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

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Authors' contributions

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

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ABSTRACT

Soil bacteria are able to synthesize a wide range of metabolites with fungicidal activity. Nine bacterial isolates were obtained from the botanical garden of university of Calabar. Preliminary examination of isolates was carried out using morphological characteristics and Gram's reaction. These isolates were designated with codes SB1, SB2, SB3, SB4, SB5, SB6, SB7, and SB8. Bacterial isolates were evaluated for their potential of antagonism against *Aspergillus niger* isolated from spoiled vegetables like tomatoes by using agar diffusion technique. Percentage inhibition of mycelial growth by these isolates recorded values as 27%, 0%, 66%, 40%, 97%, 0% and 23% respectively. Isolates were analyzed through several biochemical tests and were identified as *Bacillus sp.*, *Enterobacter spp*., *Pseudomonas spp*., *Proteus spp.*, *Escherichia coli*, *Streptococcus spp.* and *Staphylococcus spp.* respectively. These result indicated that bacterial species exhibited varying degree of antagonism against the fungus *Aspergillus niger*. *Escherichia coli* showed maximum inhibitory potential against tested fungus with reduction of up to 97% in their fungal

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growth. *Pseudomonas spp.* and *Bacillus spp.* followed with 66.7%. This result showed that *Pseudomonas spp.* and Bacillus *spp.* exhibited similar percentage of antagonism against *Aspergillus niger*. From the results obtained, it can be interpreted that test bacterial species can be used as fungal agents like *Aspergillus niger*.

Keywords: Antagonistic potential; soil bacteria; pathogenic fungi; Aspergillus niger; fungicidal.

1. INTRODUCTION

Soils are exceptionally complex and highly dynamic systems rising from the interaction of biotic and abiotic processes occurring for over billions of years [1,2]. Soils are the most important reservoir of biodiversity on the planet, which is true for microorganisms. Within the last decade a large array of soil bacteria including specie belonging to the genera *Pseudomonas*, *Ascospirillum*, *Ascobacter*, *Klebsiella*, *Enterobacter* and *Alcaligens*. *Alcaligens*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Serratia,* have been evaluated for their antagonistic effect on fungi [3,4]. Among their overall beneficial effects are their ability to deplete the immediate environment of available nutrients such as iron and to elude various metabolites thereby promoting plant growth [4,5].

Aspergillus niger are among the major pathogens of plants. Although they are generally recognized as safe by the food and drug agency, they cause several diseases in plants including black mold or black rot of onions, garlic and tomatoes, crown rot of peanuts [6]. Infection of plants by this pathogen has resulted in very low production and also commercial losses. Black mold caused by *Aspergillus* is the foremost post-harvest diseases of tomatoes and oranges. Synthetic fungicide such as imazole and thiabendazole are currently used to control post-harvest infection although this often results in fungicide residue in the fruit which may negatively affect human health [7].

In general, Antagonism refers to the reaction of any organism that suppresses or interferes with the normal growth and activity of plant pathogens [8]. These organisms which are referred to as antagonist can be used for pest control and are referred to as biological control agents, antagonistic strains of soil bacteria have been evaluated for their activity against pre and post-harvest pathogens [9,10]. An understanding of the mode of actions of antagonist 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 [11]. However, non-antibiotic modes of action are usually preferred for biocontrol of post-harvest diseases. This research evaluates soil bacteria as potential antagonist and their application as biocontrol agents. The main objectives of current study were to isolate and characterize bacterial flora of the University of Calabar botanical garden, and screen antagonists for *in-vitro* mycelia inhibition of *Aspergillus niger* and identify the most effective isolates as biocontrol agent.

2. MATERIALS AND METHODS

2.1 Sample Collection

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

Spoiled tomato fruit was also brought from one of the popular market (Watt market) in Calabar, Cross river State, Nigeria and conveyed to the laboratory in sterile polythene bag.

2.2 Serial Dilution of Samples

Ten grams (10 g) of soil (for isolation of soil bacteria) was weighed using weighing balance and added to conical flask containing 100 ml of water and shaken well to get homogenous suspension that served as aliquot. From the aliquot 1 ml of the sample was used and serially diluted up to seven dilutions. The same serial dilution was followed for the spoiled tomato sample which had fungal growth on it (black cottony growth) [12].

2.3 Inoculation Technique (Isolation of Bacteria)

Pour plate technique was applied wherein 1ml of each soil dilution (10^{-3}, 10^{-5} and 10^{-7}) were

inoculated into sterile Petri dishes and molten nutrient agar media was poured onto it and swirled carefully and allowed to solidify and the plates were incubated at 37℃ for 24 hrs. At the end of the incubation period emerging colonies were enumerated using the colony counter.

2.4 Isolation of Fungus (*Aspergillus niger*)

Pour plate technique was applied wherein 1 ml of each dilution from spoiled tomato sample (10^{-3}, 10^{-5} and 10^{-7}) were poured onto it Petri dishes. Molten Potato Dextrose Agar (PDA) medium was poured into inoculated plates and allowed to solidify. These was kept at room temperature for 72 hours. *Aspergillus niger* was identified in mixed culture using colonial morphology.

2.5 Purification of Fungi

The Potato Dextrose Agar (PDA) plates were prepared with addition of ampicillin. 0.5 mm mycelial growth with black conidia of *Aspergillus niger* was transferred from mixed culture plates into the purification plates. This was kept at room temperature for 72 hours.

2.6 Preliminary Identification of Morphological Characteristics of Bacteria Isolates

Emerging and visible colonies were enumerated using a colony counter (Stuart scientific model). The method of Barrow & Feltham [13], was used to read and describe the morphological characteristics of colonies of the isolates on plates. All discrete colonies were subjected to Gram staining and microscopy to differentiate Gram negative and Gram positive bacteria.

2.7 Evaluation of Soil Bacteria with Antagonistic Potentials

2.7.1 Multiplication of isolates

The bacterial colonies suspected of having antagonistic effect were re-cultured in a peptone broth for multiplication. Twenty five milliliter (25 ml) aliquot of peptone broth was poured into test tubes which was covered with a cap and sterilized in an autoclave for 15 minutes at 121℃. These was kept at room temperature for one hour. Isolated bacterial and fungal pathogen were then separately transferred from pure culture into test tubes containing peptone broth and kept to stand for 24 hours at room temperature. After 24 hours the antagonist and pathogen were used for evaluation of bacterial isolates.

2.7.2 Screening for potential antagonist

Agar well diffusion method was used to screen for potential antagonist. PDA plates were prepared by sterilizing the media at 121℃ for 30 minutes. Media was poured into plates and kept to solidify, using a sterile borer, three wells were made on 10 PDA plates. Using a sterilized wire loop, a loopful of the pathogen in peptone broth was streaked on these PDA 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. Plates were wrapped with foil and stored at room temperature.

2.8 Biochemical Test

Different biochemical tests were employed to effectively identify the different isolates. The biochemical tests carried out were: oxidase test, motility test, sugar fermentations, hydrogen sulphide production, gas production test, indole test, methyl red test/ vogues proskauer test, citrate utilization test, urease test and catalase test [14].

3. RESULTS

A total number of culturable bacterial isolates were obtained from soil sample of the University of Calabar botanical garden. The result as represented in Table 1 shows varied colony forming unit of bacterial isolates. Isolates were characterized by their colonial morphology and Gram's staining and were classified as Gram positive rods, Gram negative rods and Gram positive cocci. Antagonist activity was measured as the reduction in growth of fungal mycelia during the interaction with different bacterial isolates. Zones of inhibition (ZOI) were measured in millimeters. Bacterial isolates with ZOI of diameter 20 mm and above were considered effective antagonist. Percentage of mycelia reduction was calculated using the formula:

$$\% \text{ reduction} = \frac{(1 - \text{diameter of mycelium growth on plate})}{\text{Diameter of mycelium growth on control}} \times \frac{100}{1}$$

Plate 1. Reduction in fungal mycelial growth by *Escherichia coli*

Plate 2. Reduction in fungal mycelial growth by *Bacillus spp*.

4. DISCUSSION AND CONCLUSION

Result obtained from the conducted studies showed that 9 isolates were obtained from the University botanical garden. Bacteria species isolated included *Bacillus spp.*, *Enterobacter sp.*, *Pseudomonas sp.*, *Proteus sp., Streptococcus sp., Staphylococcus sp.* and *Escherichia coli.* Out of 9 isolates 7 showed antagonistic effect against *Aspergillus niger.* Percentage of mycelia inhibition were recorded as follows: *Bacillus sp.* (SB1) 27%; *Enterobacter sp.* (SB2) 0%; *Escherichia coli* (SB3) 15%; *Pseudomonas sp.* (SB4) 66.70%; *Bacillus sp.* (SB5) 66.70%; *Proteus sp.* (SB6) 40%; *Escherichia coli* (SB7) *97%, Streptococcus (SB8) 0%, Staphylococcus sp*. (SB9) 23*%.*

In this present study *Escherichia coli* recorded the highest mycelia inhibition of up to 97% followed by *Pseudomonas sp.* which recorded up to 67.7% inhibition in close relation to bacillus with 67.7% *Enterobacter sp. and streptococcus* did not show *any* antagonistic potentials against tested fungus.

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). Also observed was *Bacillus* with colony code (SB1) with higher mycelia inhibition percentage than *Bacillus sp.* with colony code (SB5).

Table 1. Plate count from soil sample

Sample	Colony count	Colony code
$S10^{-3}$	4.0x 10^4 cfu/ml	SB1
		SB2
$S10^{-5}$	7.0 x 10^6 cfu/ml	SB_3
$S10^{-7}$	6.0 x 10^8 cfu/ml	SB_4
		SB_5
		SB_6
		SB_7
		SB_8
		SB_9

Table 2. Preliminary characterization 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 in chains
SB_8	Cream circular convex dry	G+ve cocci in chains
SB_9	Cream irregular dry raised	G+ve cocci in cluster

SB ⟶ *Soil Bacteria*

Table 3. Table showing antagonistic interactions

Colony code	Mean diameter of fungal mycelia inhibition (mm)	Diameter of mycelia growth (mm)	Percentage of mycelia inhibition (%)
SB_1	16.50 ± 1.53	43.70	27.00
SB_2	0.00 ± 0.00	60.00	0.00
SB_3	5.00 ± 0.20	50.70	15.00
SB_4	25.30 ± 1.15	20.00	66.70
SB_5	24.00 ± 2.00	20.00	66.70
SB_6	21.30 ± 1.53	36.00	40.00
SB_7	30.00 ± 1.00	2.00	97.00
SB_8	0.00 ± 0.00	60.00	0.00
SB_9	8.30 ± 1.52	46.00	23.00
Control	0.00±0.00	60.00	0.00

Table 4. Biochemical characterization and identification of biochemical reaction

Colony code	LAC	MAN	GLU	CIT	IND	UREASE	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	-	+	+	+	-	-	+	+	-	-	-	+	R	R	+	*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

Previous studies have investigated the antifungal potential of *Pseudomonas fluorescence* against pathogenic fungi *Fusarium sp.* and reported against wood decaying fungi [15]. Yadav also reported that cytosolic proteins of *Escherichia coli* are responsible for antifungal potential against pathogenic strains of *Aspergillus niger* and *Candida albican* [9,16].

Another studies mentioned that the mycelia growth of many species of *Aspergillus* and *Fusarium* were inhibited by antifungal potential of *Bacillus spp.*, *Pseudomonas spp*. and *Streptococcus spp.* [6,9]. In the case of *Streptococcus,* it exhibited the least antagonistic activity against *Aspergillus furmigatus*. There was also observed differences in the growth of *Fusarium oxysporium*, where growth of *Fusarium* on *Streptococcus* treated PDA medium was noticed to be higher than the growth on control plate. The bacteria species greatly induced the growth of the above tested fungi while *Bacillus spp.* significantly reduced growth of fungi next to *Pseudomonas spp*.

The production of antibiotics by soil bacteria and their uses in the bio-control of plant pathogens have been reported in many reviews. The results from this research agree with that of other researchers in showing variation in antimicrobial potential among different soil bacteria. Among the bacteria isolates obtained in this research, seven (7) isolates exhibited antagonistic potential against the *Aspergillus niger*. 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 [9,17].

Escherichia coli, Pseudomonas spp., Bacillus spp. had more antagonistic effect on vegetative growth of tested fungi (*Aspergillus niger*). These bacteria species showed high inhibition effect on fungal spore germination. Therefore *Pseudomonas spp.* and *Bacillus spp.* could be used as biocontrol agents against the phyto-pathogenic fungi. Furthermore feasibility of plant disease management by soil bacteria can be confirmed using these microbes.

5. RECOMMENDATION

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

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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