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Isolation and Characterization of Endophytic Bacteria

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Endophytic bacteriareside with in plant hosts without causing disease. In our study, we isolates four endophytic bacteria from explants (root,stem, petiole, and leaf) of *Aloe Barbadensis, Adhatoda vasica, Phyllanthus amarus* and *Calotrophis gigantea*.Entophytic bacteria such as *Azotobacter,Xanthomonas* and *Pseudomonas* could be identified from explants through various biochemical test. Endophytic bacteria were beneficial to the plant growth and it can be proved that inoculate to Mustard plant which was showed good growth promoting effect. The endophytes contributes substances that possess biocontrol activity against *Curvularia* sp., *and Alternaria* sp.,.Thus in our study,we focus on the growth promoting effect of endophytic bacteria in mustard plant and biocontrol activity against fungal pathogens.

Key words: Endophytic bacteria, *Azotobacter* sp, *Xanthomonas* sp, *Pseudomonas* sp.

The term endophytes refers to the interior colonization of plant by microorganisms that do not have pathogenic effects on their hosts (Hallmann *et al.,*1997). Endophytic bacteria are prokaryotes that colonize internal tissues of healthy plants without causing symptoms of disease (Wilson, 1995). They are mostly the members of common soil bacteria such as *Pseudomonas, Bacillus* and *Azospirillum* (Chanway, 1996). Endophytes occur in every host species sampled to date including above 200 terrestrial and aquatic species representing 20 families of diverse taxa such as marine microalgae, mosses, ferns, gymnosperms, monocots, herbaceous and woody dicots. Specialized bacteria can live within some plants as endophyte and may be beneficial.Endophytic bacteria have been discovered in sugarcane, cotton, pears and potatoes. Some are plant pathogens that can survive for extended periods in a quiescent state. The majority have no known positive deleterious effects on plant growth or development. The use of these bacteria as microbial delivery systems in agriculture is a current topic in agricultural biotechnology (Prescott *et al.,* 2003).

Endophytes utilize nutrition and reside inside the host plant, in turn it help in the promotion of basic metabolic functions like atmospheric fixation, solubiling phosphates and increase the secretion of growth promoting hormones like auxin, cytokines etc.by plants.It also help in improving the adaptation of plants to the stressed environment like water scarcity, p^H,temperature extremities and stressagainst the invasion of pathogens.The plants with endophytes are good competitors for nutrition and space than the co-growing plants. So the endophytes help the plants to dominate ecological niche.

Much of the work with endophytic bacteria has been with agricultural and horticultural

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plant species. Endophytes may be transmitted either vertically (directialy from parent to offspring) or horizontally (from individual to unrelated individual).Vertically transmit through fungal hypha penetrating the host's seeds (e.g. *Neotyphodium*). Since their reproductive fitness is intimately tied to that of their host plant, these fungi are often mutualistic. Conversely, horizontally transmitted fungal endophytes are sexual and transmit via spores that can be spread by wind and/or insect vectors.

The present work was aimed to isolate the endophytic bacteria from different medicinal plants such as *Aloe barbadensis,Adhatoda vasica,Phyllanthus amarus* and *Calatrophis gigantea* and findout the characterization of endophytic bacteria and their growth promoting effect in mustard plants.

MATERIAL AND METHODS

Isolation of endophytic bacteria

Putative endophytic bacterial strains were defined as isolates that were obtained from surface-sterilized plants, displayed differentiable colony morphologies. Medicinal plants was collected randomly during the growth season from 10 healthy mature plants per site at four different geographical locations. Individual plants were selected aseptically 3 cm above the soil level, and the stalks were stripped of leaves, put into plastic bags, and kept on ice until further processing. In the laboratory, the stalks were wiped with 70% ethanol and flame sterilized, and each stalk was dissected into a segment containing the third, fourth, and fifth nodes. The outer stalk was removed, exposing a cylinder of tissue inside the cork borer. Plant leaves and stems were surface sterilized for 10 s with 2% sodium hypochlorite containing 0.1% Tween 20 and to remove the disinfectant, sections were rinsed five times each in two washes of nonsterile deionized distilled water and wash sterile water. The section were dried with paper. All plant samples were placed into polyethylene bags. Tissue extracts were then serially diluted in 12.5mM potassium phosphate buffer (pH 7.1)and plated in triplicate to recover any bacterial endophytes present in the plant tissue (Hall man *et al.,*1997). Morphological characterization of endophytic bacteria was identified (Krieg *et al.,* 1984).

Charcterization of endophytic bacterial isolates (Williams and Wilkins, 1994)

1. Gram Staining
2. Motility Test
3. Kovac's Oxidase Test
4. Biochemical Test
   *Indole test
   *Methyl red test
   *Voges-proskauer test
   *Nitrate test
5. Phenotypic characterization of endophytic bacterial isolates (Lindberg *et al.,*1984)

Screening of biocontrol activity of endophytic bacteria

The study on the antagonist activity of endophytic bacteria against fungal pathogens *(Curvularia sp., Alternaria sp.,)* was done by dual culture method on the PDA plates (Chen *et al.,* 2001).

The fungal pathogens was retrived in the potato dextrose agar and the mycelia disc(8mm) was taken using a cork borer at the actively growing peripheral region of the fungal mat. The inoculation of fungal disc was done under the vertical laminar airflow and incubated at ambient temperature for 3-5 days. Control was also maintained without the bacterial culture.

Isolation of genomic DNA

The genomic DNA of different endophytic bacteria could be obtained through agarose gel electrophoresis (Araujo *et al.,* 2002).

RESULTS

Isolation of endophytic bacteria

The endophytic bacteria were isolated from various explants of *Aloe barbadensis, Adhatoda vasica, Phyllanthus amarus, Calotropis gigantea* and the colony morphology was observed (Table 1).

Four endophytic bacteria were isolated from different explant viz.root, stem, petiole, leaf of selected plant which show different morphological appearance on the nutrient supplemented medium. The isolates from the leaf and petiole were in similar morphological characters.

Characterization of isolated endophytic bacteria

Biochemical test was also carried out for the characterization of the isolated bacterial culture

from the various explants of the plant. Table 2,plate 2a,plate 2b clearly indicates the various test which were done according to the "Bergey's Manual of the Determinative Bacteriology" organism was identified to the Genus level as:

01 - *Azotobacter* sp.
02 - *Xanthomonas* sp.
03 - *Pseudomonas* sp.
04 - *Pseudomonas* sp.

Table 1. Morphology of the isolated endophytic bacteria

Explant	Colony morphology
Root (O1)	Pale white, regular edges, slimy colonies flat
Stem (O2)	Pale yellow, small, raised colonies, circular edges
Petiole (O3)	Abundant, slimy, raised, regular edges and medium turned green
Leaf (O4)	Abundant, thin, white raised and media turned green

Table 2. Characterization of Endophytic bacteria

Test	Root (01)	Stem (02)	Petiole (03)	Leaf (04)
Gram's staining	Gram –ve Rods in clumps	Gram –ve Rods	Gram –ve Rods	Gram – ve Rods
Motility	Non-motile	Motile	Motile	Motile
Indole	+	+	+	+
MR	+	+	+	+
VP	+	+	+	+
Nitrate	+	+	-	-

Table 3. Effect of inoculated endophytic bacterial culture on the growth of Mustard plant Root

Culture inoculated with plants	Length of the plant growth(cm) (observation at every 7 days interval)			
	0^{th} day	7^{th} day	14^{th} day	21^{st} day
Control	1.2	1.2	1.2	1.2
Azotobacter sp.,	1.2	1.3	1.4	1.6
Xanthomonas sp.,	1.0	1.0	1.1	1.2
*Pseudomonas sp.,(*Petiole)	1.2	1.2	1.3	1.6
Pseudomonas sp.,(Leaf*)*	1.0	1.1	1.2	1.3

Table 4. Effect of inoculated endophytic bacteria culture on the growth of Mustard plant Stem

Culture inoculate with plants	Length of the plant (cm)Stem (Observation at every 7 days interval)			
	0 day	7^{th} day	14^{th} day	21^{st} day
Control	1.5	1.8	2.2	2.8
Azotobacter sp.,	1.8	2.2	2.5	2.6
Xanothomonas sp.,	1.7	2.1	2.2	2.4
Pseudomonas sp.,	1.8	1.9	2.3	2.8
Pseudomonas sp,.	1.7	1.9	2.0	2.3

Members of the genus display the following defining characteristics of microorganism *Azotobacter* , *Xanthomonas* are positive result for all the test but nitrate test both give positive result. The *Azotobacter* are nonmotile and *Xanthomonas* are motile *Pseudomonas*:Gram negative,motile, rod shaped, Indole positive, Methyl red positive, Voges-proskaller test positive ,Nitrate test negative (Krieg *et al .,*1984).

The isolated endophytic bacteria when inoculated with the plant showed the growth promoting effect. The bacterial isolates from the different part of the plant when inoculated with the mustard plants root and stem (Tables 3-5) plant-microbe interaction could be seen.

The isolates from the leaf and petiole identified as *pseudomonas sp.,* and from root identified as *Azotobacter* showed the better growth compared to that of the control (stem, root) because growth promotion due to bacterial enhancement.

*Azotobacter r*eleases growth promoting substances such as IAA, gibberllic acids, nicotinic acid, panthothenic acid etc .The substances increases the growth rate of the crops by giving a good microenvironment for their roots (Kumarasen, 2008). After all the Endophytic bacteria isolated from the explants were beneficial to the plant and provided positive result for the further studies about endophytes. Previously report on endophytes infected plants often grows faster than the non-infected ones which is due to enhance in the production of phytohormones such as IAA,Cytokine (Zou & Tan,1999).

In the case of isolates of Xanthomonas *(*stem) and *Pseudomonas* (leaf) showed no effect and the result was similar to that of the control . Cytokinins are generally essential for the initiation of plant growth (Lakshmanan *et al.,* 1997).

Finally the total plant growth was really good seen in root, petiole (*Pseudomonas, Azotobacter*) inoculated mustard plant. This shows the some endophytic bacteria show its growth

Table 5. Effect of inoculated endophytic bacteria culture on the growth of Mustard plant

Culture inoculated with plants	Length of the plant growth(cm) (Observation at every 7 days interval)			
	0^{th} day	7^{th} day	14^{th} day	21^{st} day
Control	2.7	3.0	3.4	4.0
Azotobacter sp.,	3.0	3.5	3.9	4.2
Xanothomonas sp.,	2.7	3.1	3.3	3.6
Pseudomonas sp., (Petiole)	3.0	3.1	3.6	4.4
Pseudomonas sp., (Leaf)	2.7	3.0	3.2	3.6

Table 6(a). The biocontrol activity of *Pseudomonas* sp.,

Pseudomonas concentration in ml	Length of inhibition(cm)	
	Curvularia sp.,	Alternaria sp.,
Control	Nil	Nil
0.5	2.5	4.5
1.0	3.5	6.0

Table 6(b). The biocontrol activity of Xanthomonas sp.,

Xanthomonas concentration in ml	Length of inhibition(cm)	
	Curvularia sp.,	Alternaria sp.,
Control	Nil	Nil
0.5	7.5	2.5
1.0	6.0	1.5

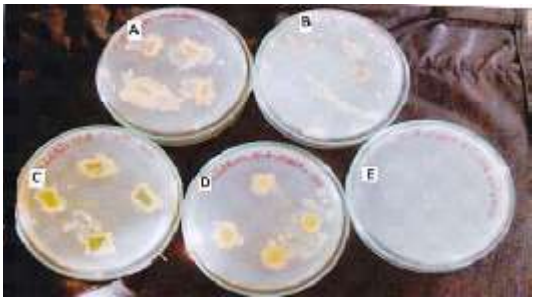
Table 6(c). The biocontrol activity of *Azotobacter* sp.,

Azotobacter concentration in ml	Length of inhibition(cm)	
	Curvularia sp.,	*Alternaria Sp.,*
Control	Nil	Nil
0.5	6.5	5.0
1.0	4.5	3.0

promoting effect in the root and petiole inoculated mustard plant.

Bio-control activity of isolated endophytic bacteria

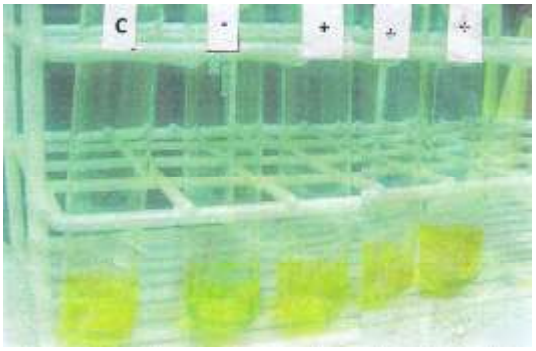
The bio-control activity shown by endophytic bacteria on the fungal pathogen of plants (Table 6 a,b,c plate 3a, plate 3b and plate 3c). All the endophytic bacteria such as *Azotobacter* sp.,*Xanthomonas* sp., *and Pseudomonas* sp.,showed resistance to the growth of the fungal pathogens namely *Alternaria* sp. and *Curvularia* sp.



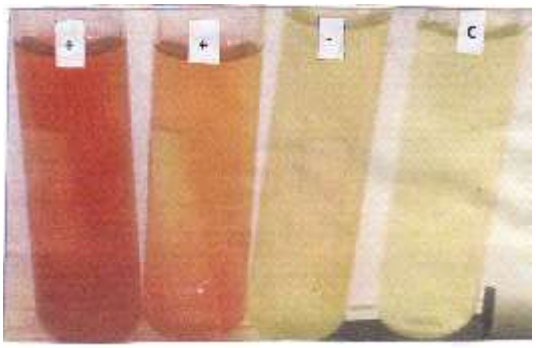
A-*Phyllanthus amarus*(Pentiole), B-*Aloe barbadensis*, C-*Calotrophis gigantea*(Leaf), D- Adhatoda Vasica(Stem), E-Control

A-*Adhatoda vasica* (Stem), B-*Phyllanthus amarus* (Pentiole), C-*Calotrophis gigantea* (Leaf), D-*Aloe barbadensis*, E-Control

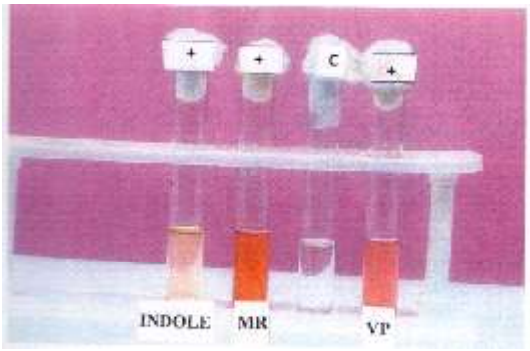
Plate 1. Colony morphology of the isolated endophytic bacteria



Indicate the positive(Xanthomonas, Pseudomonas) and negative (Azotobacter)result for motility tests



Indicate the positive *(Xanthomonas,Azotobacter)* and negative *(Pseudomonas)* result for nitrate reduction tests



Indicate the positive *(Xanthomonas, Azotobacter, Pseudomonas)* results for INDOLE, MR & VP tests

Plate 2. Characterization of endophytic bacteria isolates

Isolation of genomic DNA

Genomic DNA isolated from genus level of bacterial microbes. The genomic DNA can be obtained through Agarose gel electrophoresis process. The band seen that under UV light transilluminator. White band is indicate genomic DNA of different bacterial microbes in genus level (Plate 4).

The biocontrol properties of *P. fluorescens* bacteria might induce systemic resistance in the host plant, so it resist the invading pathogen. The bacteria might produce compounds antagonistic to other soil microbes, such as phenazine-type antibiotics or hydrogen cyanides (Hass and Defago , 2005).

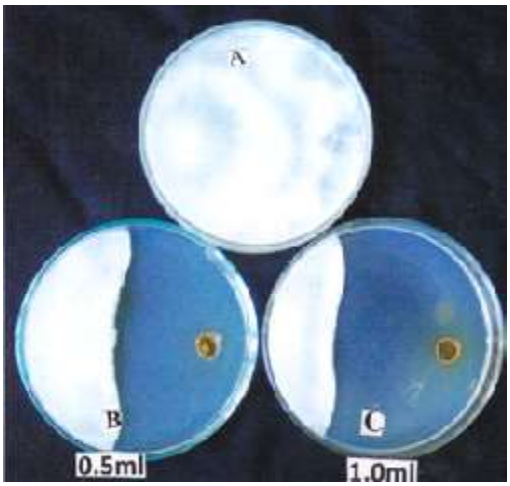


Plate 3a1.
A- Control(Culvularia SP.,)
B-0.5ml(Pseudomonas sp,)
C-1.0ml(Pseudomonas sp,)

Plate 3a 2.
A- Control(Alternia sp.,)
B-0.5ml(Pseudomonas sp,)
C-1.0ml(Pseudomonas sp,)

Plate 3a. The biocontrol activity of *Pseudomonas* sp

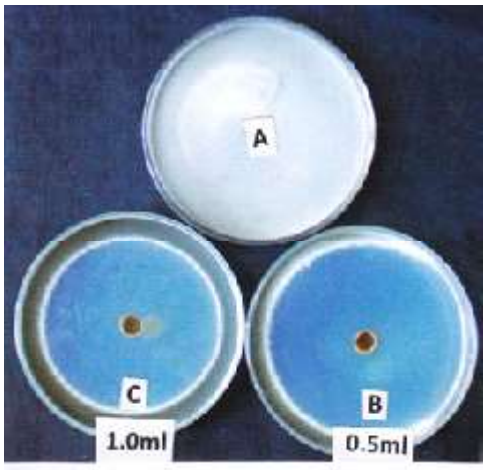


Plate 3b1.
A- Control(*Culvularia sp*,)
B-0.5ml(*Xanthomonas sp*,)
C-1.0ml(*Xanthomonas sp*,)

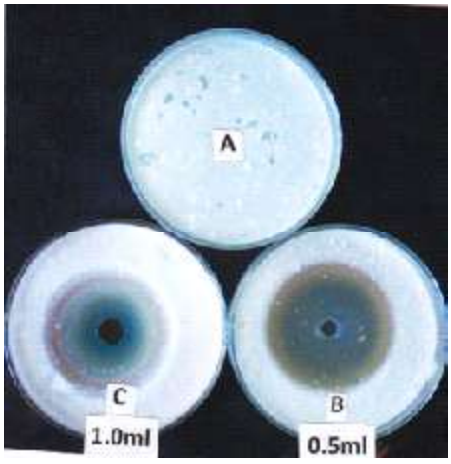


Plate 3b 2.
A- Control(*Alternia sp*.,)
B-0.5ml(*Xanthomonas sp*,)
C-1.0ml(*Xanthomonas sp*,)

Plate 3(b). The biocontrol activity of *Xanthomonas* sp

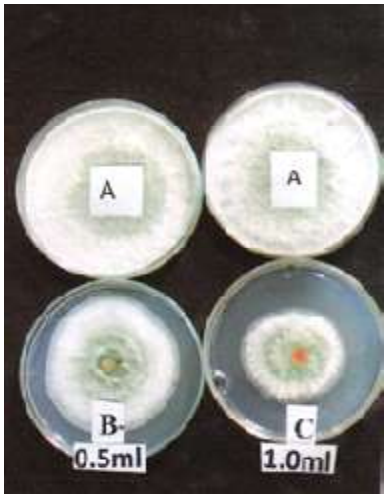


Plate 3b1.
A- Control(*Culvularia sp*,)
B-0.5ml(*Azotobacter sp*,)
C-1.0ml(*Azotobacter sp*,)

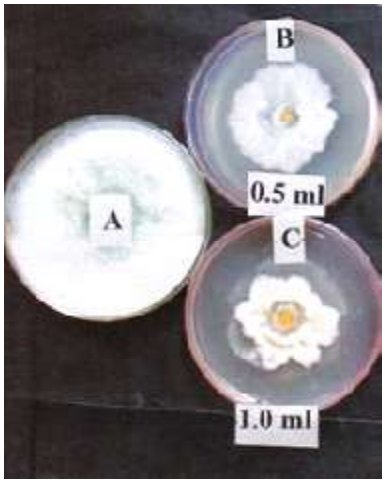
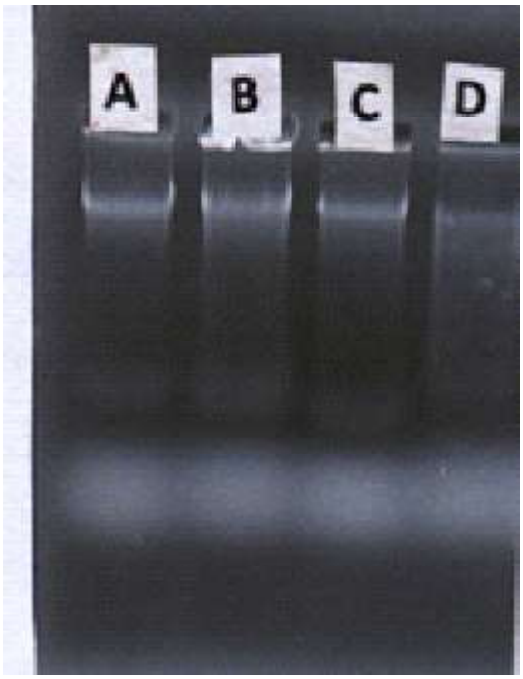


Plate 3b 2.
A- Control(*Alternia sp*.,)
B-0.5ml(*Azotobacter sp*,)
C-1.0ml(*Azotobacter sp*,)

Plate 3(c). The biocontrol activity of *Azotobacter* sp



A - *Azotobacter sp*., (Root)
B - *Xanthomonas sp*.,(Stem)
C - *Pseudomonas sp*, (Petiole)
D - *Pseudomonas sp*,(Leaf)

Plate 4. The biocontrol activity of *Azotobacter sp*.,

CONCLUSION

The growth promoting effect of those bacteria were also seen in the Mustard plant that can be further studied and applied in the economically important agricultural crops. These bacterial cultures can also be inoculated with the normal agricultural crops to enhance the nutrient uptake.

The biocontrol activity of endophytic bacteria is found to be effective. The endophytic bacteria could resist the growth of fungal pathogen like *Alternaria* sp., and *Curvularia* sp., this shows that they help the plants to fight against the invasion of pathogens.

The genomic DNA can be isolated from endophytic bacteria in genus level through Agarose gel electrophoresis,white band indicate the genomic DNA.

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