RESEARCH ARTICLE

HARNESSING THE POWER OF ENDOPHYTES: INSIGHTS INTO THEIR ROLE IN PLANT GROWTH PROMOTION, BIOACTIVE COMPOUNDS FORMATION AND SUSTAINABLE APPLICATIONS IN AGRICULTURE

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Abstract:

Endophytes are the microorganisms that live in the plant tissues without causing any harm to the host. These enhance plant growth, provide resistance to the environmental stress and also protect the host plant from the pathogens. The endophytes are bacteria, fungi and actinomycetes. These can be isolated from different plant parts on the media. Their metabolic products produced have gained significant interest due to their potential in the production of bioactive compounds with antimicrobial, anticancer and antioxidant properties. Identification of novel endophytic species is by modern techniques such as Polymerase Chain Reaction, DNA sequencing, Next-generation sequencing, Fluorescent *in-situ* Hybridization, Restriction Fragment Length Polymorphism, Metagenomics and Denaturing Gradient Gel Electrophoresis and have allowed for a deeper understanding of the genetic diversity. The bioactive compounds characterization involves the use of various analytical methods such as High-Performance Liquid Chromatography, Gas Chromatography-Mass Spectrometry, Nuclear Magnetic resonance Spectroscopy, Polymerase Chain Reaction, Metagenomics,16S rRNA gene sequencing, FTIR and Matrix-Assisted Laser Desorption Ionization. This research has broad applications in agriculture, medicine and biotechnology offering sustainable alternative to the synthetic chemicals.

Keywords: Endophytes, Bioactive Compounds, Molecular Study, Molecular Characterization.

Introduction:

The microorganisms living inside the plant tissues without causing any disease are called as endophytes Endophytes live in various plant tissues and form helpful relationship that facilitates nutrient exchange and improve other activities. The bacterial endophytes used as biofertilizers to improve crop production and significantly reduce the chemical input into the environment. (Jadhav *et al.* 2024). The term endophytes (Gr.endon=within and phyton=plant) was first coined by De Bary and an endophyte is a bacterial or fungal microorganism which spends the whole or part of its life cycle colonizing inter-

and /or intra-cellularly inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease. Endophytic bacteria originate from the rhizosphere, seeds or plant material (Soundarapandian and Dhandayuthapani, 2010).

The plants internal tissues provide a uniform and safe environment for the endophytes. The endophytes are used for more successful biological control of plant diseases. Endophytic microbes stimulate plant growth directly by producing plant hormones, nutrient uptake and resistance to the stress conditions.

The endophytes also protect the host plant from pathogenic microorganisms. Endophytes also cause associative nitrogen fixation, lower ethylene levels, production of siderophore, solubilization of nutrients, promotion of mycorrhizal functioning, decreasing pollutant (organic or heavy metal) toxicity, etc (Soundarapandian and Dhandayuthapani, 2010). Plant growth promoting rhizobacteria (PGPR) are generally exploited to a broad range of agricultural crops to enhance the growth, increase seed germination, increase the plant weight and also increase the yields. PGPR increase the plant growth by bacterial synthesis of plant hormones such as indole acetic acid, cytokinin and gibberlins as well as increased mineral and nitrogen availability in the soil. Rhizobacteria use numerous mechanisms as nitrogen fixation, phosphate and potassium solubilization, siderophore production, phytohormone production (15, 17–20), antibiotic production, volatile organic compounds (VOCs) exopolysaccharides efficient a wide range of other microorganisms (Cochard *et al.,* 2022). Endophytic bacterial communities produce secondary compounds and hormones so that the invasive plants can compete in the habitat (Mamangkey *et al.*, 2019).

Endophytic microbes can be isolated from surface disinfected plant tissues or extracted from within plants (Yuan *et al.,* 2017). *Paenibacillus polymyxa* is a plant growth promoting rhizobacterium has immense potential to be used as an environmentally friendly replacement of chemical fertilizers and pesticides. Chemical fertilizers, pesticides and herbicides addition in the soil is a worldwide issue (Khan *et al.,* 2020). Bioremediation, designing consortium, bioinoculants, concomitant approaches such as bioremediation with plant growth promotion can help to solve this issue. To overcome the environmental issue, the production of selected PGPR inoculants which can withstand the specific conditions, is required. Information and training to the farmers and staff also needed to understand and apply endophytic PGPR inoculum for specific plants to get the fruitful results (Tiwari *et al.,* 2010).

Molecular Study of the endophytic microorganisms was done by Polymerase Chain Reaction (Yadav & Chowdappa 2015), DNA sequencing (Suryanarayanan *et al.,* 2008), Next-Generation sequencing (Chaparro *et al.,* 2014), Fluorescent *in-situ* Hybridization (Kumar, 2015), Restriction Fragment Length Polymorphism (Berg *et al.,* 2005), Metagenomics (Dazzo *et al.,* 2008) and Denaturing Gradient Gel Electrophoresis (Kumar, 2015).

Molecular characterization of bioactive compounds produced by the endophytes was performed by High Performance Liquid Chromatography (Berg *et al.,* 2005), Gas Chromatography-Mass Spectrometry (Yadav & Chowdappa 2015), Nuclear Magnetic resonance Spectroscopy (Suryanarayanan *et al.,* 2008), Polymerase Chain Reaction (Kumar, 2015), Metagenomics (Chaparro *et al.,* 2014),16S rRNA gene sequencing (Suryanarayanan *et al.,* 2008), FTIR (Nausheen, 2021)

and Matrix-Assisted Laser Desorption Ionization (Dazzo *et al.,* 2008).

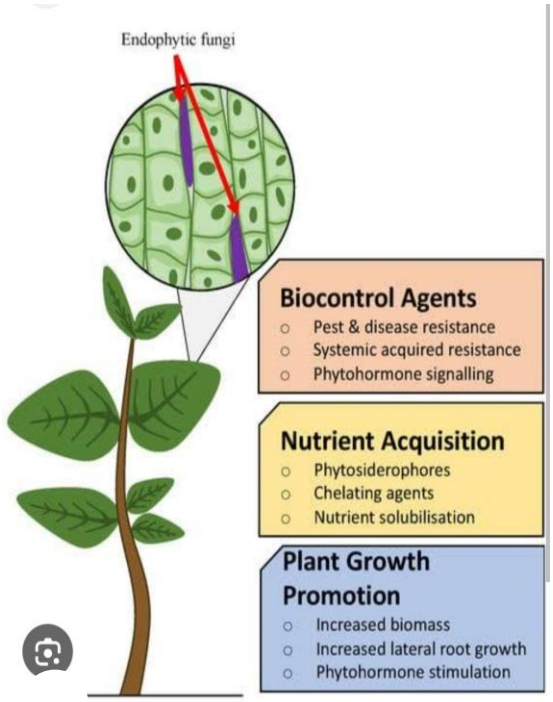

Fig. 1: Endophytic Fungi

(https://www.mdpi.com/microorganisms/microorganisms-11-01276/article_deploy/html/images/microorganisms-11-01276-g003.png)

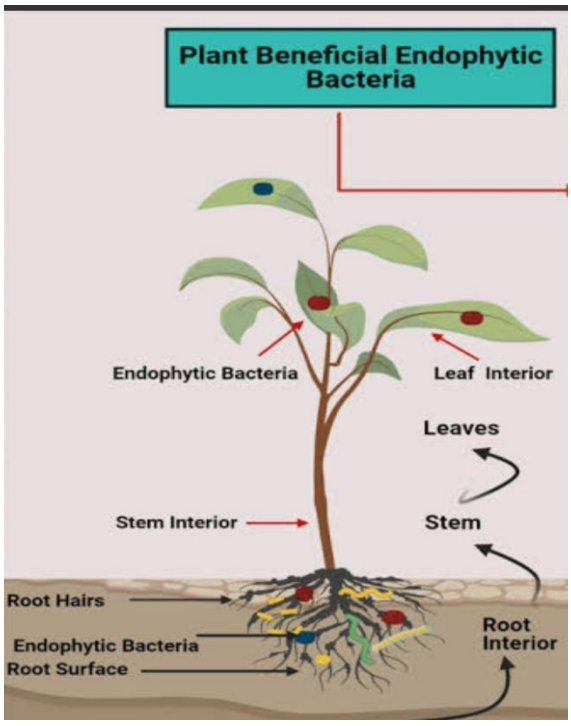

Fig. 2: Endophytic Bacteria

(https://surl.gd/ladcrh)

Plant Growth Promoting Endophytes:

1. Sample collection and isolation of endophytes: Various samples were collected as bulbs, root nodules, leaves, roots and rhizomes for the isolation of endophytic microorganisms.

1.1. **Collection of bulbs:** *Lilioum lancifolium* fresh and healthy bulbs were selected and collected for the isolation of endophytes (Wei *et al.,* 2019 and Khan *et al.,* 2020).Firstly the bulbs were washed with tap water to remove the adhered dust particles. Outermost layers were peeled off and the inner portions were washed with tap water for 5 minutes. Samples were dipped in 70 % ethanol for 1 min followed immersion in 10% NaClO solution for 20 min.Bulbs were then washed with sterile distilled water. After surface sterilization, the outer layer of bulb was removed aseptically. The bulb was then cut into approximately 1 cm x1 cm pieces and inoculated on LB agar plates. The plates were incubated at 30 ± 1° C until the bacterial growth started on the bulb pieces. After 2-3 days of incubation, the appeared colonies were inoculated separately into LB broth and incubated at 30 ± 1 ° C until the pure cultures were obtained by serial subculturing. The isolated endophytes were stored at -80 ° C (Khan *et al.,* 2020).

1.2. Collection of roots, stems and leaves: Roots, stems and leaves of *Chromolaena adorata* were collected for the isolation of endophytic bacteria. Fragments of Roots, stems and leaf (±1-2 cm) were washed under flowing tap water. Surface sterilization was done by dipping fragments into solutions as follows: 70% EtOH (3 min), NaOCl (5 min), distilled water, 70% EtOH (1 min), distilled water. Fragments were dried aseptically using filter paper. Fragments were cut into small pieces and placed on top of Tryptic Soy Agar. Then incubated at37 ° C for 16 to 18 hours. Colonies appeared were transferred to nutrient agar supplemented with ketocanazole (0.3 g/ml) (Lin *et al.,* 2023).

Bacterial isolation samples of rice plants were collected from Bangkok, Chonburi and Supanburi provinces, Thailand. Roots (Yuan *et al.,* 2017, Vinayarani and Prakash,2018, Cochard *et al.*, 2022, Yuan *et al.,* 2017, Lin *et al.,* 2023, Helal *et al.,* 2022) and stems were cleaned with running tap water and cut into small pieces. Surface sterilization was performed using 10% (v/v) NaHClO that was added with a few drops of Tween-20. Samples were rinsed with sterilized distilled water five times and ground using a mortar and a pestle. Ground tissues were placed in glass bottles containing sterilized distilled water and shaken on a rotary shaker to obtain bacterial suspension. Serial dilutions of the suspension was prepared up to the 10-3 concentration and plated on nutrient agar (NA; HiMedia) and tryptone soya agar (TSA; HiMedia) plates. Bacterial isolates were obtained after incubation at 30° C for 7 days. All isolates were purified and further grown on NA plates. Control plates were obtained by plating the water that was used for the final rinse on NA and TSA plates (Tiwari *et al.,* 2010).

Healthy and asymptomatic leaves of *O. sanctum* var. CIM-Angana were selected for the isolation of endophytic bacteria. The leaves were washed under flowing tap water for 10 min and were placed in 1% Sodium hypochlorite (NaOCl) for 10 min and rinsed 4 times with 0.02 M sterile potassium phosphate buffer pH 7.0.A 100 µl of aliquot was taken from the final buffer wash and transferred to 5 ml nutrient broth in screw capped bottle with control as sterility check. Samples were discarded if the growth was detected in the sterility check samples in nutrient broth kept in an incubator shaker (200 rpm at 28 °C) after 48 h. Each sample was macerated in a sterile pestle and mortar with sterile distilled water. 100 µl of the extract was taken and serial dilutions up to 10^{-5} were made. Each dilution was placed on three different media: nutrient agar, King's B medium and potato dextrose agar with three replications each. The plates were incubated at 28 °C for 48-72 h.The colonies on the media looking different by their cultural characteristics were isolated (Tiwari *et al.,* 2010).

The *Mangifera indica* L plant root material was collected from the outskirts of Shringaltali, District Ratnagiri (M.S.). The roots were dipped in 1 % sodium hypochlorite for 5 min. and then in 70 % ethanol for 60 seconds, followed by rinsing them in sterile distilled water. The pieces of roots were transferred into 100 ml sterile Trypticase Soy Broth (HI Media Laboratories, India) and kept on rotary shaking incubator at37^{o}C for 8-10 days. After 10 days of incubation, the broth was diluted and 1ml was spread over sterile nutrient agar and trypticase soy agar plates. The plates were incubated for 2 to 3 days at 37^{o}C, and then observed for the growth of various bacterial endophytes. These bacterial colonies were then sub-cultured on nutrient agar slants and preserved for further procedures. Selection of efficient isolates was carried out by Colony picking (Bhalerao *et al.,* 2023).

Roots of rice plant (*Oryza sativa)* have collected from nearby regions of Guhagar City, Ratnagiri district, Maharashtra state, India. The rice plant samples were kept in sterile polyethylene bags and transported to the laboratory. Roots were washed with sterile distilled water and then surface sterilized with 70% ethanol for 1 minute, followed by treatment with 2% sodium hypochlorite for 3 minutes to kill and clean the outer microflora of the roots. The surface sterilized roots used for making serial dilutions from10-1 to 10-8. The 0.1 ml of serially diluted aliquot from last three dilutions was spread on nutrient agar plates; the plates were incubated in the incubator at 370C for 24 hours. Out of total 36 isolates, 6 isolates separated on the basis of morphological characteristics and used for further studies (Jadhav *et al.,* 2024).

1.3. Collection of rhizomes: Rhizomes were used for the isolation of endophytic bacteria. The collected rhizomes were washed in running tap

water. This removes the adhered soil particles. Then rhizomes were dipped in phosphate buffer (per L:6.33 g of NaH_2PO_4; 16.5 g of $Na_2HPO_4.7H_2O$; 200 ml Tween 40).To remove foam of Tween 40, distilled water was used.Rhizhomes were further sterilized by immersion in 70% ethanol for 2 min.and in 3.5 %sodium hypochlorde for 3 min and then rinsed in distilled water.1 g of rhizome was ground in sterile motar and pistle with phosphate buffered saline and the solution was made up to 10 ml.Serial dilutions from 10^{-1} to10^{-4} were prepared.0.1 ml of aliquots were spread on nutrient agar containing Nystatin (50 mg/ml.).The plates were incubated at 37° C for 7 days.Colonies were selected and pure cultures were preserved in 40 % (v/v) glycerol solution at 20 °C (Vinayarani and Prakash, 2018*)*.

1.4. Collection of nodules: Fresh, healthy and unbroken pink root nodules of gram crop plants were used for isolation of *Rhizobium*. The medium used for isolation of the *Rhizobium* was modified Yeast Extract Mannitol Agar. Nodules were taken from the mature respective plant roots and washed with sterile distilled water to remove attached soil particles. Nodules were externally sterilized by 1% Mercuric Chloride. Again water wash treatment was done to remove the Mercuric Chloride. Cleaned nodules were crushed in sterile distilled water.The five serial dilutions were prepared from this. The last dilution was selected and used for the isolation of *Rhizobium*..A loopful of the last dilution was selected and streaked on sterile Yeast Extract Mannitol Agar (YEMA) medium modified containing 40µg/ml $ZnCl_2$ and $CoCl_2$ (Tong and Sadowsky, 1994) and 500 µg/ml $CuSO_4$. The streaked plates were incubated at 280C for 3 days. After incubation, colony characteristics were taken. Isolated *Rhizobium* species were characterized based on the standard procedures. Grams nature & morphology were detected and noted down (Joshi and Kamthane, 2017and Kamthane,2012).

2. Morphological & Biochemical Characterization of Isolated Endophytic Bacteria:

2.1. The morphological and biochemical characteristics of the isolates were examined according to Bergey's Manual of Determinative Bacteriology. Endophytic bacterial isolates were characterized based on morphological characteristics such as colony size, shape, margin, elevation, color, consistency, and opacity. The biochemical characteristics such as carbohydrate utilization tests (KB009 Hicarbohydrate kit, HiMedia), citrate utilization, indole production, methyl red test, vogue Proskauer test, catalase test, oxidase test, nitrate reduction, and H2S production have been studied (Jadhav *et al.*, 2024).

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After incubation, colony characteristics were taken (Kamthane, 2012).

2.2. Four endophytic fungi were identified from cotton roots via morphology and ITS (internal transcribed spacer region) phylogenic analysis (Yuan *et al.*, 2017).

2.3. The colony morphology, size, shape, colour and growth pattern noted down. Biochemical IMViC tests, citrate test, presence ofoxidase, catalase, succinic acid, starch hydrolysis, ammonia production, casein hydrolysis were conducted to characterize the isolated bacterial endophytes. The Gram's reaction was done as per standard procedure.T he motility was checked using hanging drop method. For the KOH solubility test, a loopful of bacteria mixed with 3% KOH solution on a clean glass slide for 1 min and observed for thread like mass (Vinayarani and Prakash, 2018).

2.4. Bacterial isolates were subjected to pasteurization step to select the spore forming bacteria (Helal *et al.,* 2022).

2.5. Various biochemical and physiological tests were carried out using QTS-24 Kit. The 18 hours old bacterial colony grown on LB plate was suspended in 6 ml sterile saline. Liquid paraffin was added to the cups of ADH and H_2S for creating anaerobiosis. The box was covered with the supplied plastic lid and incubated at 37ºC for 18-24 hours. Potato dextrose agar (PDA) was used for culturing of fungi (Maryam *et al.,* 1974).

2.6. Preparation of Fungi Culture: The endophytic fungi were cultured by using two methods as liquid culture medium method and solid culture medium method of fungi.

Liquid culture medium method of fungi: The fungi strain was cultured in liquid Czapek-Dox medium at 25ºC in a shaker incubator at 150 rpm. The concentration of spore suspension was adjusted to $1x10^7$ conidia /ml with deionized water.

Solid culture medium method of fungi: The maize-sand (V/V=1:1) medium was disinfected by high pressure steam at 121 ºC for 20 min. Five micro liters of fungi culture solution was inoculated into sterile maize-sand medium at 25ºC under static culture to generate a final spore count of$1x10^8$ conidia per gram of solid medium (Yuan *et al.,* 2017).

2.7. Preparation of cell-free culture filtrate: Fungal agar-mycelium disks (5 mm diameter) were taken from the edge of an actively growing fungal colony. The disks were inoculated to a 500 ml Erlenmeyer flask containing 300 ml liquid Czapek-Dox medium and the culture was maintained at 25ºC on a shaker incubator at 150 rpm for 5 days. The crude culture filtrate was filtered with three layers of filter paper and the filtrate was filter-sterilized through a 0.2 µm milipore filter (Wei *et al.,* 2019).

2.8. Calculation of the percentage of infection: A 0-4 scale was used to classify the plants according to the percentage of plant tissue affected by chlorosis, leaf necrosis or defoliation (0=healthy plant or plant without symptoms,1=plant affected by 1-33%,2=34-66%,3=67-99% and 4=dead plant).The disease incidence, disease index and percentage of protection were calculated as follows:

Disease incidence (%) = $[(n_1+n_2+n_3+n_4)/n] X 100$

Disease index (%) = $[(0n_0+1n_1+2n_2+3n_3+4n_4)/4n] X 100$

Protection (%) = [Disease index ($_{Control}$) - Disease index ($_{treatment}$)]/ Disease index ($_{Control}$) X 100

Where n_0-n_4 were the numbers of plants with each of the corresponding disease ratings and n was the total number of the plants assessed (Yuan *et al.,* 2017**).**

Another method is also there to calculate the DI (Disease index) based on the following criteria: healthy plants=0; less than one or part of the cotyledons turned yellow or necrotic =1; two cotyledons turned yellow or necrotic=2; one true leaf turned yellow or necrotic=3;two or more true leaves yellowing or necrosis=4.

DI= Σ (number of stages X number of plants) / (highest level value X total number of plants) X 100 %. Each pot trial was repeated three times in triplicates (Zhang *et al.,* 2023).

The disease severity index was calculated by using five point scale. The leaves were categorized in five groups on the basis of infection

to calculate disease severity index (DSI). The disease severity index was calculated by using the following formula:

$$DSI = \frac{\Sigma \text{ All rating}}{(\text{Number of observations}) \times (\text{All ratings} - 1)} \times 100$$

Percentage of infection categorized in five ratings as 0-1; 2-25; 26-50; 51-75 and 76 and above. The leaves were counted in each ratings and multiplied by 0,1,2,3,4 and 5 respectively. The total leaves were counted and this will give Σ of all ratings. Number of observations will be 100.All ratings are 5.These values were put in the formula and got the percentage of DSI. This value will give percentage of DSI of collected leaves. This will be converted for 100 leaves (Kamthane, 2002).

3. Plant growth promoting (PGP) traits of endophytic microbial isolates:

The endophytes promote the plant growth includes nitrogen fixation, phosphate solubilization, antimicrobial activity, volatile metabolites and nonvolatile metabolites production to kill pathogenic microorganisms. Microorganisms also support plant growth by producing organic acid, indole acetic acid, and phosphatase enzyme. The microorganisms also inhibit the growth of pathogenic microorganisms by a common mechanism producing cell wall degrading enzymes such as protease, cellulase. ACC deaminase, α-/β-amylase and chitinase.

3.1. Microorganisms were considered as: Diazotrophs when grown on nitrogen free medium and fix nitrogen.

3.2. The antimicrobial activity was evaluated against *Ralstonia solanacearum* as a representative for bacterial phytopathogens. Nutrient agar at 50ºC was seeded with 10% of 24 h grown *R. solanacearum* then mixed and poured into petriplates. *R. solanacearum* seeded medium was spot inoculated with each bacterial isolate and incubated at 30 ºC for 24 to 48 h. The positive results obtained when bacterial isolates show *R. solanacearum* inhibition zone around them (Helal *et al.,* 2022).

The antifungal activities against *Fusarium oxysporium* were tested using dual culture plate assay method. At the centre of the potato dextrose agar,6 mm mycelial agar disc of7-days-old fully grown *Fusarium oxysporium* was kept. A loopful of an overnight incubated each of the bacterial isolates was inoculated by streaking 3 cm away from the fungal mycelial disc. The plates were incubated at 25ºC for 5 days. The positive results obtained when inhibited fungal growth either as a contact inhibition or an inhibition zone (mm). (Helal *et al.,* 2022)

The antifungal activity of *F. solani* CEF559 filtrate was studied on sporulation and spore germination of *V. dahlia* In this procedure they added 20 ml **c**ell free filtrate of *F. solani* CEF559 to a 50 ml sterile Erlenmeyer flask and 100 µl spore suspension ($1x10^7$ spores/ml) of *V. dahlia* Vd080 cultured in liquid Czapek-Dox medium was added. In the control plate, same amount of liquid Czapek-Dox medium and spore suspension of *V. dahlia* Vd080 were added. The flasks were shaken at 150 rpm for4 days at room temperature. Spore concentrations were estimated with a hemocytometer on 4 and 6 days. This test was repeated twice (Wei *et al.,* 2019).

The percentage of growth inhibition (IR) 6 days after inoculation was calculated as follows: IR (%) = [(control colony diameter – treatment colony diameter)/control colony diameter] × 100. (Wei *et al.,* 2019 and Kamthane,2013).

3.3. *In vitro* biocontrol testing of *R. japonicum against* the fungi isolated: This was done by dual culture technique using King's B agar. Now the isolated *R. japonicum* culture was spot inoculated on one edge of the plate. On the other edge of the plate, a 5mm disc of four day old pure, isolated and identified fungal pathogen was placed. The plates were prepared in triplicates along with the control plate. In the control plate, the purified fungus was inoculated and incubated only. The

plates were prepared as mentioned above and were kept at room temperature for incubation to observe the interactions among the two organisms i.e. in between *R. japonicum* and the fungus. (Kamthane, 2002).

The percent inhibition was calculated using the following formula (Kamthane, 2013)—

Percent inhibition= (C-T) x100/C

C=Radial growth of fungus in mm in control plate.

T= Radial growth of fungus in mm on plate inoculated with *Rhizobium japonicum.*

***In vivo* assay of antagonisis:** The *A. tenuissima* causing Alternaria Blight of soybean varieties JS 71-5 and JS93-05 were isolated from the respected varieties. The *A. tenuissima* antagonist causing Alternaria Blight of soybean was tested *in vitro* and later tested *in vivo* in pot culture experiment along with control for the comparison. The fungal spore suspension adjusted to $20x10^6$ spores/ml was used for the study. Sterilized garden soil and sterilized sand in the ratio of 2:1 was used for the experiment.

The seeds are treated with *Rhizobium japonicum* isolated from the two mentioned varieties as JS 71-5and JS93-05 of soybean. This is done by mixing isolated rhizobia culture in 10 percent sugar and 40 percent gum arabic to form slurry. In this, seeds were added. With the result, a uniform coat of the *R japonicum* is formed around the seeds. The treated seeds are dried in shade and sown immediately. In the two test pots five coated seeds were sown. In the first test pot, to the seedlings five cuts were made with a disinfected scissor. In the second test pot the seedlings were without cuts. In the control pot the uncoated five seeds were sown. After a weak, the suspension of *A. tenuissima* was applied. The root nodules, roots and shoots were harvested on 28^{th} day after soybean sowing. The roots were washed with water to remove the soil particles attached. Nodules formed, shoot length as well as root length and dry weight was recorded. Shoot length as well as root length was recorded in cm and the dry weight was measured in grams. The number of root nodules in each variety were also calculated (Kamthane, 2013).

In vitro screening for antagonism: In vitro inhibition of mycelial growth of *Fusarium* species was tested by the bacterial isolate using dual culture method. The percentage of growth inhibition was calculated by using the formula:

% inhibition = [(R-r / R x100)]

r=fungal colony radius opposite to the bacterial colony and

R=maximum radius of the fungal colony away from the bacterial colony (Maryam *et al.*, 2019).

Another method was also there according to Lin *et al.* in 2023**.**

The fungus *V. dahlia* V991 was cultivated for 7 days at 25^{o}C for 7 days on PDA plates. Then200 µl of bacterial suspension was spread onto LB agar plates and incubated at37^{o}C for 12 h. A fungal block 1 cm in diameter was placed at the center of the PDA plate. Equivalent amount of tested bacterial strains and blank LB block (0.5 cm x 0.5 cm x0.3 cm) were placed on both sides of the same plate. All isolates were incubated at 25^{o}C for 3-5 days, and the fungal mycelial growth was observed.

The inhibition rate (IR) was calculated by using the formula:

(Control colony diameter-processing colony diameter) x 100% / (Control colony diameter-fungus cake diameter). All plates were repeated in triplicate.

3.4. Volatile metabolites and its inhibitory bioassay: This bioassay was tested using double dish method. In this they tested inhibition of Verticillium wilt of cotton causing *Veticillium dahlia* mycelial growth by volatiles of *Fusarium solani* CEF559 isolate. A mycelial agar plug of CEF559 was placed onto PDA in a petridish and incubated at 25^{O}C for 7 days. Another petridish containing a mycelial agar plug (5 mm diameter) of *V. dahlia* Vd080 was placed inversely over the

petridish containing 7 days old culture of *F. solani* CEF559.This double dish set was immediately sealed with paraffin. In the control plate, a PDA petridish inoculated with *V. dahlia* Vd080 was placed inversely over another petridish containing PDA but without *F. solani* CEF559 to make a double dish set. There were three replicates of double dish sets. The double dish sets were incubated at 25ºC for 7 days. The diameter of *V. dahlia* Vd080 in each double dish set was measured. The percentage of growth inhibition was calculated using:

The percentage of growth inhibition (IR) 6 days after inoculation was calculated as follows:

IR (%) = [(control colony diameter – treatment colony diameter)/control colony diameter] × 100.

The experiment was performed three times (Wei *et al.,* 2019 and Zhang et. Al., 2023).

3.5. Nonvolatile metabolites and its inhibitory bioassay: The nonvolatile metabolites production was estimated by placing a 5 mm disc of *F. solani* CEF559 mycelial disc centrally on two layers of cellophane covering PDA medium. The plates were incubated at 25ºC for 6 days before the two layers of cellophane, CEF559 culture was removed, and a 5 mm disc of*V. dahlia* Vd080 was placed at the centre. The control plates were only inoculated with *V. dahlia* Vd080.The Petridishes were again incubated for 12 days. The percentage of growth inhibition after inoculated with *V. dahlia* Vd080 was calculated by using the formula:

IR (%) = [(control colony diameter – treatment colony diameter)/control colony diameter] × 100.

Three replicates were there and the experiment was repeated twice (Wei *et al.,* 2019).

4.0. Effect of Endophytic Metabolites:

4.1. **The antifungal activity of metabolites:** The antifungal activity of *F. solani* CEF559 metabolites on spore germination of *V. dahlia* was studied. In this study, *V. dahlia* Vd080 spores were harvested 5 days post inoculation in Czapek-Dox medium. Spore concentration was adjusted to $2x10^3$ spores/ml. 100 µl spore suspension was evenly spread onto a PDA plate. To determine the effect of high temperature on the activity of *F. solani* CEF559 metabolites, a PDA plate with metabolites of *F. solani* CEF559 was autoclaved at 12ºC for 20 min and then inoculated with *V. dahlia* Vd080.In the control plate, only inoculated with *V. dahlia* Vd080.The plates were maintained at 25ºC and germination observed under microscope. The germination inhibition was calculated as follows:

GIR = (germination in control-germination in *F. solani* CEF559 treatment)/ germination in control x100 %. There were three replicate plates and the experiment was repeated twice (Wei *et al.,* 2019).

4.2. The antifungal activity of crude protein: The antifungal activity of crude protein of *F. solani* CEF559 on *V. dahlia* was studied. The cell free culture broth was added with 60% saturated ammonium sulphate (W/V) that was stirred overnight at 4ºC to allow protein precipitation. The precipitated proteins were precipitated by centrifugation at 6000 rpm for 30 min at 4ºC, dissolved in a 1/10 (V/V) phosphate buffer (0.2 M, pH 7.0) and dialyzed for 12 h to remove ammonium sulphate. Some crude proteins were dissolved in phosphate buffer (0.2 M, pH 7.0) and their antifungal activity was tested against *V. dahlia.* The PDA plate was inoculated with *V. dahlia* Vd080 spore suspension by the spread plate method. Two wells were drilled symmetrically. The PBS- phosphate buffer saline containing proteins (2 mg/ml) was added to one well and the PBS treated well as a control. At 25ºC for 3 days, the plates were incubated. There were three replicate plates and the experiment was repeated twice (Wei *et al.,* 2019).

4.3. Organic acid production assay:

Organic acids, such as acetic acid, lactic acid, and citric acid, lower the pH of the environment, creating an acidic environment that is hostile to many microorganisms. Most pathogens thrive in

neutral pH conditions, and the acidic environment impairs their ability to grow and multiply.

Organic acids can penetrate the cell membranes of pathogens, causing structural damage. This disrupts the integrity of the pathogen's cellular structure, leading to leakage of essential intracellular components and, ultimately, cell death (Khan *et al.,* 2009).

50 µl of bacterial suspension was inoculated in $MgSO_4$ (10mM) in 800 µl of sucrose tryptone medium (ST) containing sucrose (20g/L) and Tryptone (5 g/L). The medium was supplemented with 10 ml of trace elements solution. Trace elements solution contained $CuSO_4.5H_2O$ (20 mg/L), $FeCl_3$ (100 mg/L), H_3BO_3 (20 mg/L),$NaMoO_4$ (20 mg/L),$MnCl_2$. $4H_2O$ (20 mg/L), $ZnCl_2$ (280 mg/L). The samples were incubated at 30ºC for 5 days and with 200 rpm.100 µl of 0.1% alizarin red pH indicator was added to all samples to detect the organic acids (Khan *et al.,* 2020).

4.4. Indole acetic acid production: Indole acetic acid is a plant growth promoting substances.Some of the important morphological effects of IAA on plant growth are elongation of the stem, gall formation and root hair curling. IAA also enhances the root length and shoots height of the seedlings.

4.4.1. A bacterial suspension of 150 µl in 10 mM $MgSO_4$ was inoculated in 3 ml of 1/10 diluted 869-rich medium. The medium was supplemented with tryptophan with four different concentrations as 0 mg/ml, 2mg/ml, 4mg/ml and 6mg/ml.The samples were incubated at 30ºC for 4 days. Then the cultures were centrifuged at 4000 rpm for 20 min.1 ml of the supernatant was mixed with 2 ml of Salkowski's reagent (98 ml 35% $HClO_4$,2 ml 0.5 M $FeCl_3$). The development of pink colour after 20 min was considered as a positive test for IAA production. Indole acetic acid produced was measured based on the standard curve of known values (Khan et. al., 2020).

4.4.2. Nutrient broth containing 5 mM L-tryptophan was inoculated with bacteria and incubated at 30ºC for 48 hours on the rotary shaker.200µl of the supernatant was obtained by centrifugation and tested for IAA produced by using Salkowski's reagent (Raweekul *et al.,* 2016).

4.4.3. Okon's malate medium with tryptophan (100 mg/L) as a precursor of indole-3- acetic acid was inoculated with cultures. Then the cultures were incubated in a shaker at 160 rpm at 30ºC for a week. The produced indole-3- acetic acid was quantitatively determined by using $FeHClO_4$ and Fe-H_2SO_4 reagents. The bacterial cells were harvested. The supernatant obtained by centrifugation at 8000 rpm for 8 min at 10ºC. The volume of the supernatant was reduced from 70 to 15 ml using freeze dryer (Martin Christ, Alpha 1-4, Germany). The pH of the sample was adjusted to 2.8. Indole acetic acid was extracted. Equal volume of ethyl acetate was added to the cell free liquid culture medium (Supernatant) and mixed in a separating funnel. Ethyl acetate fraction was evaporated to dryness at room temperature in a fume hood. The residues were dissolved in 1 ml of ethanol. The samples were analyzed on HPL Cusing Turbochom software (Perkin Elmer, USA). The elusion was performed by using licosorb-C18 column for IAA. Ethanol: acetic acid: water (30:1:70) was used as mobile phase at the rate of 1 ml/minute for 30 minutes. IAA absorbance was detected on a UV detector at 280 nm wavelength. The concentration of IAA was calculated on the basis of peak height and peak area (Maryam *et al.,* 2019).

4.4.4.IAA produced from endophytic bacteria by using colorimetric method or colour change was determined using Salkowski's reagent.100 µl of overnight culture (OD_{600}=0.5) was inoculated into 100 ml nutrient broth with 0.2 % L-tryptophan (W/V) and incubated for 5 days at 28ºC ± 3ºC.Culture was centrifuged at 10000 rpm for 30 min at 4ºC. Supernatant was mixed with 2

ml Salkowski's reagent (0.1 M $FeCl_3$ solution + 400 ml conc. H_2SO_4+580 ml distilled water). Gently homogenized mixture was incubated in dark for 30 min till pinkish red colour formation. IAA concentrations were estimated by using spectrophotometer under wavelength A_{530} along with blank solution without bacterial culture. Standard curve of IAA obtained by following the same procedure using various concentrations as 0,30,60,90,120 and 140 ppm (Mamangkey *et al.*, 2019).

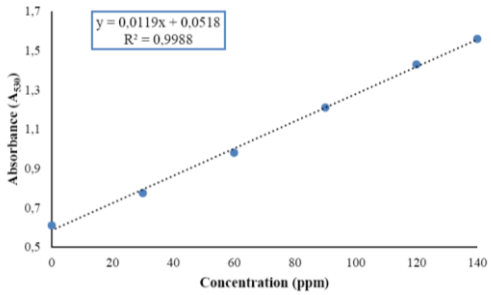

Fig. 3: Standard curve of IAA (Mamangkey *et al.*, 2019)

4.4.5. The standard range was used to allow accurate measurement at low concentrations. The range included the IAA concentrations as: 300, 150, 100, 50, 25, 12.5, 6.25, 3.125 mg/ml (Cochard *et al.*, 2022).

4.4.6. In 15 ml sterile nutrient broth with L-tryptophan in test tubes was inoculated the isolate and incubated at 28^{O}C for 72 h in the dark. Then 2ml of this broth was centrifuged at 12000 rpm for 10 min. followed by addition of 4 ml Salkowski's reagent to the 1 ml of supernatant. The tubes were incubated at 37^{O}C for 1 h. Pink or red colour formation indicated IAA production by the organism (Vinayarani and Prakash, 2018).

4.5. The Phosphate solubilization ability:

Phosphate solubilization not only supports plant growth by making phosphorus more available but also plays an indirect role in pathogen inhibition through competition for nutrients, production of antimicrobial compounds (Rodriguez and Fraga,1999), biofilm formation, the alteration of soil pH (Khan *et al.*, 2009) and alteration of pathogens environment (Sharma, and Bhatnagar, 2012). This multifaceted role of phosphate-solubilizing endophytic microorganisms makes them crucial for integrated pest management and sustainable agriculture practices.

The Phosphate solubilization ability was studied. This ability was detected by spotting the microorganisms on the Pikovskya medium containing tricalcium phosphate and incubated at 28 ± 2^{o}C for 2-3 days. Development of clear zone around the strains indicated positive result for phosphate solubilization (Vinayarani and Prakash, 2018).

5. The hydrolytic enzymes: Different scientists studied the hydrolytic enzymes production. The microorganisms inhibit the growth of pathogenic microorganisms by a common mechanism producing cell wall degrading enzymes such as protease, cellulase. ACC deaminase**,α**-/β-amylase and Chitinase**.**

5.1. **Protease and its detection:** Protease degrade proteins present in pathogens cell wall and the enzymes involved in pathogenesity (Vidhyasekaran andMuthamilan,1999). Protease play an important role in defense mechanism against the pathogens.

To detect the protease production, a loop full of bacteria was streaked on skimmed milk agar plate. The skimmed milk agar plate contained skimmed milk-100 g, peptone-5 g, agar-15 g and distilled water-1000ml.The plate was incubated at 28^{o}C for 48 hours. After incubation, the development of clear zone around the streak was considered as a positive result of protease activity (Vinayarani and Prakash, 2018).

Protease hydrolytic activity was also screened by spotting bacterial colony on skim milk agar (SMA) with composition (g/L): peptone-4 g, yeast extract-1, skimmed milk-12,NaCl-18,agar-15,in 1000 ml distilled water. The culture was then incubated at 28 ± 3^{o}C for 24 h. After incubation, the development of clear zone around the colony

was considered as a positive result of protease activity (Mamangkey *et al.,* 2019).

Protease positive when surrounded by a clear zone on nutrient agar with 10 % skim milk (Helal etal., 2022).

5.2. Cellulase and its activity detection:

Cellulases degrade cellulose, a major structural component of plant cell walls and certain microbial cell walls. Plants use cellulases to break down the cell walls of invading pathogens, particularly fungi (Zhang and Li., 2015).

Cellulose is a major polymeric constituent of fungal cell wall. Cellulase lyses the fungal cell wall and releases the protoplasmic constituents. This is one of the antifungal activities used to control phytopathogenic fungi, to protect the crop plants.

In the basal medium, carboxy methyl cellulose (CMC)was used to determine the cellulolytic activity ($NaNO_3$-1g,KCL-1g,K_2HPO_4-1g,$MgSO_4$-5g,yeast extract-0.5g,agar-15g,distilled water-1000ml).The bacteria was streaked on the medium and incubated at 28ºC for 3 days. After incubation, the plates were flooded with 0.01 % Congo red solution for 15 min and destained with 1 % NaCl solution for 5 min.The degradation of CMC was detected by the production of a clear zone and the bacteria was positive for cellulose production (Vinayarani and Prakash,**2018**).

- Cellulase activity was screened by streaking a loopful of bacterial suspension on Bushnell Has Medium (BHM) with composition (g/L): carboxy methyl cellulose (CMC)-10.0,K_2HPO_4-1.0, $MgSO_4$.7 H_2O-0.2,NH_4NO_3-1.0,$FeCl_3$.6H_2O-0.05,NaCl-18.0,$CaCl_2$-0.02,Agar-15.0 in 1000 ml distilled water. The culture was then incubated at 28 ± 3ºC for 96 h.After incubation; the plates were flooded with 0.3 % Congo red solution for 20 min.1 M NaCl was used to wash the plates. The development of clear zone around the colony was considered as a positive result of cellulase activity (Mamangkey *et al.,* 2019).

5.3. ACC deaminase and its detection:

ACC deaminase plays a vital role in pathogen inhibition primarily by by reducing the production of ethylene and by regulating ethylene levels in plants (Glick, B. R. (2005). ACC deaminase-producing bacteria help plants maintain growth, reduce stress, and enhance resistance to pathogen attacks (Zhang and Glick, 2011). ACC deaminase-producing bacteria, through their ability to modulate plant hormones and induce systemic resistance, offer significant potential for biocontrol applications, particularly in reducing plant disease severity and promoting plant health under pathogen stress (Muller *et al.,* 2015).

Endophytes ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase wasdetermined.250 µl of the bacterial suspension in $MgSO_4$ (10mM) was added to 1.2 ml of salts minimal medium (SMN) containing 5 mM ACC as a sole source of N. The cultures were incubated at 30ºC for 3 days with 150 rpm shaking. Then centrifuged at 4000 rpm for 20 min at room temperature. The supernatant was removed. The obtained pellets were resuspended in 100 µl of Tris-HCl buffer (0.1 M) (pH=8.5). Bacterial cells were disrupted by adding 3 µl tolune followed by vigorous vortexing.Further 10 µl of ACC (0.5M and 100 µl of Tris-HCl buffer (0.1 M) (pH=8.5) were added, and the samples were gently vortex for 10 min. Then incubated at 30ºC for 30 min with 150 rpm shaking.690 µl of 0.56 N and 150 µl of 0.2 % 2,4-dinitrophenylhydrazine reagent (in 2 N HCl) were added to the cell suspensions. Samples were then incubated at 30ºC for 30 min. followed by the addition of 1 ml NaOH (2N). The negative controls were without ACCC. The positive test considered when the colour change from yellow to brown (Khan et. al.,2020).

5.3.1: ACC deaminase production and its activity detection: Bacterial isolates were grown on nutrient agar plates at 30ºC for 48 hours. Cells were scraped off the medium and washed with 1

ml of Dworkin and Foster (DF) salt minimal medium by resuspension and centrifugation. Bacterial suspension was prepared by collecting bacterial cells in one ml of DF salt minimal medium.2 µl of suspension was inoculated on DF salt minimal agar containing 2 mM-amino cyclopropane -1-carboxylic acid as the sole nitrogen source. Plates were then incubated at 30°C for 04 days. Positive bacterial isolates utilize ACC as the nitrogen source. Negative and positive control groups were obtained by inoculating the suspension on DF salt minimal agar and DF salt minimal agar supplemented with 2 mM $(NH_4)_2SO_4$ respectively (Raweekul *et al., 2016*).

5.4. α/β-amylase and its activity detection: α/β amylases break down starch into sugars like maltose and glucose. These sugars, especially maltose, can influence the growth of pathogens. For instance, some pathogens rely on starch or sugars to fuel their growth. The action of amylases can starve pathogens of necessary nutrients by converting starch into smaller sugars, disrupting pathogen development. The breakdown products of starch by amylases may serve as precursors for the synthesis of antimicrobial compounds in plants. For example, glucanases and chitinases—other enzymes in the plant immune response—can be activated by the sugars released during starch breakdown, contributing to pathogen inhibition (Zhang and Li, 2015).

A loopful of bacteria was streaked on starch agar with composition (g/L): soluble starch-10,yeast extract-1.0,NaCl-18,agar-15 in 1000 ml distilled water. The culture was incubated at 28 ± 3°C for 24 h. Iodine solution (1 g iodine dissolved in 2 % KI solution) was flooded over the plate and left for 10 min. Formation of clear zone around colonies indicate a positive result ofα-amylase activity. β-amylase amylase activity was measured using same procedure by reducing soluble starch composition to 5.0 g (Mamangkey *et al.,* 2019).

5.5. Chitinase and its activity detection: Chitinase is an enzyme that degrades chitin, a major component of fungal cell walls. These enzymes impair the structural integrity of the pathogen, preventing it from growing and reproducing (Kassanis and Dempsey, 1979).

The hydrolytic activity was screened by spotting bacterial colony on Colloidal Chitin Agar (CCA) with the composition (g/L):Na_2HPO_4 (6.00),KH_2PO_4 (3.0),NH_4Cl (1.0),NaCl (0.5),yeast extract (0.05),agar (15.0),colloidal chitin 1% (w/v) in 1000 ml distilled water. The culture was incubated at 28 ± 3°C for 96h. Formation of clear zone around colonies indicate a positive result of Chitinase activity (Mamangkey *et al.,* 2019).

6. Hydrogen cyanide (HCN) and its activity detection: HCN can exert direct toxic effects on pathogens, particularly through the inhibition of their respiration and enzymatic activities. The presence of HCN disrupts the electron transport chain in mitochondria, leading to cellular dysfunction and the death of sensitive organisms, including fungal and bacterial pathogens (Bakker and Schippers, 1987).

Hydrogen cyanide (HCN) is known to effectively block the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. HCN forms stable complexes with several essential divalent metal ions (Kumari and Shrivastava, 1999).

Hydrogen cyanide production by the bacteria was detected by growing the antagonistic bacteria on King's B agar medium. A colour change from yellow to brown indicated the cynogenic activity of the bacteria. The intensity of colour was recorded visually (Maryam *et al.,* 2019).

Culture was grown at room temperature (28^0C) on a rotary shaker in Kings B broth. Whatmann Filter paper No.1 was cut into uniform strips of 10cm long and 0.5cm wide saturated with alkaline picrate solution and placed inside the conical flask in a hanging position. After incubation at 28^0C for

48 hours, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to to the amount of hydrocyanic acid evolved. The colour was eluted by placing the filter paper in a clean test tube containing 10ml distilled water and the absorbance was measured at 625nm (Reddy *et al.*, 2008).

This HCN production ability of *Rhizobium japonicum* is the biocontrol mechanism. This HCN production inhibits the host pathogen and protects the host (Kumari and Shrivastava, 1999 and Reddy *et al.*, 2008).

Nutrient agar with 4.4 g/l-l of glycine was used to determine HCN production. Cultures were streaked on agar and Whatmann No.1 filter paper stips dipped in 0.5% picric acid in 2% sodium carbonate solution were inserted from the top of each tube, sealed with parafilm and incubated at 30^{O}C for 4 days. A colour change to brown or reddish-brown was recorded as positive reaction (Vinayarani and Prakash, **2018**).

7. Siderophore and its activity detection: Siderophores are low molecular weight high-affinity ferric iron chelators, are synthesized and secreted by many microorganisms in response to iron deprivation. These compounds solubilise and bind iron and transport it back into microbial cell, usually through specific membrane receptors (Shifa *et al.*, 2022).

The functions of siderophores are to compete with pathogens for available iron. Many pathogens rely heavily on iron for their metabolism and growth. By producing siderophores that tightly bind iron, beneficial microorganisms reduce the amount of free iron available to pathogens, thereby inhibiting their growth and virulence (Schwyn and Neilands, 1987).

To investigate the biocontrol mechanism, the *Rhizobium spp* isolated was tested for the production of siderophore. *Rhizobium* reported to produce a number of siderophores. These include rhizobactin citrate, catechol, anthranilate, vicibactin. Siderophores overcome iron limitation to their growth.1 ml active culture of *Rhizobium* isolated from *chick pea* plants were separately inoculated in the 100 ml sterile succinate broth medium (Bharbhiaya and Rao,1985) containing 4 gram succinic acid, 0.1gram k_2HPO_4,3 gram KH_2PO_4,1 gram $(NH)_4SO_4$,0.2 gram $MgSO_4.7H_2O$,1000 ml distilled water. The pH of the medium was adjusted to 7 before autoclaving. 100 ml sterile succinate broth medium without inoculation was kept as a control. The flasks were incubated at 28^{o}C on orbital shaker adjusted at 120 rpm for 24 hours. After incubation, it acts as a siderophore. After incubation, the flasks were observed for production of Siderophore by performing Arnow tests.

7.1. Detection of Siderophore: Arnow test was performed to determine the production of siderophore.

Arnows assay: For this Nitrate molybdate reagent was required. It was prepared by dissolving 10 gram sodium nitratre and 10 gram sodium molybdate in 100 ml distilled water. To the 1 ml siderophore solution or supernatant, 1ml of 1 N HCl and 1 ml of Nitrate molybdate were added with proper mixing. The catechol produced a yellow colour at this point. Then 1ml of 1 N NaOH solution was added after which colour should be changed to red. The colour appeared should remain stable at least one hour (Joshi and Kamthane, 2017).

Rhizospheric and endophytic bacteria siderophore production was determined as described by Schwyn and Neilands (1987) using Chrome Azurol S (CAS) agar medium. The bacteria were spot inoculated and incubated at 30^{O}C for 3-5 days. Yellow –orange halos formation around the colonies on CAS agar was considered as a positive result (Vinayarani and Prakash, 2018).

8. Nitrogen fixation and its assay: Certain nitrogen-fixing bacteria (e.g., Rhizobium) form symbiotic relationships with plants, particularly legumes. These bacteria colonize plant roots and

provide the plants with a source of nitrogen, which boosts plant health. A healthy plant is better equipped to resist and recover from pathogen attacks (Glick *et al.,* 1999).

A single colony of *P. polymyxa* strain SK1 and *Escherichia coli* O157:H7 grown on LB medium was streaked onto solid nitrogen-deficient malate medium (NFM:0.02 g.L-1 $CaCl_2$,0.1 g.L-1 NaCl,0.01 g.L-1 $FeCl_3$,0.4 g.L-1 KH_2PO_4,0.5 g.L-1 K_2HPO_4,0.2 g.L-1 $MgSO_4.7\ H_2O$,0.002 g.L-1 $Na_2MoO_4.2H_2O$,5 g.L- Sodium malate,15 g.L-1 agar,pH7.2-7.4 using KOH) supplemented with 50 mg.L-1 yeast extract. After incubation, a resulting single colony was then streaked on NFM to confirm the ability to fix nitrogen. Plates were incubated at $28^{O}C$ for 7 days (Khan et. al., 2020).

9. Molecular study of the endophytes: Various methods are used to study endophytes and their interactions with host plants. Some key molecular techniques are as follows:

9.1. Polymerase Chain Reaction: PCR allows amplification of specific regions of DNA (e.g.16S rRNA gene for bacteria or internal transcribed spacer) to identify and study endophytes at the molecular level (Yadav and Chowdappa, 2015).

9.2. **DNA sequencing (Sanger sequencing):** DNA sequencing as Sanger sequencing is used to obtain the exact sequence of amplified DNA fragments. This method provides detail information on the genetic makeup of endophytes. This is useful for identifying species and for phylogenic studies of endophytes (Suryanarayanan *et al.,* 2008))

9.3. Next-Generation sequencing (NGS): NGS allows high-throughput sequencing, which can sequence millions of DNA fragments in parallel. This method explores the diversity of endophytic communities in complex plant tissues (Chaparro *et al.,* 2014)

9.4. Fluorescent In Situ Hybridization (FISH): FISH is used to detect and localize specific nucleic acid sequences within intact cells of endophytes. It uses fluorescently labeled probes that bind to the target DNA or RNA sequences. FISH is used to visualize the special distribution of endophytes in plant tissues. (Kumar, 2015)

9.5. Restriction Fragment Length Polymorphism (RFLP): This technique uses restriction enzymes to cut DNA at specific sites, generating fragment patterns that can be used for fingerprinting microorganisms. This technique compares genetic variation between different endophytic strains and to study genetic diversity (Berg *et al.,* 2005).

9.6. Metagenomics: This technique involves the study of genetic material recovered directly from the environmental samples. Metagenomics is useful for studying the microbial communities of plants and their endophytes without the need for culturing. (Dazzo *et al.,* 2008).

9.7. Denaturing Gradient Gel Electrophoresis (DGGE): DGGE technique separates PCR products based on their melting behavior in a gradient of denaturing chemicals. It is often used to study the diversity of microbial communities including endophytes. (Kumar, 2015).

10. Molecular characterization of bioactive compounds produced by the endophytes:

Molecular characterization of bioactive compounds produced by the endophytes is a very essential aspect of understanding their therapeutic potential. The bioactive compounds which are produced by endophytes living within plants are often studied using various molecular techniques to identify their chemical structure, biosynthesis pathway and biological activities. Some key molecular characterization methods of bioactive compounds produced by the endophytes are as follows:

10.1. High Performance Liquid Chromatography (HPLC): HPLC is commonly used to separate and quantify bioactive compounds from endophytes. HPLC identify bioactive compounds produced by endophytes such as alkaloids, terpenoids and phenolic compounds (Berg *et al.,* 2005.)

10.2. Gas Chromatography-Mass Spectrometry (GC-MS): Volatile organic compounds and secondary metabolites produced by endophytes are identified by the powerful analytical technique as GC-MS. GC-MS identify the structure of bioactive compounds such as volatile terpenes,alkaloids and fatty acids (Yadav & Chowdappa,2015).

10.3. **Nuclear Magnetic Resonance (NMR) Spectroscopy:** Nuclear spin properties analyzed to determine the molecular structure of bioactive compounds by using NMR spectroscopy.NMRgivesthe detailed structural elucidation of complex bioactive compounds such as alkaloids,flavonoids and peptides produced by the endophytes (Suryanarayanan *et al.*, 2008).

10.4. Polymerase Chain Reaction (PCR) and gene cloning: These are molecular biology techniques used to amplify and characterize the genes involved in the biosynthesis of bioactive compounds in endophytes. These genes are often related to the enzymes like polyketide synthases (PKS) or non-ribosomal peptide synthetases (NRPS). PCR amplification of biosynthetic gene clusters (such as PKS and NRPS genes) and their cloning is used to study the molecular mechanisms underlying the production of secondary metabolites in endophytes. (Kumar, 2015).

10.5. **Metagenomics:** This sequencing allows the exploration of microbial communities in plants without culturing, providing a comprehensive analysis of genes responsible for bioactive compounds biosynthesis. Metagenomics is applied to investigate the biosynthetic gene clusters for bioactive compounds in endophytes, allowing researchers to discover novel metabolites (Chaparro *et al.*, 2014).

10.6. Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectroscopy: MALDI-TOF is a type of mass spectroscopy used to analyze the molecular composition of bioactive compounds. This technique ionizes the bioactive compounds with a laser beam and measures the resulting fragments. This technique analyzes peptides, proteins and other metabolites produced by the endophytes (Dazzo *et al.,* 2008).

10.7.16S rRNA gene sequencing (for bacterial endophytes): This is used to identify bacterial endophytes and to study their potential for producing bioactive compounds. This method identifies and cataloge bacterial species in plant tissues that might produce bioactive compounds (Suryanarayanan *et al.,* 2008.).

10.8. Fourier Transform Infrared Spectroscopy (FTIR): It is most useful for identifying chemicals that are either organic or inorganic. It can be utilized to quantitate some components of an unknown mixture and for the analysis of solids, liquids, and gases. The term Fourier Transform Infrared Spectroscopy (FTIR) refers to a development in the manner in which the data is collected and converted from an interference pattern to a spectrum. It is a powerful tool for identifying types of chemical bonds in a molecule by producing an infrared absorption spectrum that is like a molecular "fingerprint". The wavelength of light absorbed is characteristic of the chemical bond and can be seen in this annotated spectrum.

Confirmation of synthesized biomolecules was done by FTIR The product was identified by FTIR.The FTIR spectrum showed prominent peaks. These obtained peaks were matched with standard one and the groups were identified. These peaks denote carbonyl (C = O) and asymmetric C-O-C, -CH3, -CH2, -CH, C-O, C-C and O-H groups (Nousheen, 2021).

Concluding Remarks:

It is evident that there is an increasing demand for sustainable agronomy to meet the needs of the growing population and enhance agronomic yield without disturbing ecological components. It is important to explore plant-associated endophytic microbial communities. These endophytes

promote the plant growth by nitrogen fixation, phosphate solubilization, antimicrobial activity, volatile metabolites and nonvolatile metabolites production to kill pathogenic microorganisms. Endophytic microorganisms also support plant growth by producing organic acid, indole acetic acid, and phosphatase enzyme. The endophytic microorganisms also inhibit the growth of pathogenic microorganisms by a common mechanism producing cell wall degrading enzymes such as protease, cellulase. ACC deaminase,α-/β-amylase and chitinase.

Endophytes could help the crops with less fertilizers, fungicides, insecticides or herbicides.

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