



Biocontrol potential; antifungal activity and plant growth promoting activities of endophytic bacteria from *Raphanus sativus*

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ABSTRACT

The study was aimed to find an alternate approach for chemicals used in agriculture to avoid microbial infections. Fungal pathogens cause different types of plant diseases and affect a majority of edible crops by destroying the tissues of the plant in a direct or indirect mechanism. So, an alternative approach led to the development of biocontrol agents using endophytic bacteria. A total of 8 endophytic bacteria were isolated from the root, stem, and leaves of radish (*Raphanus sativus*). The antagonistic activity of these bacteria against the 2 isolated plant pathogenic fungi was determined *in vitro*. Two out of eight bacteria showed more than 50% inhibitory activity against one fungus, were further characterized using the 16s rRNA sequencing method. On the basis of the phylogenetic tree of the 16s rRNA method, the endophytic bacterial samples were identified as *Tonsilliphilus suis* and *Exiguobacterium aurantiacum* against plant pathogenic *Aspergillus flavus* isolated from *Raphanus sativus*, which makes them highly suitable as an alternative for chemical fertilizers to provide resistance to plant pathogenic fungi. The cell wall degrading activities such as protease activity, amylase activity, and plant growth-promoting properties such as Hydrogen cyanide (HCN), Indole acetic acid (IAA), ammonia production of these endophytic bacteria were evaluated. The results show that *T. suis* is the most effective strain for radish growth development.



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INTRODUCTION

In modern agriculture, a higher yield of several food crops is achieved by using external agents such as chemical fertilizers, pesticides, insecticides, and antifungal agents. The plants, soil, as well as ani-

mals consuming these crops are affected by different types of diseases (Durán-Lara *et al.*, 2020). Furthermore, humans are highly affected in a large population due to the consumption of these crops and are prone to several illnesses such as digestion issues, kidney and bone disorders. Even though disease tolerant crops are produced by implementing genetic engineering techniques, nutritional values and plant reproduction are significantly affected (Key *et al.*, 2008).

Out of all the present phytopathogens, fungal pathogens invade the edible crops majorly during both pre-harvest and post-harvest conditions (Pétriacoq *et al.*, 2018). Over 8000 types of plants are shown to be affected by fungal pathogens. They are transmitted from the soil, infected plants, and also poor growth conditions. They cause different types of wilt diseases, which may remain in the plant even after cleaning and proper cook-

ing (AUSVEG, 2020). They develop different ways to survive during unfavorable environmental conditions, in the absence of a suitable host, grow, spread, infect and reproduce (Koike, 2016).

Raphanus sativus is a cruciferous vegetable that is consumed by several people of India and also other Asian and western countries. It is also considered one of the major edible crops in Asia. They are highly rich in various nutrients, including potassium, calcium, sodium, iron, magnesium, phosphorus, zinc. Radish possesses anti-oxidant and anti-inflammatory properties due to the presence of Vitamin C. They also contain A, B and K. Radish can be used for the treatment of stomach, intestinal and liver disorders, bile duct problems, gallstones. The root of the radish may stimulate digestive juice and regulate bile flow. They also contain metabolites that have the ability to kill tumor cells and deduct the levels of blood sugar and cholesterol. Radish has been reported as a host for more than 18 pathogenic fungi. Plant diseases caused by fungal pathogens affect agricultural practices and crop yield by producing mycotoxins which leads to the food spoilage and rotting of food crops (Shuping and Eloff, 2017). Though agrochemicals play a vital role in plant disease management, intensive use of chemicals causes adverse effects on humans and the ecosystem. This leads to the development of alternative biological approaches (Meena et al., 2020).

Endophytes are an endo-symbiotic group of microorganisms that provides many benefits to hosts and helps them to withstand various abiotic and biotic stresses, which can challenge the invasion of several pathogenic fungi. The stress tolerating environment of the various resistant crops is colonized by the endophytic bacteria. They are indulging in antioxidant activity by producing immune responses (Eid et al., 2019). The endophytic genes related to the particular metabolites aid in the promotion of plant growth either by enhancing the production of auxins, gibberellins, and other plant hormones. They are also achieved by producing plant protective chemicals that will provide resistance to pathogens, tumor developments, and insect pests that is economically safe and eco-friendly, as suggested by the metabolomic research (Ek-Ramos et al., 2019).

Endophytes which are isolated from the different plant parts of radish such as leaves, stem, and roots induce a plant defence mechanism known as induced systemic resistance (ISR), which leads to a greater tolerance for fungal pathogens and is also considered as a tool for disease control in modern agriculture (Miliute et al., 2015). The isolated endo-

phytes are analysed using 16s rRNA sequencing for classification and identification. In a sample containing many species, the commonly used tool to target genes were the 16s because of the highly conserved region and transcriptional machinery.

In this study, potential endophytes showing anti-fungal activity are identified using the agar overlay method. The cell wall degrading activities and plant growth, promoting properties of the isolated endophytic bacteria are studied and proved to be showing antagonistic activity to the pathogenic fungi. Assays such as protease and amylase enzyme activities have proved that the endophytic bacteria possess the ability to degrade the fungal cell wall, thereby inhibiting the spread to other parts of the plant. The release of compounds like Indole acetic acid (IAA) and gases such as ammonia and hydrogen cyanide during the invasion of the pathogenic fungi to the plant surface is found to be having plant growth-promoting properties because the compounds contain toxicity inducing agents that suppress the growth of pathogenic fungi (Olanrewaju et al., 2017).

MATERIALS AND METHODS

Collection of samples

Healthy radish (*Raphanus sativus*) plants, including root, stem, and leaf, were bought from the local market for the isolation of endophytic bacteria and the infected radish plant, which has visible symptoms like blights, wilts and spots were used for the isolation of plant pathogenic fungi.

Isolation of endophytic bacteria

The root, stem, and leaves of a radish plant were cleaned and the surface was disinfected using 70% ethanol and 1% sodium hypochlorite for 15 mins and approximately 0.2 cm from margin were removed using a sterile knife. The samples were then triturated using a mortar and pestle with 2ml of Phosphate Buffered Saline of pH 7.2 (Seo et al., 2010). The filtered mixture was serially diluted and was inoculated on nutrient agar plates. Plates were incubated at 30°C for 72 hrs. The bacteria were isolated and grouped according to their colour, colony characteristics, and morphology. Prefatory morphological identification was performed using the gram staining method. The pure cultures were stored in slants for future use.

Isolation of plant pathogenic fungi

The infected parts of the plants were surface sterilized using 70% ethanol and 1% sodium hypochlorite. They were then inoculated on Potato dextrose agar plates and were incubated for 5 days at 35°C.

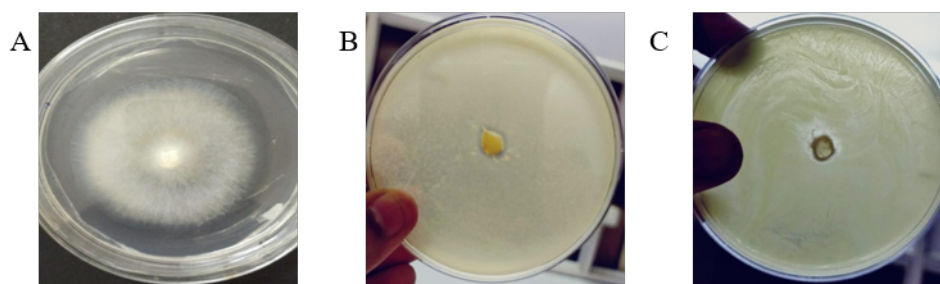


Figure 1: In vitro screening for antagonistic activity of isolated endophytic bacteria. (A) Control without endophytic bacteria, (B, C) Antagonistic activity of bacterial isolate 1, 2 against isolated pathogenic fungi

The fungal cultures were purified based on their colour and colony characteristics. The prefatory morphology was examined using a lactophenol cotton blue stain.

Screening for antagonistic activity

The antagonistic activity of bacteria towards fungi were evaluated by using the agar overlay technique (Bahroun *et al.*, 2018). The bacterial plate was prepared and incubated for 48 hrs. A five mm agar disc containing a twelve-day old culture of fungi was placed in the centre of bacteria plates. The plates with a fungal disc, without bacteria, served as a positive and negative control, respectively. The plates were then incubated at 35°C for 3 days. The antifungal activity was determined by the formation of a zone of inhibition developed around the fungal disc.

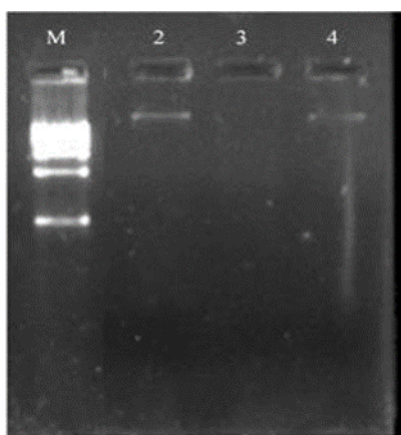


Figure 2: Gel Electrophoresis LANE 1 contains Marker; LANE 2 contains DNA from bacterial isolate 1; LANE 3 contains DNA from bacterial isolate 2; LANE 4 contains DNA isolated from fungi

Molecular characterization using 16S rRNA sequencing

DNA isolation

Bacterial isolates were suspended in 0.5 ml of saline. The suspensions were centrifuged at 10,000 rpm for

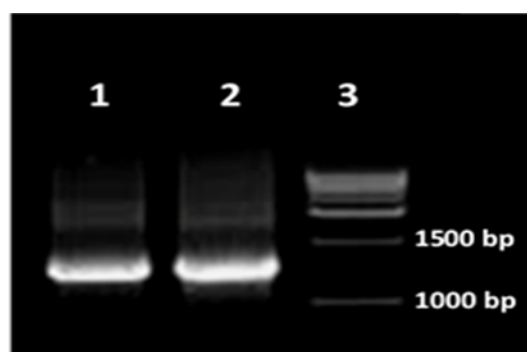


Figure 3: PCR amplified products of isolated endophytic bacteria with primers LANE 1, 2 contains PCR amplified products ~1000 bp; LANE 3: Marker.

10 to 12 mins. The supernatant was removed, and the remaining pellet was resuspended in 0.5 ml of InstaGene Matrix (Bio-Rad). The suspension was incubated at 56°C for 30 mins and then heated at 100°C for 10 mins. The supernatant was cooled and used for the PCR analysis.

Polymerase chain reaction

1 μ l of the template DNA was added to 20 μ l of the PCR reaction solution. 518F/800R primers were used to perform PCR for bacterial isolates. 35 amplification cycles were done at 94°C for 45 sec, 55°C, and 72°C at 60 sec. Approximately 1400bp of DNA fragments were amplified for the bacterial isolate.

PCR product purification

A montage PCR pack up kit was used to purify the amplified PCR products by removing unincorporated PCR primers and dNTPs.

Sequencing

BigDye terminator cycle sequencing kit was used to sequence 1400 bp of purified PCR products and to resolve the sequenced products, a 3730 XL automated DNA sequencing system was used.

Construction of the phylogenetic tree

BLAST analysis was done to identify the phylogenetic

ically similar type strains sequence from the GenBank database. By the neighbour-joining method, the phylogenetic tree was constructed by multiple sequence alignment and trimming the sequences to similar nucleotide lengths using MEGA 6 (Swofford, 2001). Nodes in the phylogenetic tree indicate the level of bootstrap. Low values (below 50%) were not indicated in the phylogenetic tree.

Cell wall degrading activity

Protease activity

The bacteria were inoculated onto the skim milk agar and incubated at 37°C for 48 hrs. The plate without bacteria serves as a control. The zone of hydrolysis developed after incubation indicates the presence of protease activity by the endophytic bacteria (Strauss *et al.*, 2001).

Amylase production

The bacteria were inoculated onto starch agar and incubated at 37°C for 72 hrs. The grown culture medium was flooded with Lugol solution for 15 mins after incubation. The formation of a transparent halo zone around the bacterial colony, indicating amylase production by the endophytic bacteria (Strauss *et al.*, 2001).

Evaluation of plant growth-promoting activities

IAA Production

Gordon and Weber's method was used to quantify IAA production (Gordon and Weber, 1951). The isolated bacteria were inoculated in the nutrient broth medium, which contains 0.2% (v/v) L tryptophan at 28°C for 72 hrs. The OD_{600nm} of bacterial isolates were maintained at 0.3. The medium with tryptophan and without bacterial inoculum was used as control. The cultures were centrifuged at 12,000 rpm for 5 mins; for 1 ml of the supernatant, 1 ml of Salkowski reagent was added. The samples were incubated in the dark for 15 mins. The optical densities of the samples were measured at 530 nm. The formation of red colour in the samples indicates IAA production.

Detection of Hydrogen Cyanide Production

Sulfocyanate colorimetric method was used for the qualitative estimation of HCN production (Alstrom and Burns, 1989). The bacterial suspension was inoculated in nutrient agar 4.4 g/L with glycine. The 7 cm diameter of Whatman filter paper was soaked in 1% picric acid for 1 min and was placed on Petri dishes. The plates were then incubated at 29° C for seven days. The colour change from yellow to orange indicates HCN production.

Ammonia Production

The bacterial culture was added with 0.5 ml of Nessler's reagent. The formation of brown colour indicates the presence of ammonia in the bacterial suspension (Cappuccino and Sherman, 2005).

RESULTS AND DISCUSSION

Isolation of endophytic bacteria and plant pathogenic fungi

The endophytic bacterial diversity of radish plants was evaluated in root, stem, and leaves. Eight bacterial strains were isolated from the *Raphanus sativus* sample and were differentiated using their colour and colony characteristics. The pure cultures were stored at optimum temperature at 28°C in slants for future use. Pathogenic fungi were isolated from infected radish leaves on Potato dextrose agar. The phenotypic characteristics of the fungal isolate were identified. Further, the pure culture of the pathogenic fungi was stored at an optimum temperature which is 28°C for future studies.

In vitro screening for antagonistic activity

The agar overlay method was performed to analyze the antagonistic activity of bacteria towards the isolated fungi isolate. Two bacterial strains showed the inhibition of mycelium growth in fungi. This can be detected by the transparent halo zone produced around the fungal disc on the plate after the incubation period Figure 1. The control plate did not produce the transparent halo zone around the fungal disc.

Molecular identification of Isolated Fungi and Bacteria

The genomic DNA was separated and purified by Gel Electrophoresis, as shown in Figure 2. PCR was performed to amplify the target region of DNA. The forward primer 5'-GAGAGTTTGATCCTGGCTAG-3' and reverse 5'-CGGTGTGTACSSGGCCCCGGAACG-3' were used, as shown in Figure 3. Molecular phylogeny of the isolated endophytic bacterial strains was performed by 16S rRNA gene sequencing and their best match type strains from the NCBI database were generated to construct phylogenetic trees.

The Phylogenetic dendrogram elucidates that *Tonsilliphilus suis* is the isolated bacteria belonging to the family of Dermatophilaceae and it is represented in Figure 4. The phylogenetic position of the bacterial isolate was determined using the 16S rRNA gene sequencing technique.

The phylogenetic position of the bacterial isolate was found using the 16S rRNA gene sequencing method. The phylogenetic dendrogram showed that

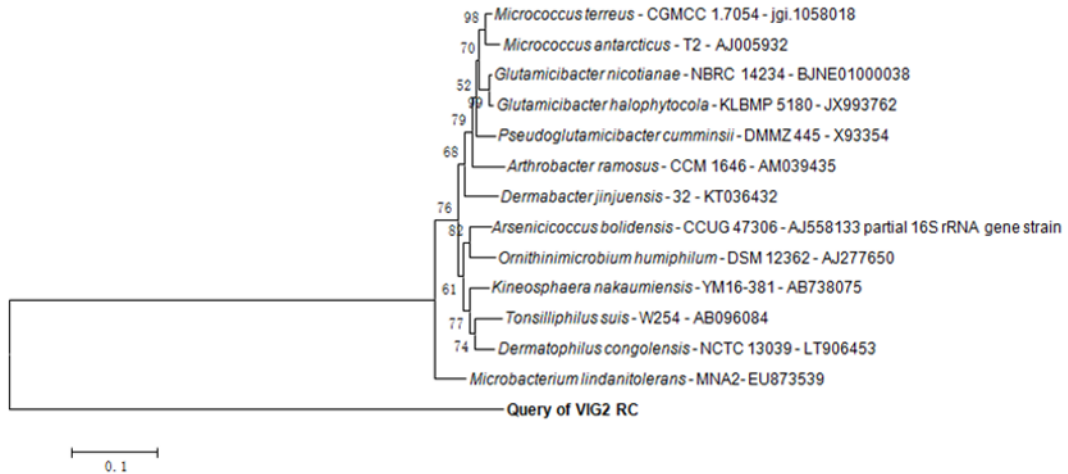


Figure 4: Phylogenetic tree of endophytic bacterial sample 1 isolated from the radish. Bootstrap values greater than 50% were indicated. Scale bar indicates the number of substitution per site.

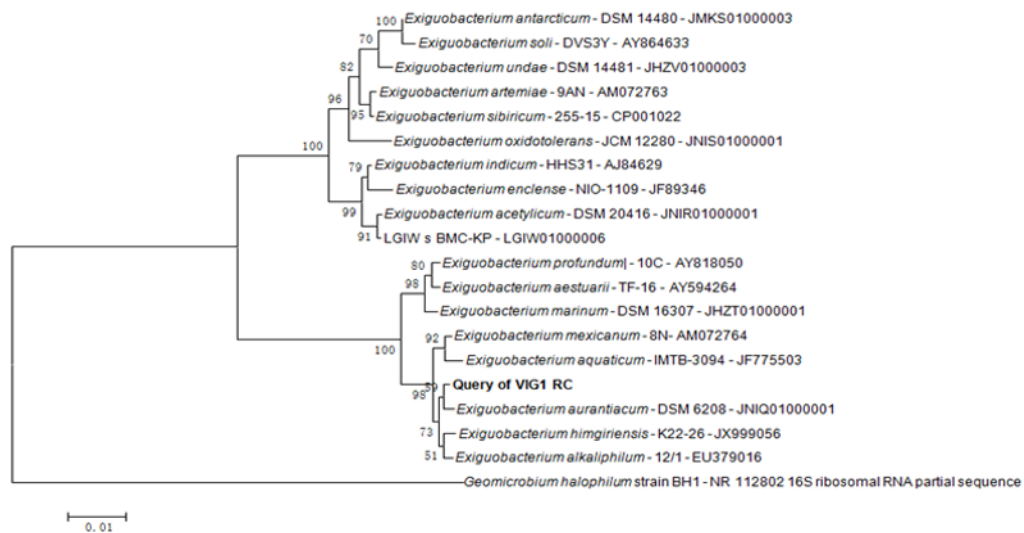


Figure 5: Phylogenetic tree of endophytic bacterial sample 2 isolated from the radish. Bootstrap values greater than 50% were indicated. Scale bar indicates the number of substitution per site.

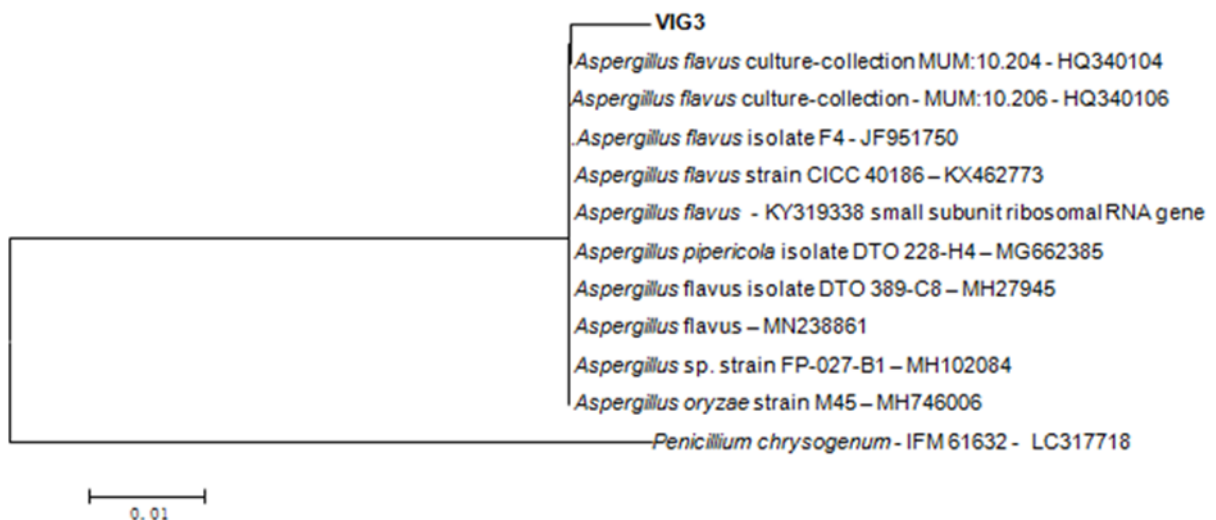


Figure 6: Phylogenetic tree of pathogenic fungi isolated from the radish. Bootstrap values below 50% were excluded.

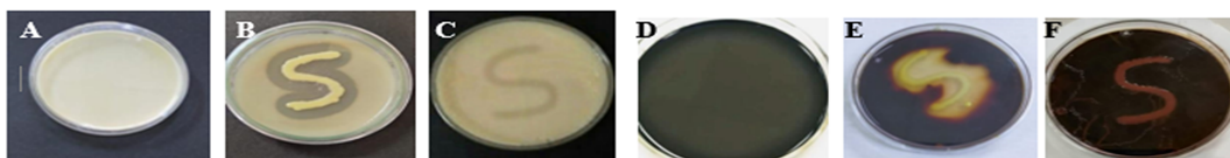


Figure 7: Cell wall degrading activity of antagonistic endophytic isolates. Protease activity (A) control without bacterial inoculum, (B) T.suis, (C) E. aurantiacum; Amylase activity (D) control without bacterial inoculum, (E) T.suis, (F) E.aurantiacum

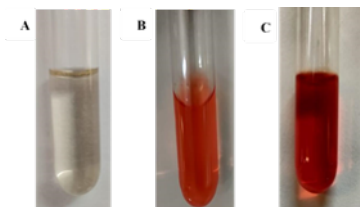


Figure 8: Detection of IAA production in isolated antagonistic bacterial strains (A) Control without bacterial inoculum (B) T.suis (C) E.aurantiacum

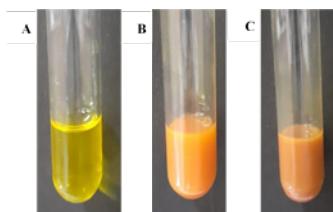


Figure 9: Screening for the production ammonia by bacterial strains (A) Control without bacterial inoculum (B) T.suis (C) E.aurantiacum

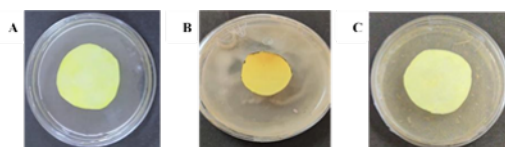


Figure 10: Screening for the production of HCN by bacterial strains. (A) Control without bacterial inoculum (B) T.suis (C) E.aurantiacum

the isolate was most similar to the sequence from the *Exiguobacterium aurantiacum* strain (99.66%). Thus, the strain belongs to the genus *Exiguobacterium* and identified as *Exiguobacterium aurantiacum*, as shown in Figure 5.

The diagram shows the dendrogram of a fungal strain isolated from infected radish. The strain was characterized on the basis of the ITS1-5.8S-ITS2 region of rRNA and alignment of the sequences from NCBI databases. Closely related nucleotide sequences of the fungal isolate were shown in the phylogenetic tree as indicated in the phylogenetic tree that the isolated fungal strain could belong to *Aspergillus* sp. *Aspergillus flavus* 3357 could be the isolated fungi strain due to the maximum number and topmost hits in BLAST, as shown in Figure 6.

Cell Wall Degrading Activity

Protease activity

The transparent halo zone formed on the skim

milk agar plate indicates the production of protease enzyme in *T. suis*. *aurantiacum* did not produce any transparent halo zone, which indicates that the *E. aurantiacum* does not produce the protease enzyme. The plate without inoculum serves as a control and it is represented in Figure 7.

Amylase activity

The clear zone is obtained around *T. suis*, which exhibits positive results, and was absent in *E. aurantiacum*, which represents a negative result which was represented in Figure 7, respectively.

Plant Growth Promoting Activities

IAA Production

Gordon and Weber's method used to determine the production of IAA by the endophytic bacterial isolates. Colour change in the samples indicates the biosynthesis of IAA by the bacterial strains *T. suis* and *E. aurantiacum* and it is shown in Figure 8,

respectively.

Ammonia Production

Colour change in the test tubes (B) and (C) indicates the production of ammonia by both the bacterial strains, and it is represented in Figure 9, respectively.

Hydrogen Cyanide Production

The sulfocyanate colorimetric method was used to detect the volatile HCN production by the bacteria. The colour change from yellow to orange indicates the production of HCN in *T. suis* strain shown in Figure 10. *E. aurantiacum* shows a negative result for the HCN production is represented in Figure 10.

The endophytes are an important bioresource for present-day agriculture, as it benefits its host by promoting its growth, improving nutrient supply, acting as a biocontrol against pathogens, and increasing the disease resistance (Omomowo and Babalola, 2019). In this research, we developed a biocontrol technique using potential endophytic bacteria to suppress the pathogenic fungi, which caused the infection to the radish plant. Two bacterial strains from the isolated bacterial strains were found to inhibit the pathogenic fungi, *Aspergillus Flavus*. *Flavus* infection occurs in hosts while pre-harvest but does not show any symptoms. This pathogen does not only inevitably decrease the crop yield but also causes contamination of seeds by secreting aflatoxins that lead to health issues to humans or animals known as aflatoxicosis.

Endophytic bacteria are also proved to be stimulating anti-fungal metabolites such as cell-wall degrading enzymes when the pathogenic fungi colonize on the surface of the plant. These enzymes break the fungal cell-wall units by using various mechanisms (Mohamad et al., 2018).

Protease and amylase activity was performed for the antagonistic bacterial strains to determine their fungal cell wall degrading capability. Amylase is an enzyme that catalyses the hydrolysis of starch, and the protease enzyme is responsible for proteolysis. To determine the enzyme activity of the isolated endophytic bacterial strains, the (Strauss et al., 2001) method was used. *T. suis* strain showed positive for cellulase and amylase activity. This indicates the ability of *T. suis* to hydrolyze the casein protein, which is found in the fungal cell wall by producing protease enzyme and the ability of the bacteria to hydrolyze starch by producing amylase. For *E. aurantiacum* strain, negative results were obtained. This shows *E. aurantiacum* could not produce either amylase or protease enzyme.

In addition to the antagonistic activity, both the

strains were found to produce ammonia and Indole acetic acid, which suggests that the endophytes are not only biocontrol agents but also plant growth promoting organisms. IAA is a plant hormone of auxin class. Auxins are identified as plant growth hormones because of its ability to help in differential plant growth with the response to gravity (Teale et al., 2006). The colour change is due to the Tris-Iron (III) complex formation due to the reduction of Fe^{3+} ions. IAA is an important auxin involved in plant physiological processes like cell signalling, plant growth, and plant defence systems. Studies indicated that IAA production by endophytic bacteria plays a vital role in host plant root development. Volatile HCN, produced by the bacteria, reacts with the picric acid (yellow) and forms isopurpuric acid (orange). HCN is considered as one of the biocontrol agents against plant pathogens (Whankaew et al., 2014). *B*-cyanoalanine produced by plants, detoxify the cyanide released by bacteria (Kosma, 2005). The remaining non-toxic HCN concentration plays a role in plant growth development. Ammonia contains nitrogen, which promotes plant growth by playing a key role in photosynthesis. Ammonia controls the pH of the soil, which helps in the overall uptake of nutrients from the soil.

In this study, the two bacterial isolates *E. aurantiacum* and *T. suis* showed antagonistic activity against the pathogenic fungi *A. flavus* isolated from *Raphanus sativus*, which makes them highly suitable as an alternative for chemical fertilizers to provide resistance to plant pathogenic fungi. These strains expressed a range of plant growth-promoting properties, including IAA production, ammonia production, and HCN production. The results show that *T. suis* is the most effective strain for radish growth development.

CONCLUSIONS

The isolated strains, *E. aurantiacum* and *T. suis*, have a vital role in the biological control of *A. flavus* infection on radish plants and improve their growth development. The isolated endophytic bacteria are proved to be a great tool as a biocontrol agent and a great alternative for conventional fungicides and other harmful chemical treatments. Futuristic work based on the *in vitro* growth analysis of the radish plant by the seed bacterization method is required to examine the significance of these endophytic bacterial isolates.

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Conflict of Interest

The authors declare that there is no conflict of interest for this study.

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