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Antagonistic activity of secondary metabolites from rhizofunctional bacteria extracts against *Fusarium* species

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Abstract:

Background: *Fusarium* species remain important fugal pathogens that produce several mycotoxins with adverse effects on both plant and animals. This work aimed to identify biocontrol agent from rhizofunctional bacteria and assess its antagonistic activity against *Fusarium* sp. using dual culture technique.
Methodology: Briefly a circular disc of the *Fusarium* sp. was inoculated at the center of Potato Dextrose Agar (PDA) plate and incubated for three days. The bacterial isolates were then inoculated about 2cm from the *Fusarium* hyphal tips and incubated for three days, and zone of inhibition was examined. Isolates that showed antagonistic activities against the fungi were subculture in nutrient broth for three days and the metabolites were extracted using ethyl acetate. The metabolic extracts were tested against the fungi using the agar disc diffusion method.
Results: Of the 20 rhizofunctional bacterial isolates screened for antagonistic activities against *Fusarium* sp., 5 showed active antagonism against the fungi with observed clear zone of inhibition in the dual culture, and microscopic examination of the fungal hyphae showed excessive and diffused hyphal branching with hyphal swelling. Ethyl acetate extracts from nutrient broth cultures did not show any zone of inhibition in dual culture against the *Fusarium* sp. All the 5 bacterial isolates were Gram positive strains but only 2 isolates (2a and 3K) were lipase positive, which may indicate that the mechanisms of antagonism could be due to the production of enzymes that have the ability to hydrolyze the cell wall and membrane lipids of the fungi.
Conclusion: The rhizoplane and rhizosphere of plants could be great sources of biocontrol agents and that bacterial isolates 2a and 3K have the potential to be used as antifungal agents against *Fusarium* sp. Molecular identification of 2a and 3K bacterial isolates to the species level is recommended.

Keywords: antagonistic; secondary metabolites; rhizofunctional; bacteria; *Fusarium*

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Activité antagoniste des métabolites secondaires d'extraits de bactéries rhizofonctionnelles contre les espèces de *Fusarium*

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Résumé:

Contexte: Les espèces de Fusarium demeurent d'importants agents pathogènes fongiques qui produisent plusieurs mycotoxines ayant des effets néfastes sur les plantes et les animaux. Ce travail visait à identifier un agent de lutte biologique à partir de bactéries rhizofonctionnelles et à évaluer son activité antagoniste contre *Fusarium* sp. en utilisant la technique de double culture.
Méthodologie: Brièvement un disque circulaire de *Fusarium* sp. a été inoculé au centre d'une plaque de Potato Dextrose Agar (PDA) et incubé pendant trois jours. Les isolats bactériens ont ensuite été inoculés à environ 2 cm des extrémités des hyphes de Fusarium et incubés pendant trois jours, et la zone d'inhibition a été examinée. Les isolats qui ont montré des activités antagonistes contre les champignons ont été sous-cultivés dans un bouillon nutritif pendant trois jours et les métabolites ont été extraits à l'aide d'acétate d'éthyle. Les extraits métaboliques ont été testés contre les champignons en utilisant la méthode de diffusion sur disque d'agar.
Résultats: Sur les 20 isolats bactériens rhizofonctionnels criblés pour les activités antagonistes contre Fusarium sp., 5 ont montré un antagonisme actif contre les champignons avec une zone claire d'inhibition observée dans la double culture, et l'examen microscopique des hyphes fongiques a montré une ramification excessive et diffuse des hyphes avec des hyphes gonflement. Les extraits à l'acétate d'éthyle des cultures en bouillon nutritif n'ont

montré aucune zone d'inhibition en double culture contre Fusarium sp. Tous les 5 isolats bactériens étaient des souches Gram positives mais seulement 2 isolats (2a et 3K) étaient positifs pour la lipase, ce qui peut indiquer que les mécanismes d'antagonisme pourraient être dus à la production d'enzymes qui ont la capacité d'hydrolyser la paroi cellulaire et les lipides membranaires des champignons.
Conclusion: Le rhizoplan et la rhizosphère des plantes pourraient être d'excellentes sources d'agents de lutte biologique et les isolats bactériens 2a et 3K ont le potentiel d'être utilisés comme agents antifongiques contre *Fusarium* sp. L'identification moléculaire des isolats bactériens 2a et 3K au niveau de l'espèce est recommandée.

Mots clés: antagoniste; métabolites secondaires; rhizofonctionnel; bactéries; *Fusarium*

Introduction:

Fusarium is a large genus of hyaline filamentous molds best known as the most important group of mycotoxigenic plant pathogens. This group of fungi are able to produce different toxins such as deoxynivalenol, nivalenol, T2, zearelenone, fusaric and moniliformin with adverse effects on both plants and animal (1). They have also emerged over the past three decades as opportunistic pathogens of immunocompromised hosts. Infections in healthy individuals typically remain localized and include keratitis, especially in association with ocular trauma, onychomycosis of the toenails or fingernails, allergic sinusitis, paronychia, and dermatomycoses. When crops such as wheat, barley, oats, rice and maize are infected with *Fusarium*, it leads to yield loss through low growth rate, reduction of grain size and weakening of the straw.

In the United States of America, *Fusarium* outbreak in the 1990's resulted in losses in the region up to $3 billion (2). The fungus produces a mycotoxin known as deoxynivalenol that poses a significant threat to domestic animals and humans (3). The strain that specifically attacks banana is called *Fusarium oxysporum* f. sp. *cubense*. Apart from field infections, *F. culmorum* is also known to cause storage rot of sugar beet, potatoes and apples (4). Parasitism mediated by degradation of cell wall of pathogenic fungi relies on extracellular lytic enzymes. Several *Bacillus* species produce enzymes that degrade chitin, an insoluble linear polymer of 1,4-N-acetylglucosamine, which is a major component of most fungal cell wall. Among these species, *B. circulans* (5), *B. licheniformis* (6), *B. cereus* (7) and *B. thuringiensis* have been implicated as potential biocontrol agents. These species are reported to secrete chitinases and the role of these chitinolytic enzymes in the biocontrol of fungal pathogens has been elucidated in experiments involving bacterial and fungal antagonists (8, 9).

Several basic mechanisms of the bacterial-induced biocontrol of plant pathogenic fungi have been described, particularly concerning the *Pseudomonas* genus (7); antibiosis, fungistasis, competition for nutriments, modification of the biophysical root environment, active exclusion of pathogenic fungi from the rhizosphere, detoxification of pathogen virulence factors and induction of plant disease resistance. In recent years, it has become apparent that bacteria coordinate their interactions and associations with higher organisms by using intercellular communication systems that rely on small diffusible molecules in a process known as quorum sensing.

Screening microbial secondary metabolites is an established method to identify novel biologically active molecules. Microbial extracts have been and continue to be productive sources of new biologically active molecules for drug discovery. It is well known that production of secondary metabolites by microorganism is influenced by fermentation conditions. In a study of 29 *Nodulisporium* strains, Li et al., (10) reported that synthesis of secondary metabolites was directly influenced by fermentation conditions and that there were differences of up to 400-fold in the concentrations of secondary metabolites between conditions. In addition, rare metabolites were consistently reported in extracts containing larger numbers of secondary metabolites (10). There are a number of previously developed methods which use direct chemical measurement to classify microorganisms but most previous studies focused on characterizing microorganisms by detecting the presence of known secondary metabolites. Frisvad et al., (11) used high-performance liquid chromatography (HP LC) diode array detection and flow injection analysis together with Electrospray Ionization Mass Spectrometry (ES-MS) to detect secondary metabolites characteristic of fungal strains responsible for spoilage of stored cereals. There has also been much success in obtaining biological control of plant pathogens using bacterization techniques. Bacteria used as inoculants are mostly *Pseudomonas fluorescens-putida* types obtained from soils and plant surfaces (12).

Investigations on microbial metabolites is gaining greater momentum in the agrochemical industry as a source for the development of new pesticide products. Several such products have been developed and used as bactericide, fungicide, acaricide or insecticide in agriculture. *Fusarium* species cause a lot of diseases in a lot of plant and crop thereby reducing the yield and quality of crop products. Hence an effective biocontrol approach such as the use of bacteria that are antagonistic to the growth of the fungi could be adopted to prevent or control the devastating effect of the fungi on crops. The main purpose of this

research is to identify rhizo-functional bacteria that possess antagonistic activity against *Fusarium* sp. and to test extracts of secondary metabolites from potent bacterial isolates against *Fusarium* sp.

Materials and method:

Sources of bacterial and *Fusarium* isolates:

Pure culture of *Fusarium* sp. was obtained from the Department of Molecular Biology and Biotechnology, University of Cape Coast, Ghana. Twenty rhizofunctional bacterial previously isolated from the rhizosphere of *Carica papaya* were obtained from the same department.

Sub-culturing of bacteria isolates and storage:

The 20 rhizo-functional bacterial previously isolated from the rhizosphere of *Carica papaya* and stored in 10% glycerol at 40°C were allowed to thaw at room temperature and vortexed for 10 sec. The streak plate method using Potato Dextrose Agar (PDA) medium was used to subculture the bacterial isolates under aseptic conditions. The inoculated plate was incubated at 28°C for 18-24 hours. The purified isolates were then transferred into 1.5ml Eppendorf tubes containing 10% glycerol which were then stored in the refrigerator at –40°C.

Sub-culture of *Fusarium* sp.

A 5-mm diameter sterile cork-borer was used to create several agar discs at the tips of the young hyphae of *Fusarium* growth on PDA medium. A sterile inoculation needle was then used to transfer the agar discs containing the *Fusarium* onto the surface of fresh PDA agar and incubated in the dark at room temperature for 3-7 days.

Determination of antagonistic activity of bacterial isolates against *Fusarium* sp.

A 5-mm mycelial disc from a 7-day old *Fusarium* culture was obtained using a sterile cork-borer was transferred onto the surface of a sterile PDA plate. The culture was then incubated at room temperature for 2 days. A single colony of pure bacterial cultures from the streak plate was picked with sterile inoculation loop and transferred onto the PDA plate containing the 2-day old pure culture of *Fusarium* sp.

Five bacterial isolates were inoculated 2cm away from the growing *Fusarium* sp. and about 2cm apart between them. A control experiment was set up without any bacteria growing concurrently with the *Fusarium* culture. Bacterial isolates able to inhibit the growth of the fungus were considered as having an antagonistic activity and were therefore selected for further screening. Such bacterial isolates were further subjected to a second screening to determine the most potent antagonist using the same approach described. The zone of inhibition between the fungus and the bacteria was measured with a ruler and the bacterial isolate that produced the widest induced zone of inhibition was selected for metabolite extraction in nutrient broth.

Extraction of crude metabolites using ethyl acetate:

Inoculum from the most potent antagonistic bacterial isolate was aseptically transferred into a conical flask containing 100ml of sterile nutrient broth and incubated on an orbital shaker at 120 rpm at 28°C for 7 days. After the incubation period, the culture was centrifuged at 10,000 rpm for 15 minutes to separate the supernatant from the bacterial cells (pellets). The crude metabolites were then extracted from the supernatant by partitioning with equal volume of ethyl acetate. Various concentrations of the crude metabolite extract were then prepared; 0.1%, 1%, 2%, 3%, 5%, and 100% and their antifungal potential was tested against a three-day old *Fusarium* culture using the disc infusion method.

Briefly sterile paper disc was impregnated with 0.5ml of the prepared concentrations of the metabolite extract and placed on an agar plate containing a 3-day old *Fusarium* culture such that each disc was 2cm away from the fungi. The culture was incubated at 28°C for 3 days. Observations were made after every 24-hour period. A negative control was set-up using filter paper disc impregnated with ethyl acetate only.

Biochemical and morphological characteristics of bacterial isolates:

The bacterial isolates exhibiting antagonistic activity were biochemically characterized using amylase, lipase, catalase and hydrogen cyanide production tests and morphologically using Gram stain and motility test.

Microscopic examination of fungi growth:

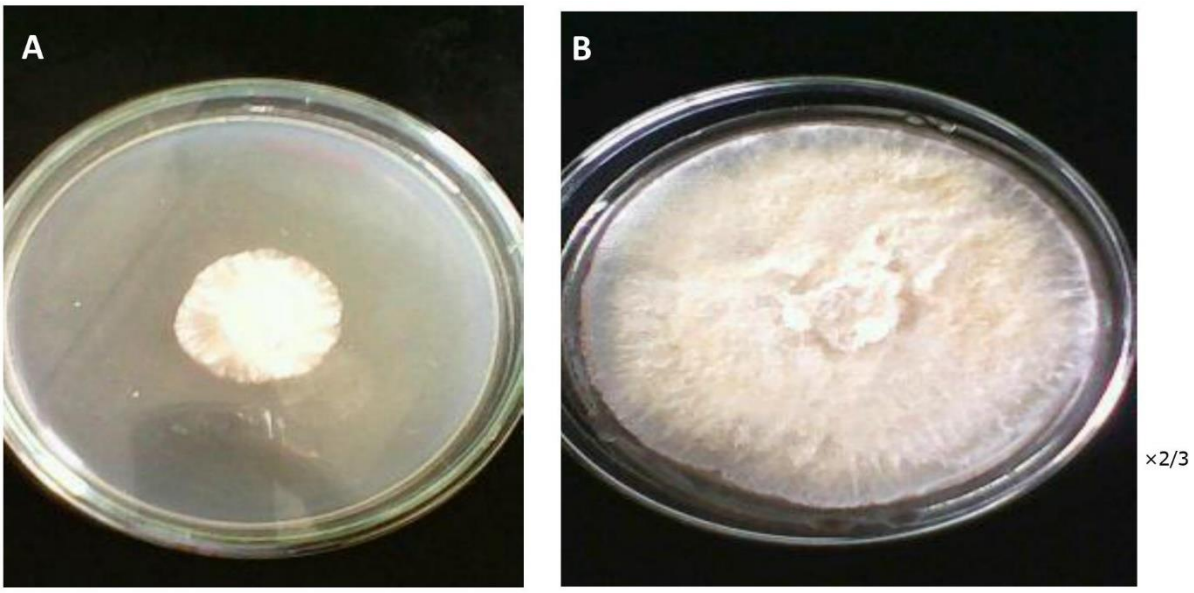
The effect of the antagonist bacterial isolates on hyphal and mycelial growth was examined using a compound light microscope.

Results:

Of the 20 bacterial isolates screened, 5 showed antagonistic activity against *Fusarium* species. The zones of inhibition varied from one bacterial isolate to the other with the bacterial isolate designated HSG2 having the highest inhibition zone (0.92 mm), followed by isolates 3k, 1a, and 2a. The bacterial isolate 4f recorded the least zone of inhibition (0.61mm) (Table 1). Pure cultures of *Fusarium* sp used for antagonistic screening are shown on Plate 1, with photographs of the first and second antagonistic screening on Plate 2.

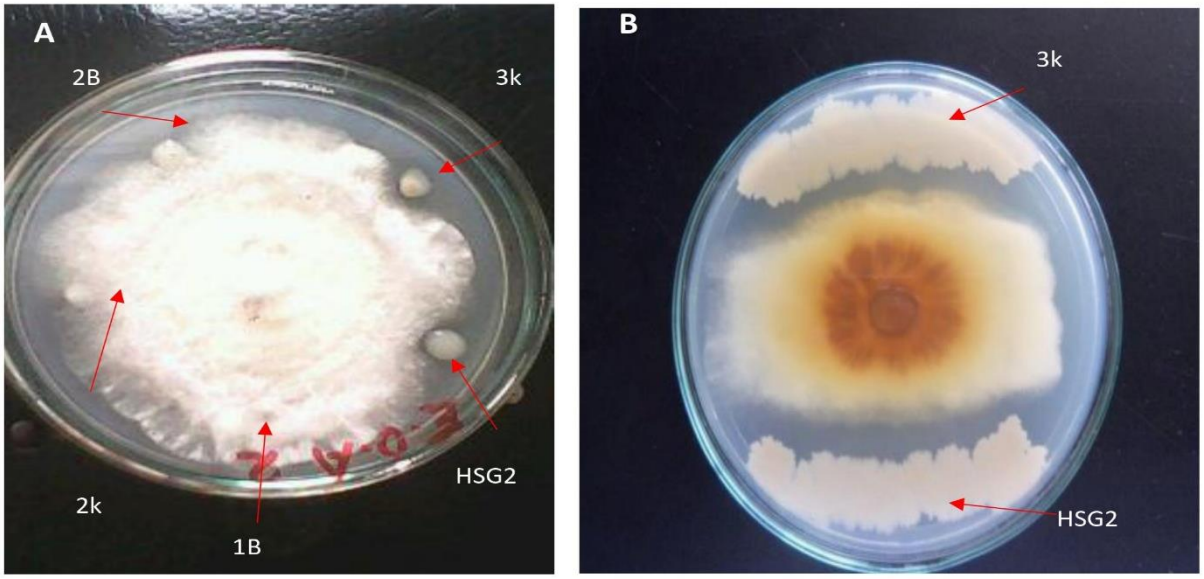
Table 1: Zone of inhibition (mm) induced by five antagonistic bacterial isolates against *Fusarium* species

Bacterial isolate	Zone of inhibition (mm)
2a	0.64
4f	0.61
1a	0.70
3k	0.71
HSG2	0.92



A: 3day old culture; B: 7day old culture

Plate 1: Pure cultures of *Fusarium* species on potato dextrose agar in different incubation periods at 28°C



A: First screening Fusarium sp + bacterial isolates (2b, 3k, HSG2, 1b and 2k); B: Second screening (*Fusarium* sp + bacterial isolates HSG2 and 3k)

Plate 2: Screening for antagonism showing mixed cultures of *Fusarium* species concurrently on potato dextrose agar after 7 days incubation at 28 °C

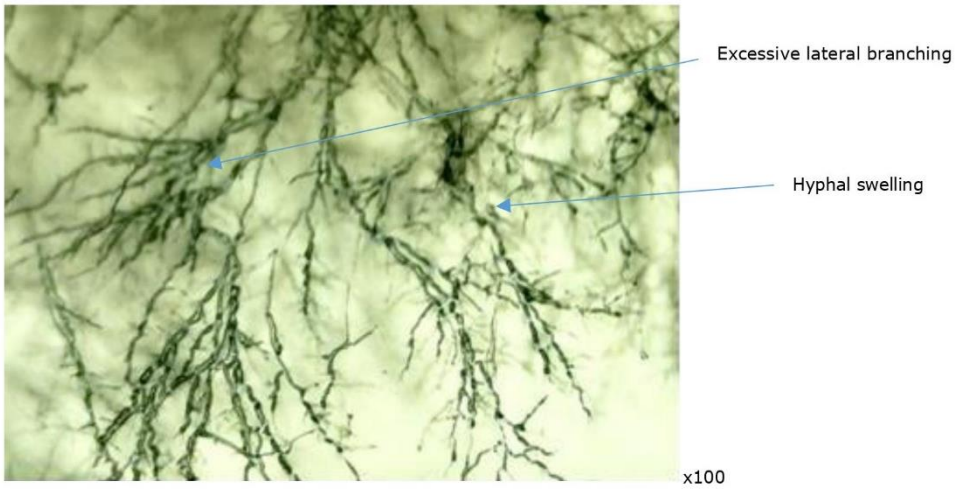
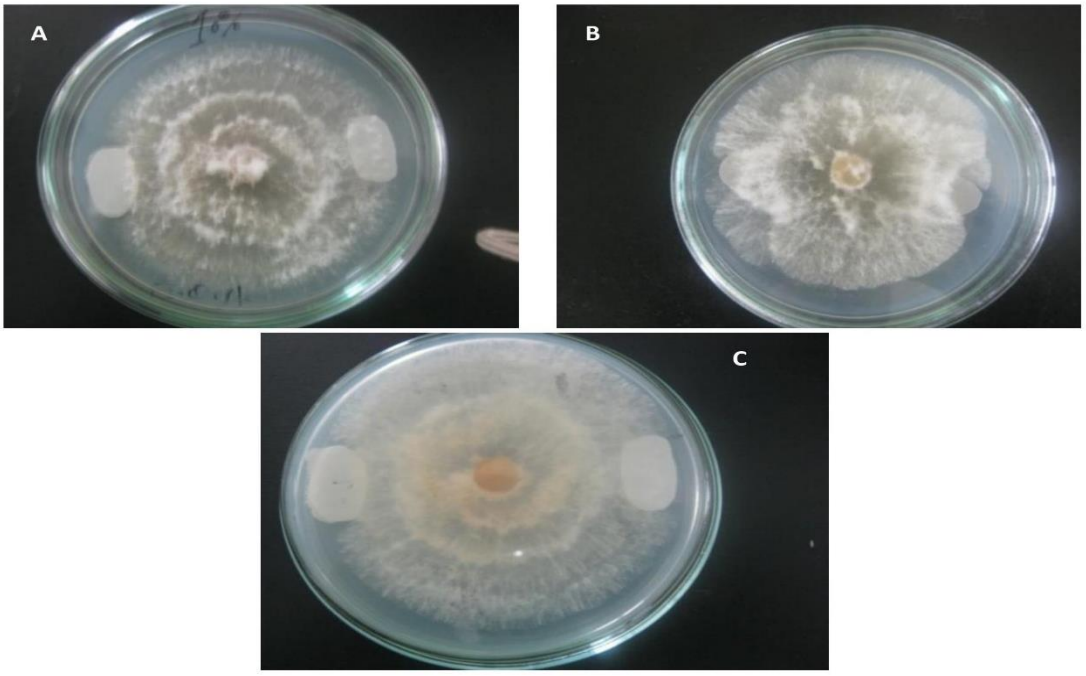


Fig 1: Morphological changes in *Fusarium* hyphae in response to antagonistic bacterial isolates

Microscopic examination of fungi growth: Both the portion of *Fusarium* mycelium close to the zone of inhibition and that far from the zone of inhibition were observed under a light microscope. Morphophysiological changes including excessive lateral branching and hyphal swellings were observed in *Fusarium* hyphae in response to the antagonistic activity of the bacterial isolates (Fig 1).

Antifungal property of crude metabolite extract: No zone of inhibition was recorded when the filter paper discs impregnated with the various concentrations of ethyl acetate crude metabolite extracts were placed on the Potato Dextrose Agar plate containing the *Fusarium* culture (Plate 3).



(A) 1% crude metabolites extract + *Fusarium* culture; (B) 100% crude metabolites extract + *Fusarium* culture (C) Ethyl acetate + *Fusarium* culture (control)

Plate 3: Results after 7-day old culture of *Fusarium* sp from the action of the filter paper discs impregnated with 1% and 100% crude metabolite extracts

Table 2: Biochemical and morphological characteristics of the five rhizobacterial isolates

Bacterial isolate	Biochemical characteristics				Morphological characteristics	
	Amy	Lip	Cat	Hcn	Mot	Gram stain
2a	-	+	+	X	+	Gram +ve rod
4f	-	-	+	X	+	Gram +ve rod
1a	-	-	+	X	-	Gram +ve rod
3k	-	+	+	-	+	Gram +ve rod
HSG2	-	-	+	x	+	Gram +ve rod

Amy=Amylase, Lip=Lipase, Cat=Catalase, Hcn=Hydrogen cyanide, +ve=Positive, -=negative, +=positive, x=not performed, Mot=Motility

Biochemical and physiological characteristics of bacterial isolates:

Data obtained from biochemical and physiological tests are presented in Table 2. All the 5 bacterial isolates were amylase negative and 2 of the isolates were lipase positive and while the other 3 were lipase negative. Furthermore, all 5 isolates were catalase positive. The hydrogen cyanide test was performed only on one isolate (3k) and this isolate gave a negative reaction. The Gram staining revealed that all 5 isolates were Gram-positive rods. The motility test however revealed that 4 out of the 5 isolates were motile and only one was non-motile.

Discussion:

This research aimed to identify biofungicidal agents against phytopathogenic *Fusarium* sp. All the five most bacterial antagonists were Gram-positive rod shaped and catalase positive. This is because these isolates produced the enzyme catalase that is able to hydrolyse hydrogen peroxide into water and oxygen leading to the evolution of white bubbles. This helps the bacteria to survive under hydrogen peroxide polluted environment. The lipase positive bacteria, 2a and 3k, produce the enzyme lipase that act on lipid and hydrolyse it into glycerol and fatty acids. This property would enable the bacteria to act directly on the phospholipid bi-layer of the cell membranes or the cell wall of other microorganisms causing disintegration in their cell wall. This therefore enables the bacteria to antagonize the growth of other competitors in its environment. The bacterial isolate 3k profoundly inhibited radial growth of the hyphae and also induced morpho physiological changes including excessive lateral branching, hyphal swellings and cytoplasmic extrusion at the tips of the hyphae as revealed by the light microscope.

According to Asante et al., (13), abnormal nuclear divisions occur at the sub-epical region of the hyphal tip before excessive lateral branching occurs, and according to Semighini and Harris (14), hyphal branching in fungi is due to mitotic cell division. On the other hand, the affected hyphal tip extrusion was most likely to be cytoplasmic substances that became extruded through the cell wall of the hyphae. This phenomenon likely reflects the exclusive targeting of exocystic vesicle laden with components required for cell surface expansion and cell wall deposition to the hyphal tip at the expense of potential branching site (3,14). This may also be due in part to the production of the enzyme lipase by the bacterial isolate 3k, which might have affected the transport of cytoplasmic substances responsible for the growth and extension of the fungal hyphae across the cell wall to the tips leading to the accumulation of cytoplasmic substances resulting in the observed hyphal swellings. This therefore supports the finding that in fungi, branching forms a central development of mycelial colony and also appears to play a major role in fungal interaction with other organisms and that there are two partings of hyphal branching: apical and lateral branching (15).

Schmid et al (3) affirmed that in fungi, branching is dominant at the apical tip and hence turn to suppress the formation of lateral branching. Therefore, the inhibition of apical branching induced by the antagonistic bacteria might have accounted for the observed excessive lateral branching in the *Fusarium* sp. in the dual culture assay. This would reduce the ability of the fungi to colonize the environment, which in turn would reduce its ability to effectively utilize the nutrients in its vicinity. This is because the hyphal tip is involved in the production of enzymes necessary for nutrient break down and assimilation as well as hyphal growth and extension. On the contrary, bioefficacy studies on the various concentrations of the crude metabolites against the *Fusarium* sp. in a dual culture did not produce any antagonistic activity. This might have been due to insufficient dissolution of the metabolites by the extraction solvent, ethyl acetate hence, the optimum inhibitory concentration was not attained.

The bacterial isolate (3k) probably occupies the rhizoplane of the plant and likely plays a role in host defense using other mechanism(s) such as hydrogen cyanide production or ion depletion mechanism either than its secondary metabolites. Among the alternative means of assimilating iron are surface reduction to the more soluble ferrous species, lowe-

ring the pH, utilization of heme, or extraction of protein-complex metal. Thus, bacterial isolate 3k might have produced small molecules called siderophores that are high affinity iron chelators or undergone iron homeostasis to capture and to use different forms of bond iron by using molecules containing iron, such as lactoferrin, ferritin or heme. Heme utilizing bacteria produce the enzyme called heme oxygenase that oxidatively cleaves the heme molecules to form intracellular ferrous iron called biliverdin and carbon mono-oxide resulting in protoporphyrin ring degradation (15). Mathieu et al., (16) again found that Gram-positive bacteria exhibit functional redundancy in iron transporter mechanism, that is, siderophore-mediated iron uptake, heme uptake and /or ferrous iron uptake, (15). Therefore, the test bacterial isolate 3k being Gram-positive may have the ability to use this mechanism to antagonize the growth of the *Fusarium* sp. by utilizing and making the iron unavailable to the fungus.

This alternative possibility for the antagonistic relationship between the *Fusarium* sp. and the bacteria is supported by the fact that, the role of iron in the virulence mechanism of some pathogenic organisms attacking crops and animals is well established. For example, the siderophore system of *Yersinia enterocolitica* is correlated with the virulence of the organism (16). In addition, the potency of common antibiotics has been elevated by building into the molecules the iron-binding functional groups of siderophores. The objective here is to take advantage of the high affinity, siderophore-mediated iron uptake system of the bacteria. Also in agriculture, the ability of some bacteria to produce chemicals such as pseudobactin or pyoverdine type siderophores has been applied to improved plant growth either through a direct effect on the plant or through control of noxious organisms in the soil. This may also be due in part to the production of the enzyme, lipase by the bacterial isolate 3k which might have affected the transport of cytoplasmic substances responsible for the growth and extension of the fungal hyphae across the cell wall to the tips leading to the accumulation of cytoplasmic substances resulting in the observed hyphal swellings. This therefore supports the finding that in fungi, branching forms a central development of mycelial colony and also appears to play a major role in fungal interaction with other organisms and that there are two partings of hyphal branching; apical and lateral branching (15).

The finding of this study suggests that, the rhizosphere and the rhizoplane is inhabited by bacteria species that could be utilized as biocontrol or bio-fungicidal agents against *Fusarium* sp. This study therefore recommends that the potent bacterial isolate be tested on other pathogenic fungi to determine the spectrum of action. Further biochemical analysis should be conducted to identify the isolate to the species level and elucidate their mechanism of inhibition including iron utilization. In addition, different extraction solvents should be tested for their efficacy to ensure efficient extraction of the metabolites.

Contributions of author:

The author performed the bench work and writing of the manuscript.

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Conflict of interest:

No conflict of interest is declared.

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