Endophytic bacteria: Potential biocontrol agents against plant pathogens

By

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SEMINAR REPORT

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DEPARTMENT OF PLANT PATHOLOGY
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CERTIFICATE

This is to certify that the seminar report entitled **"**Endophytic bacteria: Potential biocontrol agents against plant pathogens**"** has been solely prepared by **ASWATHY PEETHAMBARAN (2018-11-019)**, under my guidance and has not been copied from seminar reports of any seniors, juniors or fellow students.

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DECLARATION

I, Aswathy Peethambaran (2018-11-019) declare that the seminar entitled **"Endophytic bacteria: Potential biocontrol agents against plant pathogens"** has been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

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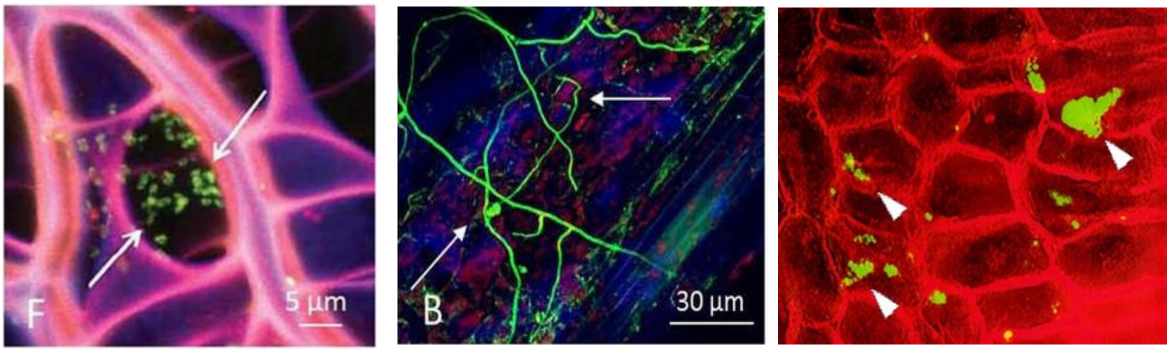
1. Introduction

The research on biological control of plant diseases has been mainly focused on rhizosphere microorganisms. However, increasing knowledge of plant associated microbes has widened the scope of biological management of plant pathogens. In this scenario, one of the promising approaches is the use of endophytes, which have the potential advantage over other biocontrol agents, as they share an ecological niche similar to that of pathogenic microbes. They colonise the plant internally and thereby stay protected from environmental stresses and fluctuations. Sustainably transmit to other plant parts away from application site and also to next generations. Endophytes are microorganisms, which are culturable or not and inhabit inside the plant tissues, without any harm to the host and do not develop external structures (Azevedo *et al*., 2000). The term endophyte is derived from two Greek words, *endon* and *phyton* meaning inside the plant. Every plant on earth is a host of one or more endophytes.

1.1 Types of endophytes



Endophytic bacteria — Endophytic fungi — Endophytic actinomycetes

Plate 1. Types of endophytes

2. Diversity of endophytic bacteria

Eventhough there are more than 16 phyla of bacteria 3 major phyla are studied intensively by various researchers which include phylum proteobacteria which is the largest phylum followed by phylum Firmicutes and phylum Actinobacteria. The phylum Proteobacteria contains genera like *Burkholderia, Herbaspirillum* and *Pseudomonas*. The phylum Firmicutes contain genera like *Bacillus* and *Paenibacillus*. The phylum Actinobacteria contains genera like *Clavibacter, Nocardiodes, Streptomyces etc* (Hallman, 1997).

3. Distribution in plant kingdom

They are found in almost all the crop plants studied.

Crop	Endophytic bacteria
Cereals	*Pseudomonas* sp
(Rice, Wheat, Maize, Sorghum)	*Herbaspirillium seropedicae*
Vegetables	*Mycobacterium* sp
(Tomato, chilli, onion, potato, pulses)	*Bacillus* sp
Spices	*Pseudomonas* sp
Black pepper	*Burkholderia phytofirmans*
Turmeric	*Bacillus* sp
Fruits	
Strawberry	*Pseudomonas* sp
Grapes	*Pseudomonas fluorescens*

Table 1. Distribution of endophytic bacteria in plant kingdom

4. Where do they come from?

They mainly originates from rhizosphere and also from phyllosphere which constitute phylloplane, caulosphere and carposphere. The bacteria present in these regions can enter the plant either passively or actively (Lamb *et al*.,1996).

5. Mode of entry and colonization

There are two modes of entry of endophytic bacteria into host plants.

5.1 Active entry

It occurs through the release of cell wall digesting enzymes like cellulose, glucanase *etc.*

5.2 Passive entry

It occurs mainly through roots, the small cracks developed at the site of lateral root emergence and root hairs. It can also enter through natural opening present on aerial plant parts like stomata and hydathodes.

Some endophytic bacteria can enter through wound also. Many colonization studies suggested that natural cracks at the lateral root emergence site are the most common entry sites for endophytic bacteria.

The rhizosphere attracts a variety of microorganism due to the presence of rhizo-deposits and the root exudates which are released during seed germination and root development.The rhizospheric bacterial communities develop and are attracted to the rhizosphere due to the carbon that is formed, which is rich in nutrients when the exudates are released. Bacteria get their carbon source from the root exudates through the breakdown of organic compounds within the root exudates. The movement of bacteria towards the root exudate is facilitated by presence of flagella. Then they get attached to the roots. This attachment is facilitated by the formation of biofilm. Biofilm is nothing but bacteria embedded in water molecules. Then they will gain entry into the plants and will colonise inside various plant parts. In order to enter and colonise successfully in plant the endophytes have to overcome various defense mechanism exerted by the plants (Reinhold-Hurek *et al*., 1993)

6. How endophytic bacteria overcome host defense mechanism?

Plants maintain surveillance of potential invading microbes by recognising certain conserved molecules characteristics of microbes called Microbe Associated Molecular Patterns(MAMPs). These MAMPs will be identified by Pattern Recognising Receptors(PRR) present on the plant and it will exert defense to prevent the entry of microorganism. In case of bacteria flagellin is the most studied MAMPs. But in case of endophytic bacteria they are very smart, so that the MAMPs produced by them cannot be identified by PRR. So they will overcome host defense mechanism. Plants will produce reactive oxygen species (ROS) and the endophytic bacteria counteract it by release of ROS scavenging enzymes like catalase, peroxidase etc.

Among the secretion systems, type 3 (T3SS) and type 4(T4SS) secretion systems are pivotal for pathogens to deliver effector proteins into the plant, which can induce effector-triggered immunity. T3SS and T4SS may be either absent or present in low abundance in endophytic bacteria and therefore, these bacteria do not seem to elicit significant plant defense responses.

7. Plant parts colonized

Bacterial endophytes most often occupy intercellular spaces in the plant, most likely because these areas have an abundance of carbohydrates, amino acids, and inorganic nutrients. They likely exclusively colonize the intercellular spaces of various plant parts including roots, leaves, stems, flowers, and seeds. Colonization can be localized at the tissue level or systemically throughout the plant body.

8. Transmission

There are two modes of transmission of endophytic bacteria

8.1 Horizontal transmission

8.2 Vertical transmission

Most endophytic bacteria are likely to be horizontally transmitted. First, the diversity of bacteria in seeds and seedlings raised under sterile conditions is typically lower than the diversity in plants grown in soil suggesting that a majority of endophytes are acquired from the environment.

Vertical transmission of bacterial symbionts from parent to offspring is, indeed, common in systems where the symbiont provides an indispensable function.

9. Isolation

9.1 Direct isolation

9.2 Vacuum extraction

9.3 Trituration or homogenisation method.

9.1 Direct isolation

Leaf and stem samples were washed twice in distilled water. Surface sterilised by immersion in 70 per cent (v/v) ethanol for one minute, four minutes in sodium hypochlorite(three per cent (v/v) available chlorine) and 30 seconds in 70 per cent (v/v). Washed three times in sterilised distilled water for one minute. After surface sterilisation, samples were cut into 5-7 mm pieces and aseptically transferred to plates containing potato dextrose agar (PDA) (Garner,1982)

9.2 Vacuum extraction

Vacuum extraction apparatus used for isolation of endophytes. The sap extracted using the apparatus was used for spread plating on suitable medium (Bell *et al*., 1995)

9.3 Homogenisation/ Trituration method

- Surface-sterilization of the healthy plant sample.
- Sample is homogenised with 12.5 mM potassium phosphate buffer.
- Serial dilution in same buffer.
- Plating on nutrient agar (NA).
- Incubation at 28 for 72 hrs. (Petrini, 1986)

10. *In vitro* evaluation of antagonistic potential of endophytic bacteria

10.1 Against pathogenic fungi

The antagonistic ability of endophytic bacteria against fungal pathogen can be done by dual cuture in which pathogen will be placed at the centre of plate and endophytic bacteria will be inoculated on both sides of the plate about 2 cm away from the periphery. Control consist of pathogen alone

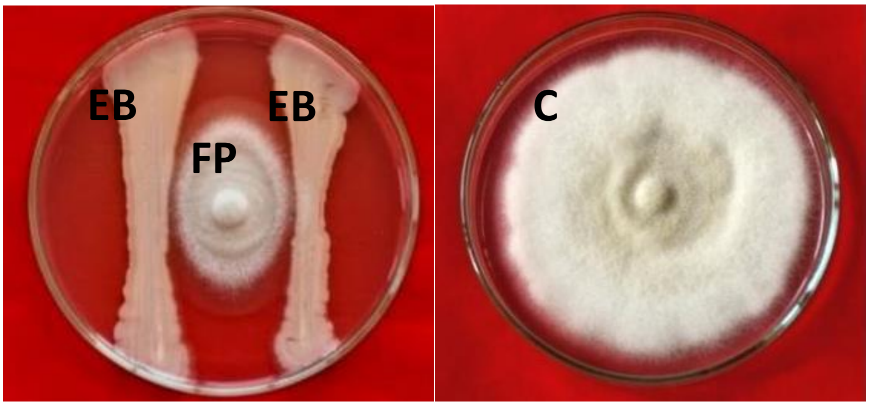


Plate 2. Dual culture technique

10.2 Against pathogenic bacteria

The antagonistic ability of endophytic bacteria against pathogenic bacteria can be done by inhibition zone technique in which the sterile filter paper disc dipped in endophytic bacterial suspension can be placed on bacterial lawn of pathogenic bacteria. . Control consist of bacterial lawn and filter paper disc dipped in sterile water.

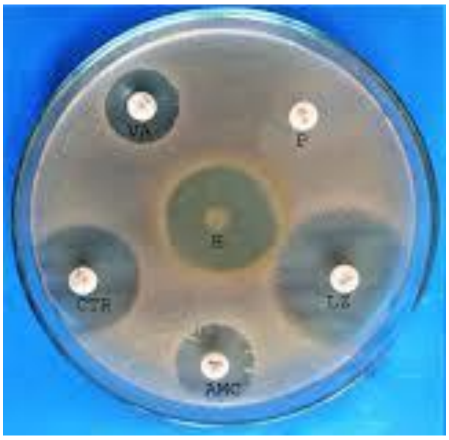


Plate 3. Inhibition zone technique

The percentage inhibition of pathogens by endophytic bacteria can be calculated using the following formula

$$\text{Per cent inhibition} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}}$$

Bamon *et al.* in 2018 evaluated *in vitro* efficacy of bacterial endophytes against *Pythium* sp causing soft rot of ginger in Meghalaya. Water soaked lesions at the emerging base and the collar region was the typical symptoms observed on infected plants. Sprout appeared pale yellowish and withering off of plant was seen and such rhizomes were rotten and emitted foul smell in which diseased plants came off with a gentle pull. Four potential bacterial endophytes viz., *Alcaligenes* sp. (GE-1), *Bacillus cereus* (GE-2), *Bacillus* sp. (GE-3), *Bacillus thuringiensis* (GE-6) were evaluated by dual culture assay and endophytes were successful in inhibiting the mycelial growth of the *Pythium* sp. causing soft rot of ginger. Of which, *Alcaligenes* sp. (GE-1) showed highest per cent inhibition of 80.98% followed by *Bacillus* sp. (GE-4) and *Bacillus* sp. (GE-5) of 78.89% and 77.33% inhibition respectively. The least inhibition was observed in *Bacillus* sp. (GE3) with 64.89%. In vitro evaluation found that four isolates were highly effective as it gave more than 70% inhibition in growth of *Pythium* spp. Therefore these isolates can be used in consortia mode in field condition against ginger soft rot.

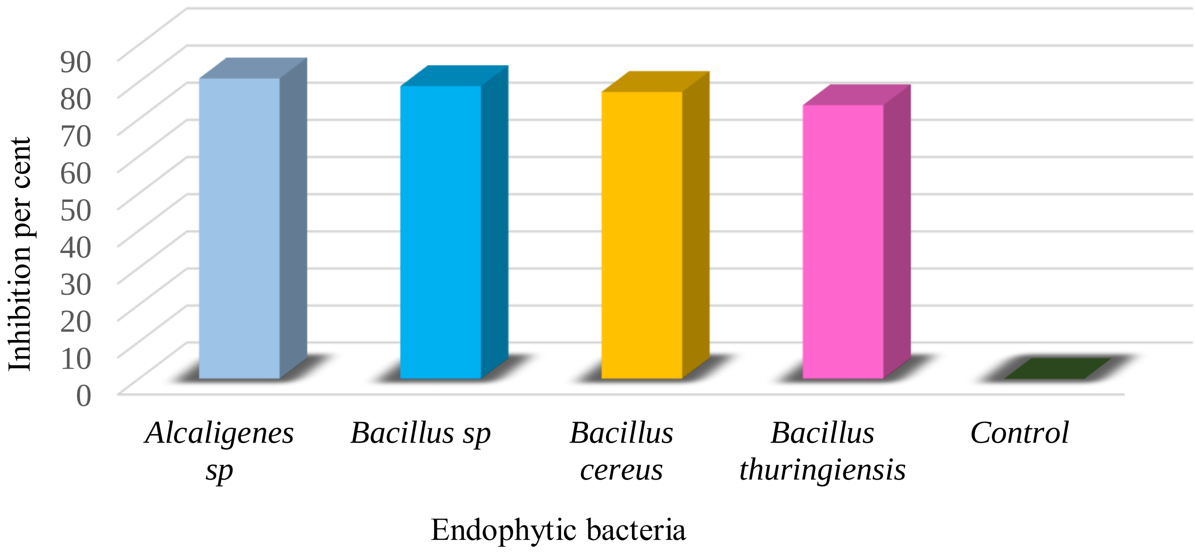


Fig 1. Effect of different endophytic bacteria on the pathogen *Pythium* sp

11. Mechanism of antagonism

Since the endophytic bacteria occupy a niche similar to that of phytopathogens they can be effectively used in the biocontrol of plant pathogens. Presumably, endophytic bacteria use similar mechanisms as that of other biocontrol agents for the control of plant pathogens.

The different mechanism employed involves:

11.1 Competitive root colonisation

Bacteria having the potential to become endophyte will be present in the rhizosphere region. Rhizosphere also contains other microorganisms including pathogen. So these endophytic microorganism will competitively exclude the pathogens and prevent them from entering the plant. The success of endophytic bacteria in preventing the entry of pathogen is mainly due to their ability to sense and quickly respond to the signals that are produced by plants (Whipps, 1997)

11.2 Lysis

Some bacteria produce fungal cell wall lytic enzymes. They can lyse fungi and use the dead material as food. This mechanism is called Predation and Parasitism (P&P). Endophytic bacteria from potato roots express high levels of hydrolytic enzymes such as cellulase, chitinase, and glucanase which can degrade fungal cell wall components. Chitinase is considered as the major responsible enzyme for fungal cell wall degradation and several examples of chitinolytic endophytes which protect against plant diseases are known.

The hyphae of *Phytophthora sojae* was lysed by the endophytic *Bacillus* sp by releasing enzyme cellulase.

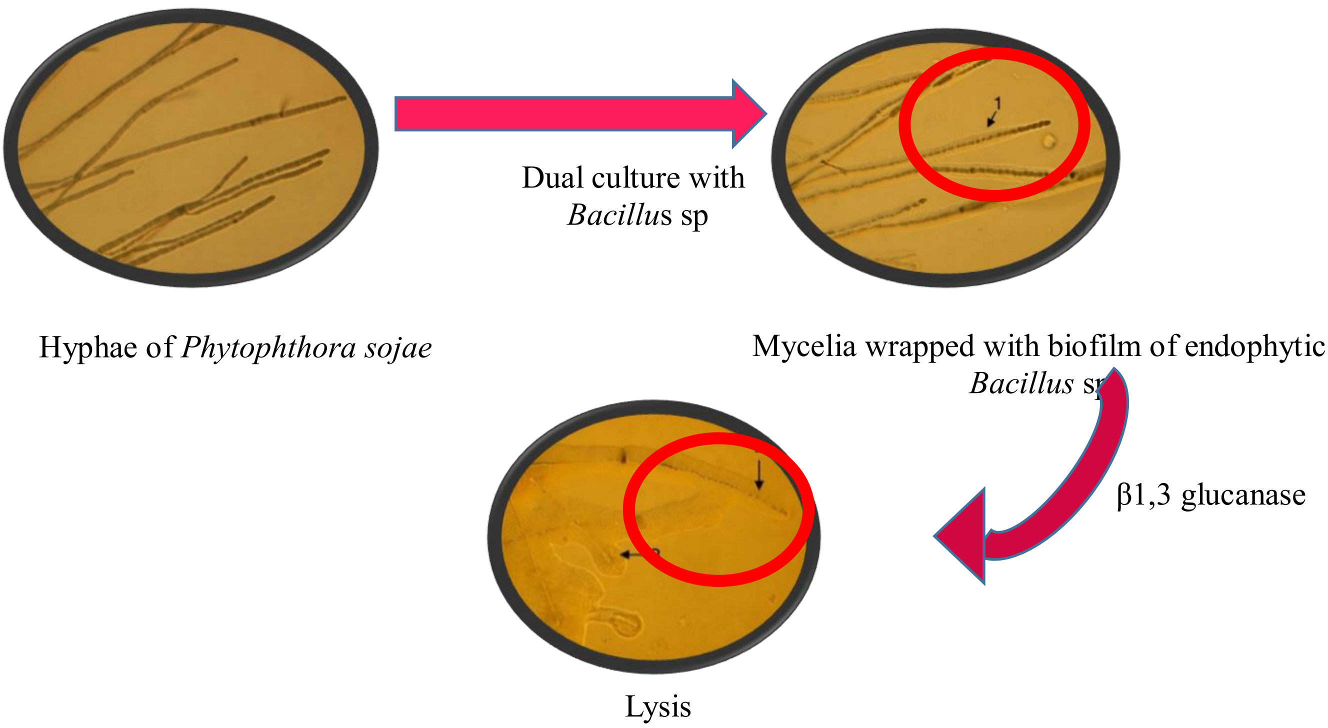


Plate 4. Lysis of *Phytophthora sojae* by endophytic *Bacillus* sp

11.3 Antibiosis

It is achieved through the release of antibiotics and volatile compounds. A significant fraction of the indigenous endophytic bacteria in plant roots is able to produce antibiotics towards fungal pathogens *in vitro* and therefore might use the mechanism of antibiosis for disease control (Fravel *et al*., 1988)

Eg. *Bacillus* sp in apple produce fengycin against *Botrytis cinerea*.

Pseudomonas sp in rice produce phenazines against *Xanthomonas oryzae*.

Gao *et al*. in 2017 conducted double plate assay to investigate the antagonistic potential of *Bacillus velezensis* ZSY-1 against *Botrytis cinerea* and *Alternaria solani*. They observed 93 per cent inhibition in mycelial growth of *Botrytis cinerea* and 83 per cent inhibition of *Alternaria solani* . GC-MS revealed that volatile compound like 2,5 – dimethyl pyrazine, benzothiazole ,4-chloro-3-methylphenol, 2-tridecanone produced by endophytic bacteria inhibited the growth of fungal pathogens.

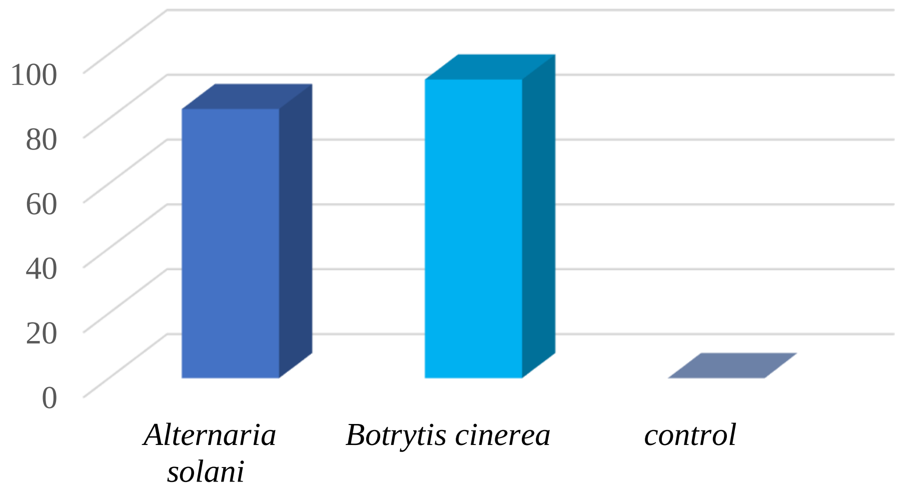


Fig. 4 . Inhibition of fungal pathogens by volatile compounds

11.4 Siderophore production

Siderophores are produced by microorganisms under iron limiting conditions. The siderophore secreted by endophytic bacteria bind to most of the Fe^{3+} that is available in the rhizosphere and as a result effectively prevent any pathogens in the immediate vicinity from proliferating because of a lack of iron. Control of *Erwinia amylovora* by *Pseudomonas fluorescens.*

11.5 Induced systemic resistance

Some rhizosphere bacteria can induce resistance towards pathogenic fungi, bacteria and viruses at sites in the plant where the applied beneficial bacterium is not present. Apparently these bacteria trigger a reaction in the plant that gives rise to a signal that spreads systemically through the plant and enhances the defensive capacity of distant tissues to subsequent infection by some pathogens. The mechanism used by these bacteria is designated as Induced Systemic Resistance. ISR is different from Systemic

Acquired Resistance (SAR) in several physiological and biochemical phenotypes . ISR can be induced by many different bacterial surface molecules, secreted metabolites, and volatiles. Examples of bacterial endophytes which have been suggested or claimed to induce ISR are *Bacillus amyloliquifaciens*, *Bacillus pumilus*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Pseudomonas syringae* and *Serratia marcescens* (Kamilova *et al*., 2006).

12. Endophytic bacteria against various plant pathogens

12.1 Endophytic bacteria against fungal diseases

Disease	Pathogen	Endophyte
Tomato wilt	*Fusarium oxysporum* fsp *lycopercisi*	*P. fluorescens*
Rice blast	*Magnaporthe grisea*	*Pseudomonas* sp
Strawberry-anthracnose	*Colletotrichum gloeosporioides*	*Bacillus amyloliquefaciens* S13-3

Table 2. Endophytic bacteria employed against fungal pathogens

Elanchezhian *et al*. 2018 studied the multifaceted benefits of *Bacillus amyloliquefaciens* strain FBZ24 in the management of wilt disease in tomato caused by *Fusarium oxysporum* f. sp. *lycopersici.* A total of 6 endophytic bacterial strains were characterized at both biochemical and molecular levels and were screened against *F. oxysporum* f. sp. *lycopersici* by dual culture technique. Strain FZB24 recorded the maximum per cent inhibition (48.3%) of *F. oxysporum* f. sp. *lycopersici* under *in vitro* conditions.

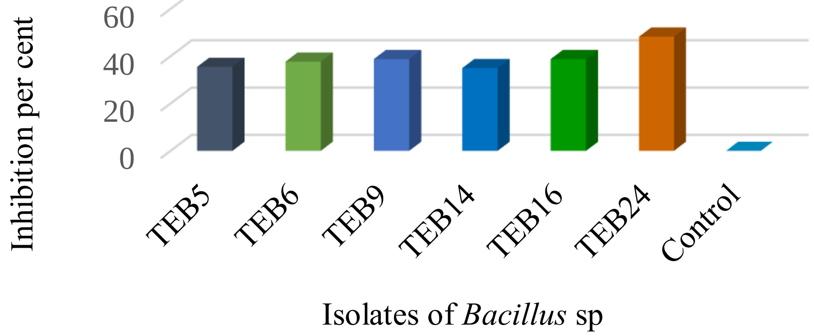


Fig.5. Inhibition of *Fusarium oxysporum* by endophytic bacteria

They also conducted a field experiment with the isolate TEB24.

Treatments (TEB24)	**Per cent disease incidence (PDI)**	**Fruit yield (t/ha)**
ST @ 4g/kg of seed	23.41	51.50
SD @ 4g/l of water	27.57	50.99
SD @ 4g/l + FS@500g/ha	30.8	51.49
ST @ 4g/kg + SD @ 4g/l+ SA 500g/ha + FS @ 500g/ha	**15.22**	**59.21**
ST with carbendazim @ 2g/kg of seed+ SD @ 0.05%	9.67	54.01
Untreated control	45.96	38.79

Table 3. Application of isolate TEB24 against *Fusarium* wilt in tomato

ST: Seed treatment, **SD**: Seedling dip, **FS**: Foliar spray, **SA**: Soil application

FZB 24 was found to be effective when applied as a combination of seed treatment @ 4 g/kg + seedling dip @ 4 g/l + soil application @ 500 g/ha + foliar spray @ 500 g/ha in reducing disease incidence. This treatment also recorded higher defense enzyme activities, maximum plant growth promotion and higher yield.

12.2 Endophytic bacteria against bacterial diseases

Disease	Pathogen	Endophyte
Tomato wilt	*Ralstonia solanacearum*	*Pseudomonas* sp
Pierces disease of grape vine	*Xylella fastidiosa*	*Pseudomonas* sp

Table 4. Endophytic bacteria employed against bacterial diseases

Balan *et al*. 2014 evaluated the anatgonistic ability of endophytic microorganism against *Xanthomonas axonopodis* causing bacterial blight of athurium. *In vitro* evaluation of 8 different isolates of endophytic bacteria against pathogen was done and was compared with KAU reference culture .They found that the isolate EB15 caused maximum inhibition of pathogen.

Treatment	**Treatment details**	**Per cent disease incidence**
T1	EB12	90
T2	EB13	90
T3	**EB15**	**61**
T4	EB26	90
T5	EB31	72
T6	*Pf* (KAU)	90
T7	Streptocycline (300 ppm)	67
T8	Turmeric + sodium bicarbonate mixture (10:1)	95
T9	Control	100

Table 5. Application of isolate EB15 against bacterial blight of anthurium

12.3 Endophytic bacteria against viral diseases

Harish *et al* .2009 studied the defense related enzymes such as peroxidases, polyphenol oxidases and phenylalanine ammonia lyase were activated in plants treated with endophytic bacteria, thus inducing resistance against viral diseases like banana bunchy top.

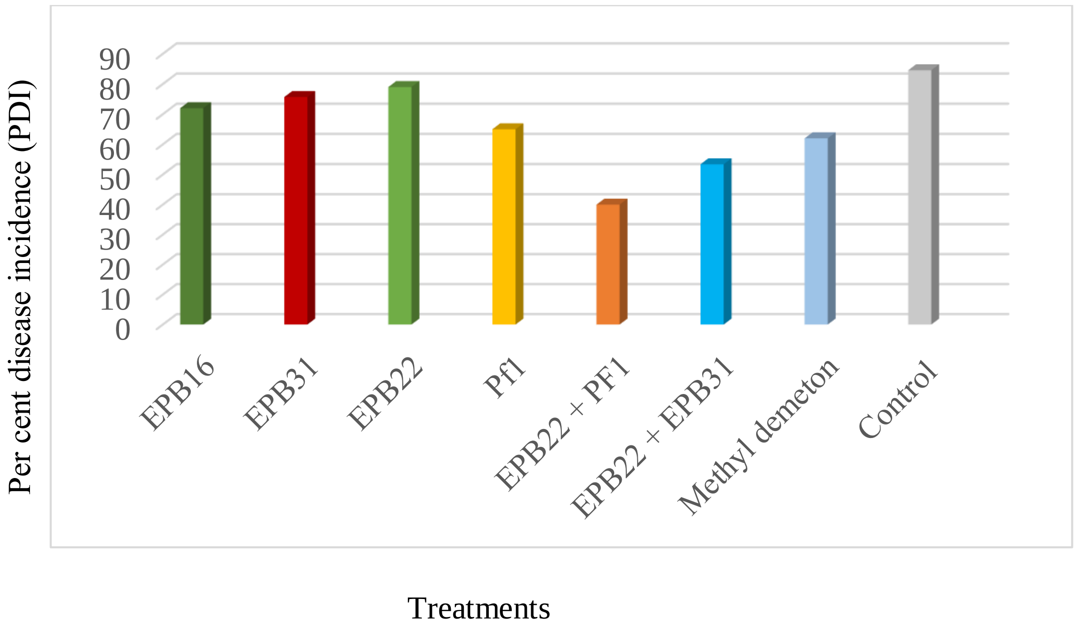


Fig .6. Effect of endophytic and rhizosphere bacteria against banana bunchy top

13. Formulations

In case of endophytic bacteria commercial formulations are not available. But different researchers have developed solid formulations and microencapsulations like alginate beads for field level application. For increasing the efficiency of biocontrol ability appropriate method of application of endophytic bacteria are to be selected.

14. Method of application

It is similar to other biocontrol agents and includes seed treatment, soil application and foliar spraying. In order to get an efficient control of pathogens the organisms should reenter the plants when applied externally. Their reentry when applied externally can be studied using colonisation studies.

15. Colonisation studies

15.1 Colonisation study using Green fluorescent protein (GFP)

Uses a marker sysyem which encodes for Green fluorescent protein. Endophytic bacteria is transformed with a gene which codes for the protein. It is then inoculated on to suitable host plant (Akbari *et al*., 2017)

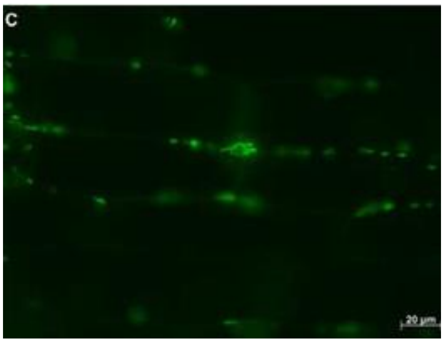
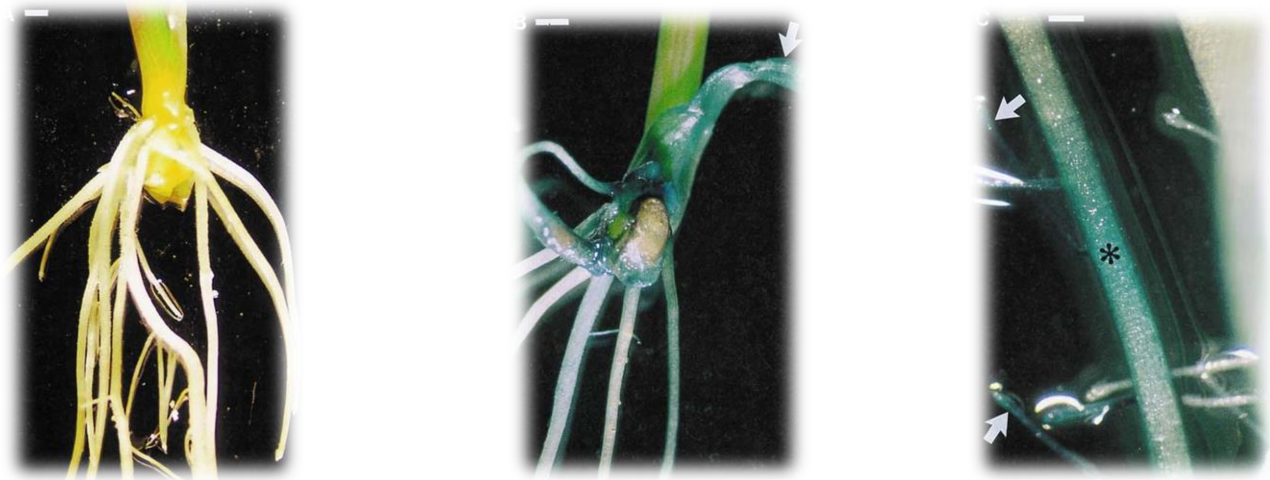


Plate 5. Images of maize root colonised with *Burkholderia* sp

15.2 Colonisation studies using GUS staining

Gus gene will be incorporated into the bacteria. It is then inoculated into the crops. Later observed using optical microscopy (Pinski *et al*., 2019)



Uninoculated control

Inoculated with GUS labelled *Herbaspirillum* sp.

Lateral root junction showing strong GUS staining

Plate 6. Images of GUS staining of *Herbaspirillum* sp

15.3 Colonisation studies using Fluorescent *in situ* hybridisation.

Fluorescently labelled probe complementary to 16 S region of endophytic bacteria will be incorporated into the endophyte. It is then observed under uv light (Verma, 2007)

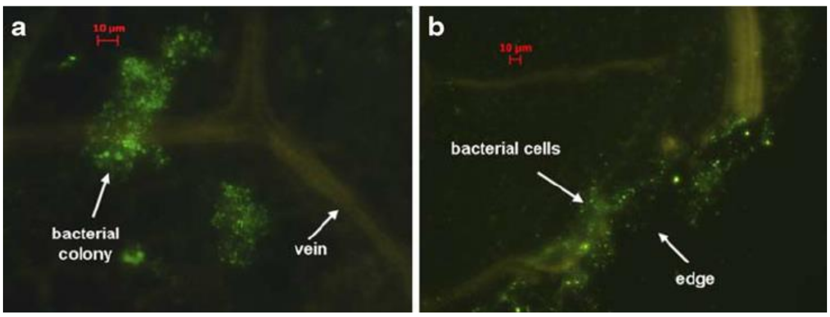


Plate 7. Images of endophytic bacteria in grape vine leaves

15. 4 Colonisation studies using Fluorescent *in situ* hybridisation.

Kurian *et al*. 2011 conducted colonisation studies using radioactive labelling. Half mature cacao pods collected from a 10-year-old cacao tree were used for inoculation. The 32P-labelled bacterial suspension (500 µl) was carefully applied into the depression around the pedicel using a micropipette and allowed to airdry. The inoculated plants and pods were kept in the greenhouse attached to RTL for two days, in order to allow the labelled bacteria to enter and colonize the tissues. Two days after inoculation, the aerial plant parts of the cacao seedlings were detached by cutting at the base of the stem in the case of root and leaf-inoculated plants. In the case of plants inoculated on the stem, it was cut at 3 cm above the point of inoculation. For pods, the inoculated part was cut and removed from the pods using a sharp knife. Then three thin slices were taken from the pod, one from the centre and the other two from the two sides. The plant parts were then arranged on absorbent paper in their original position, labelled and secured with adhesive tape.. Specimens taken from the pods were dried in a hot-air oven for two days at 50°C and then air dried as they contained more moisture in the form of mucilage. The pressed and dried specimens were autoradiographed by placing on X-ray films in the dark and covered with smooth paper and pressed. The X-ray films were exposed for 10 days in the press. The plant parts were removed and the film was developed using a commercial X-ray film developer solution.

Application of 32P-labelled endophytic bacteria on cocoa pods and roots

Autoradiogram showing 32P-labelled EB-35 (*Bacillus subtilis*)

Plate 8. Colonisation studies using radioactive labelling

16. Future prospects

- Identifying genes responsible for the endophytic nature.
- Genome sequencing of endophytic bacteria.
- Intracellular colonization to be studied.
- Formulations of endophytic bacteria are to developed for large scale application.

- Genetic engineering of endophytic bacteria is to be done.

17. Challenges

Endophytes, from the rhizosphere may include opportunistic human/animal pathogens so toxicological studies are to be conducted.

18. Conclusion

Presumably, all plant species have associations with endophytic bacterial communities which confer an array of benefits to the host plants including protection against plant pathogens. They have immense potential in the biocontrol of plant diseases since they are bestowed with the capacity to harmoniously coexist with plants.

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20. Discussion

1. Whether endophytic bacteria used for pest control?

Yes, many endophytic bacteria are used for pest control. Examples include *Bacillus megatherium* which is used to control many sucking insect pest.

2. MAMPs produced by endophytes cannot be recognised by the plant defense why?

B. subtilis enhances its colonization in plant through minimizing the stimulation of the defensive response in *A. thaliana* by producing lantibiotic subtilomycin to bind self-produced flagellin.

3. What are the other uses of endophytic bacteria?

Nitrogen fixation, stress tolerance, phosphate solubilisation, phytoremediation etc.

4. How endophytic bacteria used in control of post harvest diseases?

Applied at the time of flowering.

5. When endophytic bacteria releases cell wall digesting enzymes, will it cause harm to plants

No, the enzymes are produced in small quantities.

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Department of Plant Pathology
Pl. Path 591: Master′s Seminar

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Major Advisor : Dr. Sainamole Kurian P. — **Time** : 9:15 am

Endophytic bacteria: Potential biocontrol agents against plant pathogens

Abstract

The research on biological control of plant diseases has been mainly focused on rhizosphere microorganisms. However, increasing knowledge of plant associated microbes has widened the scope of biological management of plant pathogens. In this scenario, one of the promising approaches is the use of endophytes, which have the potential advantage over other biocontrol agents, as they share an ecological niche similar to that of pathogenic microbes. Endophytes are microorganisms, which are culturable or not and inhabit inside the plant tissues, without any harm to the host and do not develop external structures (Azevedo *et al*., 2000). The term endophyte is derived from two Greek words, *endon* and *phyton* meaning inside the plant. Every plant on earth is a host of one or more endophytes which include bacteria, fungi and actinomycetes.

Among the endophytes, bacteria are predominant and they originate mainly from the rhizosphere and phyllosphere. Endophytic bacteria enter the plant tissues either passively through natural openings or actively using hydrolytic enzymes and colonize within the intercellular spaces of roots, stems, leaves, flowers and seeds. Different methods like direct isolation, vacuum extraction and trituration or homogenization are employed for the isolation of endophytic bacteria.

Competitive root colonization, lysis, siderophore production, antibiosis and induced systemic resistance are the mechanisms used by endophytic bacteria to control plant pathogens. Defense related enzymes such as peroxidases, polyphenol oxidases and phenylalanine ammonia lyase were activated in plants treated with endophytic bacteria, thus inducing resistance against viral diseases like banana bunchy top (Harish *et al*., 2009). According to Gao *et al*. (2017), endophytic bacterium, *Bacillus velezensis* ZSY1 inhibited the growth of fungal pathogens, *Alternaria solani* and *Botrytis cinerea* by the production of different volatile organic compounds.

Endophytic bacteria can be applied as seed treatment, soil drenching or as foliar spray for the control of plant diseases. Significant reduction in the incidence of *Fusarium* wilt of tomato by soil drenching of corn starch based formulation of endophytic *Bacillus subtilis* has been reported by Elanchezhiyan *et al.* (2018). Balan *et al.* (2014) reported that foliar application of endophytic bacteria reduced the incidence of bacterial blight of anthurium when compared to other conventional biocontrol agents and chemical treatments.

Presumably, all plant species have associations with endophytic bacterial communities which confer an array of benefits to the host plants including protection against plant pathogens. They have immense potential in the biocontrol of plant diseases since they are bestowed with the capacity to harmoniously coexist with plants.

References

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