iJRASET
International Journal For Research in Applied Science and Engineering Technology

INTERNATIONAL JOURNAL FOR RESEARCH

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

Volume: 7 **Issue:** I **Month of publication:** January 2019

DOI: http://doi.org/10.22214/ijraset.2019.1061

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International Journal for Research in Applied Science & Engineering Technology (IJRASET)
ISSN: 2321-9653; IC Value: 45.98; SJ Impact Factor: 6.887
Volume 7 Issue I, Jan 2019- Available at www.ijraset.com
DOI : 10.22214/ijraset.2019.1061

Isolation, Characterization of Endophytic Bacteria from Psidium Guajava Plant and Screening of their Antibiotic Activities

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Abstract: ***Endophytic bacteria defined as those bacteria that can be isolated from within the plant and that do not harm the plant visibly. These bacteria are thought to be virulent plant pathogens but have recently been discovered to have many beneficial effects on host plants, such as plant growth promotion, increased resistance against plant pathogen, parasites, etc. Besides gaining entry into the plants through natural openings or wounds, endophytic bacteria appear to actively penetrate plant tissues using hydrolytic enzymes like cellulose and pectins. Since these enzymes are also produced by pathogens, more information on their mechanism is needed to distinguish endophytic bacteria from plant pathogen. In general, an endophytic bacterium occurs in less number than pathogens, and at least some of them do not induce a hypersensitive response in the plant, indicating that they are not identified with the plant as pathogen. Six months old healthy plant of Psidium guajiava roots and stems were collected from the cultivated plant near Avulahalli, Bangalore, Karnataka. These plant samples were collected plant in sterile clean plastic bags brought to laboratory and used for further experimental purpose. These bacterial colonies were isolated from roots and one is isolated from the stem. The four bacterial colonies isolate were found to be may same species and they were shown common morphological and biochemical tests. An antibiotic sensitivity (or susceptibility) test is done to help choose antibiotic that will be most effective against the specific types of endophytic bacteria, root colony one and stem colony shown similar minimum inhibitory concentration (MIC).***

Keywords: Endophytic bacteria, Psidium guajiava, Antimicrobial sensitivity, Biochemical tests, Stem, Root, MIC.

I. INTRODUCTION

The term “endophyte” comes from two Greek words that is “endon” meaning within, and “phyton” meaning plant. These were the microorganisms such as bacteria and fungi that reside in the plant endosphere during all or part of their life without producing any harm to the host plant. In recent years, many endophytes have been identified through culture-independent approaches such as sequencing of the 16S rRNA gene, the internal transcribed spacer regions, ITS1 and ITS2, or through whole genome sequencing of endophyte communities. Additionally, some bacterial endophytes carry genes necessary for biological nitrogen fixation (BNF), potentially enabling them to convert dinitrogen gas (N2) into usable forms of nitrogen such as ammonium and nitrate within the host plant. Bacterial endophyte strains promote plant growth by synthesizing phytohormones including indole-3-acetic acid (IAA), cytokinins and gibberellins or through regulating internal hormone levels in the plant body. IAA produced by endophytes within plants increases the number of lateral and adventitious roots, facilitating access to nutrients, and improving root exudation, offering more resources for soil microbes to interact with roots.
Some endophytic microbes have been shown to protect plants from herbivores or to be responsible for the synthesis of novel and useful secondary products such as Azorhizobium caulinodans, Burkholderia cepacia, Enterobacter cloacae, Klebsiella variicola, Pseudomonas putida, from plant Rice, Yellow lupine, Citrus plant, Sugar cane, Carrot, respectively. The attachment or adhesion of bacterial cells to the plant surface is considered the first step of the colonization process. Bacteria in the vicinity of the plant roots most likely swim towards the roots, using chemotactic affinities for root exudates. This is followed by attachment to the root surface, which is likely important in getting access to potential entry sites at lateral root emergence areas or other openings caused by wounds or mechanical injuries. The

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exopolysaccharides (EPS) synthesized by bacterial cells may facilitate the attachment of bacterial cells onto the root surface and may be important in the early stages of endophytic colonization.

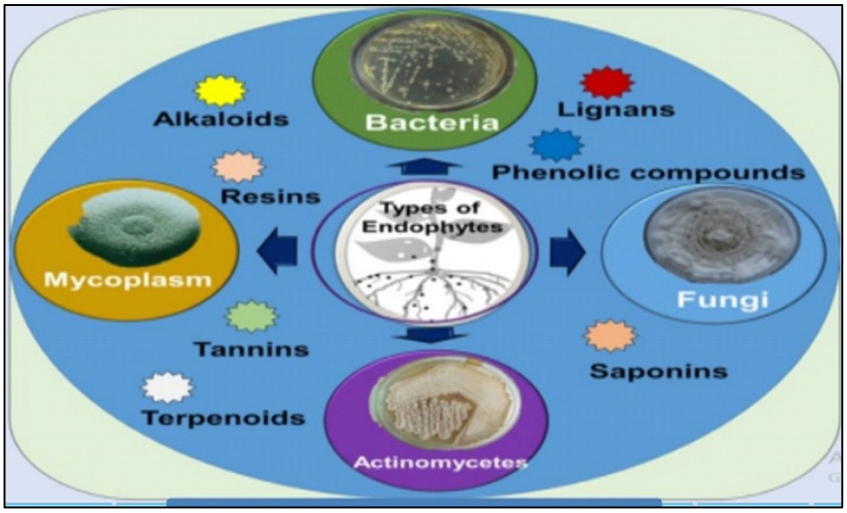

Figure 1. Hypothesized colonization cycle of bacterial endophytes in the host plant. (a) Mobilization of seed endophytes in germinating seedlings. (b) Recruitment of alien endophytes from the soil in developing seedlings. (c) Colonization by alien and inherited endophytes. (d) Whole plant colonization by various endophytes. (e) Variation of endophyte communities in the host plant in response to different biotic and abiotic stresses. (f) Vertical transfer of endophytes into seeds.

Endophytic bacteria those that can live inside the plant tissue and are not harmful for the host, these are few and different from those which are present in external environment. These bacteria may have the beneficial role in the enhancing plant growth, resistance against the pathogen, stress, to produce varieties of enzymes and bioremediation. The *Psidium guajada* (Guava) is edible fruit yielding and medicinal shrub or tree.

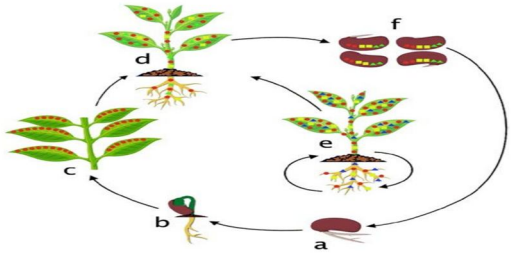

Figure 2. Introduction to endophytic bacteria

Phyto-symbiotic strain of Methylbacterium which was isolated from hybrid poplar trees (Populus deltoids x nigra)able to biodegrade many nitro-aromatic compounds such as 2,4,6- trinitrotoluene. .The engineered Burkholderia cepacia G4 studied to increase plant tolerance to toluene and decrease the transpiration of toluene to the atmosphere . Toluene is one of the component four for BTEX 19 contamination, and this has the potential to improve phytoremediation by decreasing toxicity and increasing degradation of the xenobiotic component . Endophytic bacteria plays an hopeful role in the remediation of contaminants as well as their degradation**.**

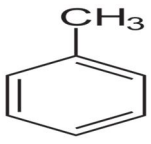

Figure 3. TOLUENE

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The plant response to the presence of endophytes seems to be conditioned, to a great degree by the plant genotype . Plant genes may be modulated by the existence of the endophytic bacteria, and these genes provides the effects of endophytes in plants. Endophytic bacteria provide useful and rich models to study the genetic expression of bacteria in their natural niches or habitats (inside plants), which are more structured and variable than culture media under controlled laboratory conditions . Antiobiotic peptide produced by the endophytic bacteria provides the host plant protection from many of the plant pathogens. Such property being plasmid encoded serves both as additional markers in recombinant selection as well as genes beneficial to the plant.

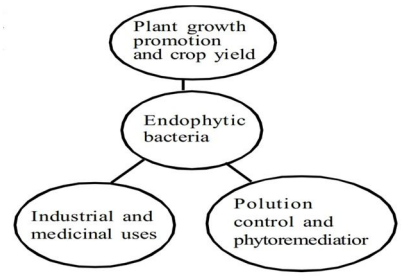

Figure 4. Schematic representation of applications of bacterial endophytes.

Table-1. Overview of Plants and associated endophytic bacteria.

Plant Type	Endophytic bacteria Identified	Reference
Strawberry	Psudomonas fluorescens, Psudomonas corrugata, Pseudomonas tolaasii, Pseudomonas paucimbilis.	Tanprasert and Reed, 1997
Red clover(Trifolium pretense L.)	Serratia, Agrobacterium, Rhizogenes, Arthrobacter, Bacillus , Enterobacter, Pseudomonas spp., Klebsiella, Micrococcus.	Sturz et al.,1998
Citrus plant	Alcaligen spp., Bacillus cereud, B. pumilus,B. subtilis, Enterobacter closeae, Pantoea agglomerans.	Araujo et al, 2001
Rough Lemon(Citrus jambhiri)	Pseudomonas spp.,Enterobacter spp., Lactobacillus spp., Seratia spp.	Gardner et al, 1982
Sugarcane	Herbaspirillum serotedicae	Dong et al, 1994
Saccharum officinarum	H. rubribalbicans, Acetobacter diazotrophicus	Dong et al, 1994
Cotton (Gossypium hirsutum L.)	Agrobacterium sp., Bacillus spp., Serratia spp., Pseudomonas spp. ,Enterobacter sp.	Mclnroy, Kloepper et al, 1995
Alfa alfa(Medicago sativa L.)	Pseudomonas sp., Erwinia sp.	Gagne et al., 1987
Corn (Zea mays)	Pseudomonas sp.,Bacillus sp.,Enterobacter sp., Klebsiella sp., Vibrio sp.	Lalande, Fisher, et al, 1989,1992
Cucumber (Cucumis sativis L.)	Pseudomonas sp., Bacillus sp. Enterobacter sp., Agrobacterium sp.	Mahaffee and Kloepper, 1997
Sugar Beet (Beta vulgaris L.)	Bacillus sp.,Pseudomonas spp., Lactobacillus spp., Xanthomonas sp.	Jacobs et al , 1985
Potato (Solanum tuberosum)	Bacillus sp., Micrococcus sp., Pseudomonas sp., Xanthomonas sp.	Hollis, Copeman, 1951 and 1974
Grapevine	Enterobacter sp., Pseudomonas sp., Bacillus sp., Xanthomonas sp. , Klebsiella sp.	Bell et al., 1994
Tomato	Pseudomonadaceae	Samish et al, 1963
Canola (Brassica napus L.)	Flavobacterium sp., Micrococcus sp., Bacillus sp., Rathayibacter sp.	Bell et al, 1994
Wheat (Triticum aestivum)	Azorhizobium sp.,	Webster et al., 1997
Rice (Orzya sativa L.)	Pseudomonas sp., Agrobacterium sp., Bacillus sp., Azosprillium sp.	Stoltzfus et al., 1997

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II. MATERIALS AND METHODS

A. Collection of plant sample.

Collection of plant samples was done in sterile clean plastic bags, from the cultivated field near Avalahalli, Old Madras road, Bengaluru and Karnataka. These plants were maintained under controlled environmental conditions in well insulated plastic pots.

B. Isolation Of Endophytic Bacteria From Psidium Guajada (Guava) Roots And Leave Tissue

1) *Surface Sterilization:* The root and leave sample of the Guava plant were taken and processed separately. Healthy and undamaged leave and root were collected from the plant grown under controlled environmental conditions. These were than washed under running water and dried followed by surface sterilization. Surface sterilization was done first washing in 1% savlon for 5 minutes. Subsequently these leaves and pieces of roots were treated with 0.1 % mercuric chloride for surface sterilization. Two sets of experiment with different timing for the sterilization were prepared to standardize the surface sterilization efficiency.

2) *Isolation Of Endophytic Bacteria:* The samples of leaves and roots were cut into small pieces and macerated separately in phosphate buffer of pH 7.2 with a sterile pestle and mortar. Tissue extract were then prepared for tenfold dilution in sterile saline. Serial dilutions (10^{-5}, 10^{-6}, and 10^{-7})were prepared from this extract. For inoculations 0.1ml of the aliquot was used on Nutrient Agar medium. The inoculations were done in triplicates separately for both roots and leaf tissue extract. These plates were then incubated at 37^0 C. Observations were taken after 48 to 72 hrs .Bacterial colonies were differentiated on the basis of morphological colony characters. Bacterial isolates were picked from plates and purified by streaking techniques and incubated at 37 ^{0}C. The isolation process repeated till monocultures were obtained for further experimentations .The media used for isolation was Yeast Manitol Agar, Ashbeys and Picovasky's medium.

C. Characterization Of Endophytic Bacteria

1) *Morphological Characterization*

a) *Gram Staining Tests:* The Gram stain procedure was originally developed by the Danish physician Hans Christian Gram to differentiate pneumococci from Klebsiella pneumonia. In brief, the procedure involves the application of a solution of iodine (potassium iodide) to cells previously stained with crystal violet or gentian violet. This procedure produces "purple colored iodine-dye complexes" in the cytoplasm of bacteria. The cells that are previously stained with crystal violet and iodine are next treated with a decolorizing agent such as 95% ethanol or a mixture of acetone and alcohol. The difference between Gram-positive and Gram-negative bacteria is in the permeability of the cell wall to these "purple colored iodine-dye complexes" when treated with the decolorizing solvent. While Gram-positive bacteria retain purple iodine-dye complexes after the treatment with the decolorizing agent, Gram-negative bacteria do not retain complexes when decolorized. To visualize decolorized Gram-negative bacteria, a red counter stain such as safranin is used after decolorization treatment.

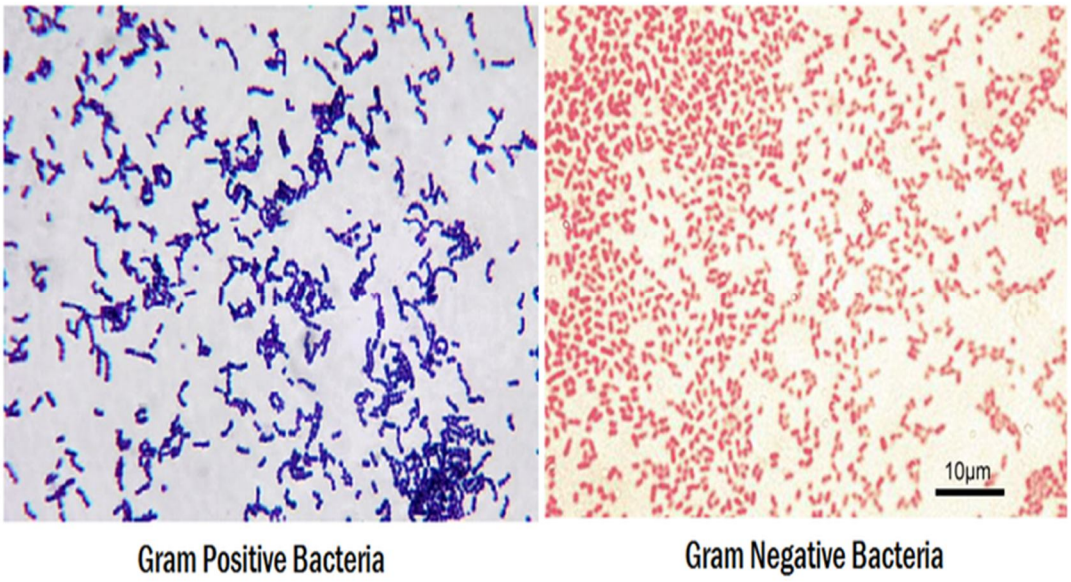

Figure 5. Gram Staining

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b) Motility Test: There are a variety of ways to determine motility of a bacterium—biochemical tests as well as microscopic analysis. Microscopy is the most accurate way to determine motility, assuming that you have a fresh culture of bacteria. Not only can motility be identified, but also the organization and number of flagella.

Motile bacteria move about with structures called flagella (a few exceptional bacteria move with the help of axial filaments, which cannot be seen in the microscope). Nonmotile bacteria without flagella are called atrichous.

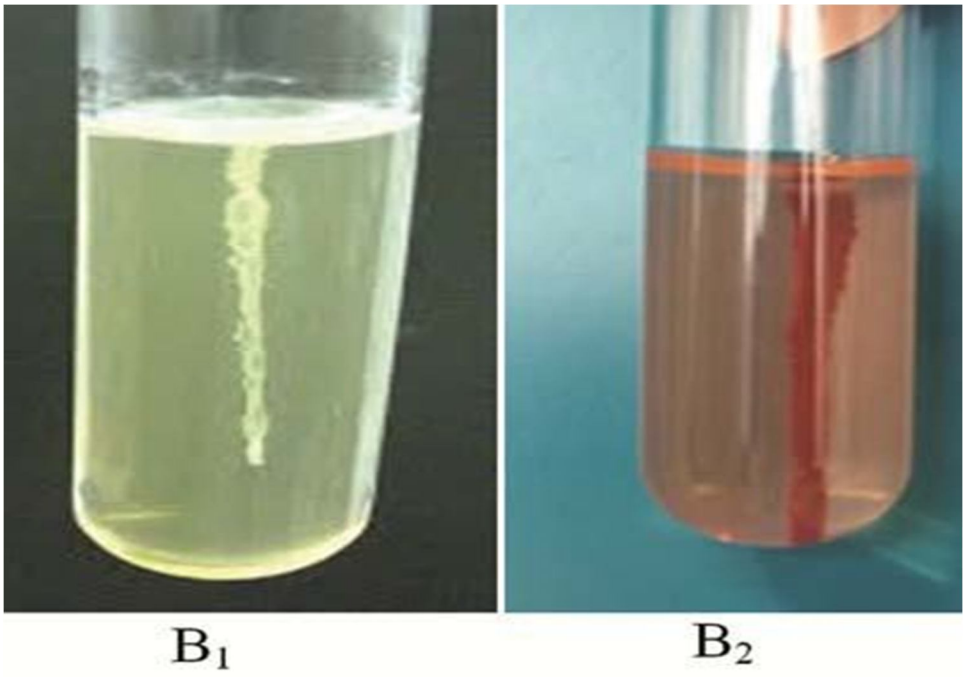

Figure 6. $B_{1:}$ Nonmotile; $B_{2:}$ Motile

2) Biochemical Characterization

a) Catalase Test

Enzymes that decompose hydrogen peroxide into water and oxygen. Hydrogen peroxide forms as one of the oxidative end products of aerobic carbohydrate metabolism. If this is allowed to accumulate in the bacterial cells it becomes lethal to the bacteria.

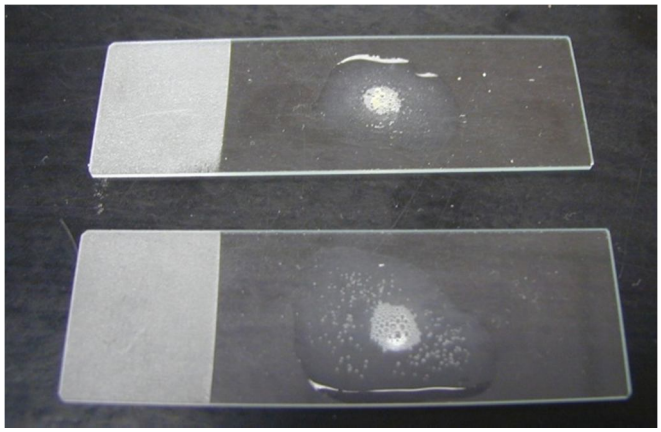

Figure 7. Catalase Test

b) Oxidase Test: Determine the presence of bacterial cytochrome enzyme oxidase. Cytochromes in aerobic respiration transfer electrons (H) to oxygen to form water. The reagent used is a dye p-phenylenediamide dihydrochloride (PPDD) acts as an artificial electron accepter substituting the oxygen. In the presence of enzyme cytochrome oxidase dye is oxidized to indophenol blue which is a dark purple colored enad products.

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Figure 8. Oxidase Test

c) IMViC Tests

IMViC: it's a group of tests used mainly to identify Enterobacteriaceae members which include:

i) *Indole Test:* Indole is a component of the amino acid tryptophan. Some bacteria have the ability to break down tryptophan for nutritional needs using the enzyme tryptophanase. When tryptophan is broken down, the presence of indole can be detected through the use of Kovacs' reagent. Kovac's reagent, which is yellow, reacts with indole and produces a red color on the surface of the test tube.

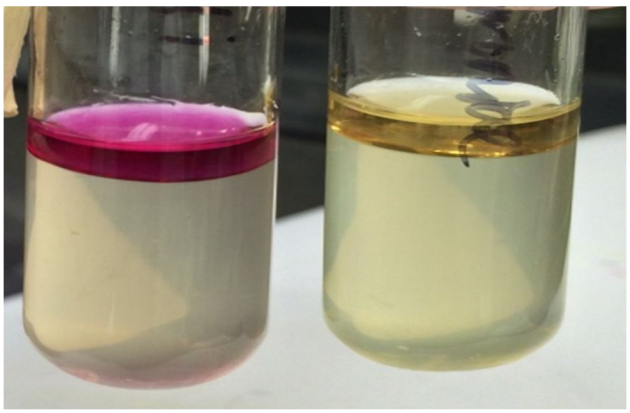

Figure 9. Indole Test

ii) *MR-VP test:* MR test Principle to test the ability of the organism to produce acid end product from glucose fermentation, this is a qualitative test for acid production.

Figure 10. MR Test

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iii) VP Test: To determine the ability of the organisms to produce neutral end product (acetoin) from glucose fermentation.

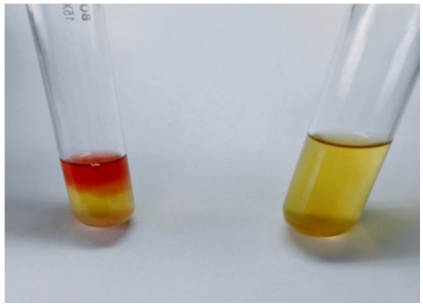

Figure 11. VP Test

3) Citrate Utilization Test: Simmons Citrate agar is a defined medium containing sodium citrate as the sole carbon source. The pH indicator, bromthymol blue, will turn from green at neutral pH (6.9) to blue when a pH higher than 7.6 is reached (alkaline). If the citrate is utilized, the resulting growth will produce alkaline products changing the color of the medium from green to blue. (Blue color= positive reaction eg; Klebsiella) ;(green color=negative reaction eg; E.coli) .

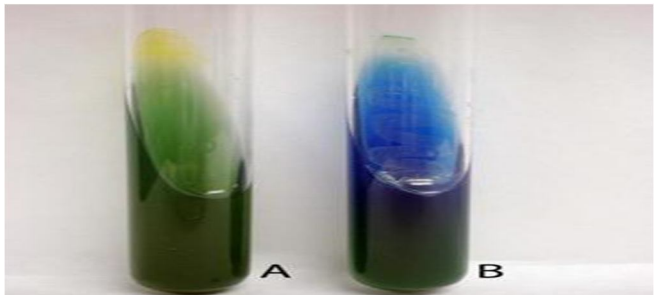

Figure 12. A. Negetive; B. Positive

4) Antibiotic Sensitivity Test: The isolates to be screened for their sensitivity for antibiotics were cultured on to the nutrient agar plate. Antibiotic Kanamycin of varying concentration (5µg/ml, 50µg/ml, and 100µg/ml) used for treating the culture by using well technique. The protocol was repeated for Ampicillin and Amoxycillin antibiotic with concentration – 5µg/ml, 50µg/ml, 100µg/ml on separate plates. Cultures inoculated for 24 hrs at 370C. The growth of the bacterial colonies was observed and zone of inhibition measured.

III. RESULT AND DISCUSSION

The given plates are showing the result of endophytic bacteria isolation and characterization of *Psidium guajava* plant of their stem and root we are taken as to identify the endophytic bacteria morphology. The Inhibition zone or colonies are formed due to the petriplate containing media which is suitable for the endophytic bacteria of the taken sample.

Interpretative chart of zone sizes

Antibiotic	Diameter of zone inhibition (mm)		
	Resistant	Intermediate	Susceptible
Tetracycline	<14	15-18	>19
Chloramphenicol	<12	13-17	>18
Cotrimoxazole	<10	11-15	≥16
Nitrofurantoin	<14	15-16	>17
Erythromycin	<13	14-22	>23
Gentamycin	<12	13-14	>15

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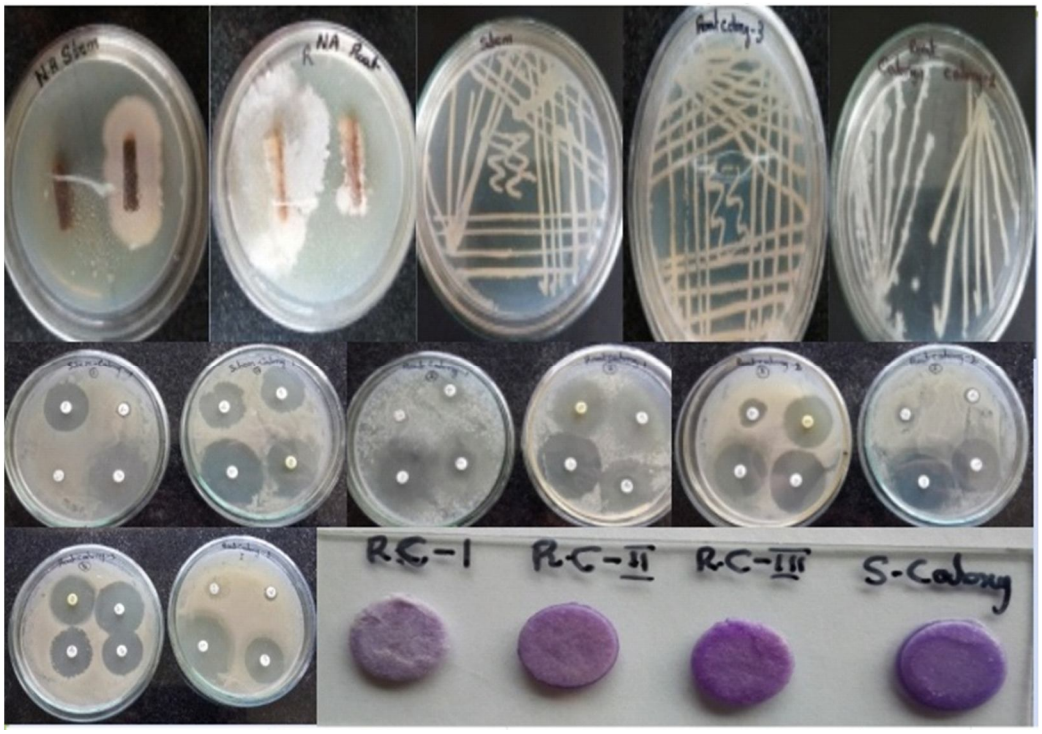

Figure 13. Screening And Antibiotic Test of *Psidium guajava* Plant

The colony characteristics should be done by the taken of *Psidium guajava* plant of different part of root and different part of stem as per the characteristics the colony will be form variety of shape, colour, margin, opacity, elevation and consistency and also motility.

Table 1: Characterisation of Endophytic bacteria

Colony characteristics	Root Colony-1	Root Colony-2	Root Colony-3	Stem Colony-1
Shape	Circular	Circular	Circular	Circular
Colour	Milky white	Milky white	Milky white	Creamish
Margin	Entire	Entire	Entire	Entire
Opacity	Opaque	Opaque	Opaque	Opaque
Elevation	Flat	Flat	Flat	Flat
Consistency	Smooth	Smooth	Smooth	Smooth
Gram Characters	Gram Positive	Gram Positive	Gram Positive	Gram Positive
Motility	Motile	Motile	Motile	Motile

Table 2: Isolation of Endophytic bacteria

Isolate	Root Colony-1	Root Colony-2	Root Colony-3	Stem Colony-1
Catalase Activity	+	+	+	+
Oxidase	+	+	+	+
Indole	-	-	-	-
Methyl Red	-	-	-	-
Voges Proskauer	+	+	+	+
Citrate Utilization	+	+	+	+
Starch Hydrolysis	+	+	+	+

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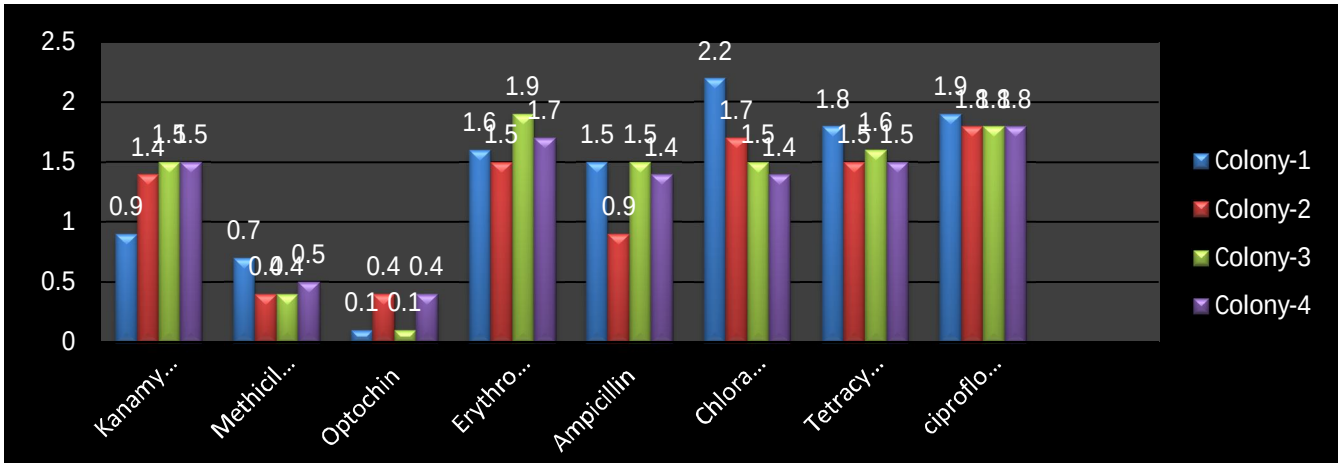
The Given Bar Chart showing the Antibiotic Sensitivity test of *Psidium guajava*



IV. CONCLUSION

Endophytes are fungi or bacteria occurring inside plant tissues without causing any apparent symptoms in the host. A majority of undescribed bacterial and fungal species exists within economically important plants. Ongoing research and latest developments in research associated with endophytic microorganisms to draw the attention of the research community to ward this emerging field and possible exploitation of the available sources for their therapeutics uses in various fields, such as medical, pharmaceutical, food and cosmetics. The first attempt to use endophytic bacteria for the improvement of phytoremediation processes have been used. The construction of endophytic bacteria with a new catabolic function, natural gene transfer is a great potential. A considerable research field is also required to design a particular strategy of reinoculation of endophytic bacteria. The Focus of the research articles that the colonization, recruitment, attachment and the entry to the distribution of bacterial endophytes in the plant of *Psidium guajava.* . Endophyte– Plant interactions can increase the health quality of plant and can significant for economic growth of plant especially the crops. The total mechanism of these endophytic bacteria is 1st it make interaction with plants which plays an essential role to biotechnological field and a beautiful relation between agronomic plant–bacterial relationship for a wide range of biotechnological and economical applications. The emerging field of endophytic bacteria could be the source for therapeutic uses to various field such as medical, pharmaceutical, food and cosmetics also.The research on endophytes future studies is to devlope endophytes to increase the production of biomass and bioenergy in plants and crops also for economical and therapeutic benefits in living worlds. Thats why, screening and isolation of endophytic bacteria with antimicrobial properties which investigated which can be used in basic and applied fields of science. Further it can be easily applied in the agricultural, medical and pharmaceutical field .The isolation and characterization and antibiotic sensitivity results showed the ample population of endophytic bacteria associated with plants which provide greater benefits to the plant-endophyte mutualism relation. its also necessary to identify the mutualism relationship of endophytic bacteria with the plant and its benefits and also the metabolic activity of plants and the biochemical characters of endophytes combines to shows an outstanding combination of bioactivity to plant which acts as a defense mechanism for plants .

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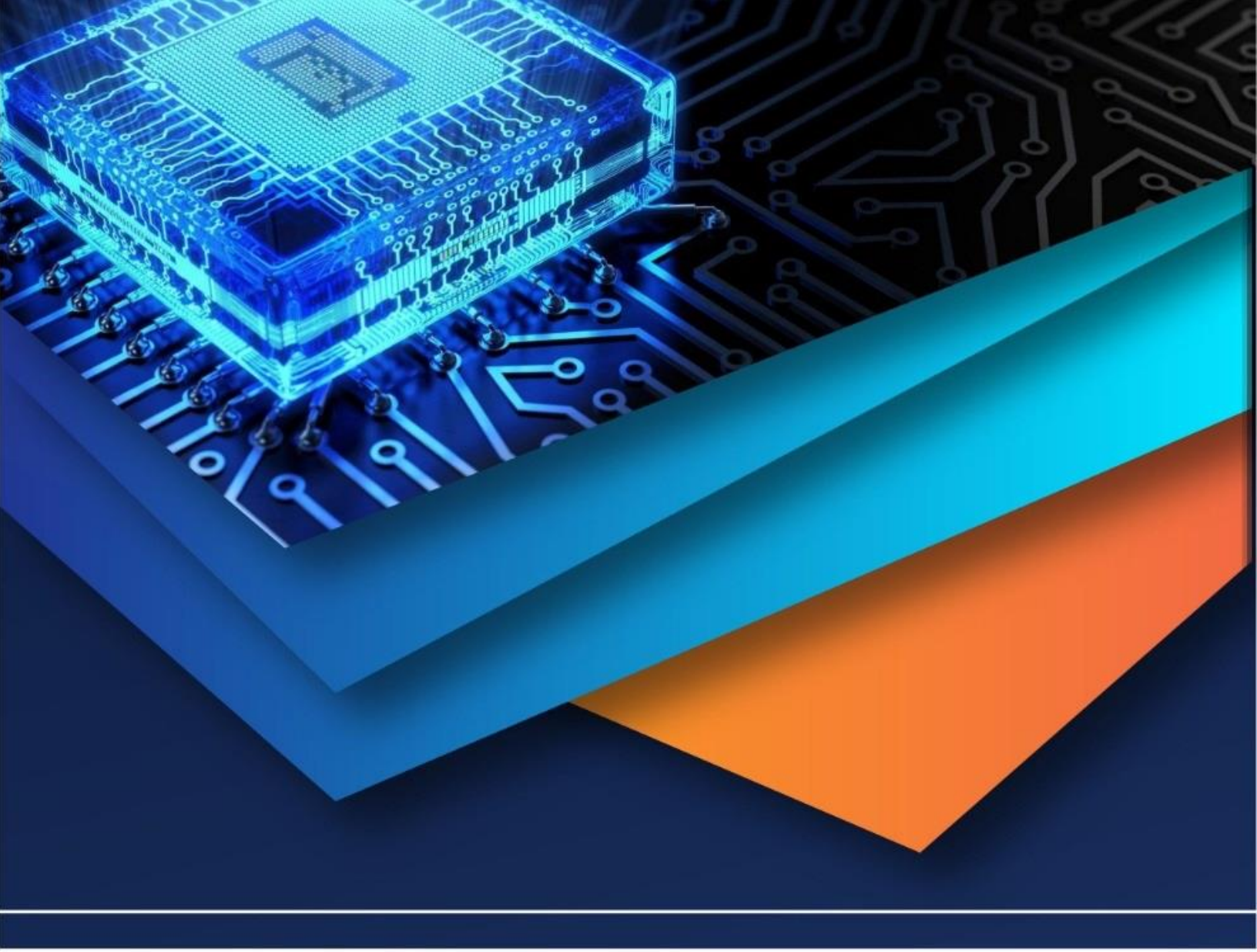

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