.0 ± 0.0	60mm
SB_9	8.3 ± 1.52	46mm
Control	0.0mm	60mm

The inhibition of fungal growth may be due to the production of antifungal metabolites. This bye product by the high proportion antifungal strain may be associated with an ecological role playing a defensive action to maintain their niche or enabling the invasion of strain into an established microbial community [10].

5. CONCLUSION

In the present work high levels of in vitro control of *A. niger* have been verified by *E. coli* isolates. However, in future investigations molecular studies should be carried out confirming that the isolates obtained from *E. coli* are not pathogenic to humans. *Pseudomonas* spp and *Bacillus* spp could be used as bio-control agents against the phyto-pathogenic fungi they also showed a reasonable inhibitor effect on fungal spore germination.

6. RECOMMENDATION

Use of Bacterial antagonist to manage plant diseases seems to be a promising alternative strategy and should be adopted for the control of some diseases on different plants and crops. However this study should investigated more extensively for food safety before commercialization.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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