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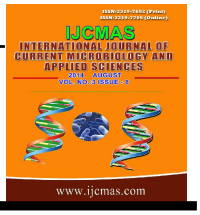


Original Research Article

Identification and characterization of Endophytic bacteria from fruits like Avacado and Black grapes

M.P.Prasad* **and Sunayana Dagar**

Department of Biotechnology, Sangenomics Research Labs, Domlur Layout, Bangalore, India
**Corresponding author*

Keywords

Endophytic bacteria, Siderophore, IAA, HCN, *Bacillus sps*., Lipase.

A B S T R A C T

Plants are hosts to one or more endophytic microorganisms. However the endophyte plant interaction is one of the least studied biochemical systems in nature. Endophytes include the fungi, bacteria, and actinomycetes that primarily reside in the tissues beneath the epidermal cell layers and the host tissues are transiently symptomless and inconspicuous. In the present study fruits such as Avacado and grapes were selected as they are known to be rich in antioxidant properties and are less explored in terms of endophytic bacteria. From the two types of fruits studied, six isolates were selected for further studies based on their dominance as well as uniqueness or differences with others in colony morphology. All the bacterial isolates were Gram positive and belonged to the genus Bacillus. All the endophytic isolates were found to produce IAA with maximum production by isolates from Avacado. All the 6 isolates were found to produce ammonia and two of them HCN. Three isolates from Avacado and one from Black Grapes were found positive for Siderophore Production. The isolates were tested for enzyme production assays also and were found positive for catalase and lipase enzyme. Two isolates from black grapes were protease positive. The work indicates the importance of the endophytic bacteria which can bring out changes in the phytochemical properties of the fruits and also a good source for industrial exploitation.

Introduction

Plants are generally associated with diverse microorganisms. Endophytic organisms are those that colonize the plant internal tissue showing no external sign of infection or negative effect on their host (Schulz & Boyle, 2006). Plants constitute vast and diverse niches for endophytic organisms. Of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes (Strobel *et al*., 1993). But most likely, there is not a single plant species devoid of endophytes. Only a few of these plants have ever been completely studied relative to their endophytic biology. Consequently, the opportunity to find new and beneficial endophytic microorganisms among the diversity of plants in different ecosystems is considerable.

The term endophyte (Gr. endon, within; phyton, plant) was first coined by De Bary (De Bary, 1866) 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 (Wilson, 1995). The presence of endophytes was reported by Vogl in 1898 who revealed a mycelium residing in the grass seed *Lolium temulentum*.

Endophytic bacteria have been isolated from a large diversity of plants. Organisms like *Bacillus, Enterobacter, Klebsiella, Pseudomonas, Burkholderia, Pantoea, Agrobacterium, Methylobacterium* spp. constitute the endophytes commonly isolated from diverse plants such as rice, wheat, maize, cotton, clover, potato, sugarcane, tomato, cucumber, and wild grasses (Bacon and Hinton, 2006). The precise role of endophytes in plants is not yet known. However, their capability to thrive within the host tissues away from microbial competition and environmental degradation has made endophytes potential candidates for use in agriculture.

The role of endophytic microorganisms in plants can be divided into two categories based on types of activity: growth promotion and disease control. Endophytic bacteria are believed to elicit plant growth promotion in one of two ways: Directly by producing phytohormones such as auxin or cytokinin or by producing the enzyme 1-aminocyclopropane- 1-carboxylate (ACC) deaminase, which lowers plant ethylene levels and indirectly by preventing pathogen infections via antifungal or antibacterial agents, by outcompeting pathogens for nutrients by siderophore production, or by establishing the plant's systemic resistance. Other beneficial effects of endophytes to plants include by helping plants acquire nutrients, via nitrogen fixation, phosphate solubilization or iron chelation, increased drought resistance, thermal protection, survival under osmotic stress (Bacon and Hinton, 2006). A particular bacterium may affect plant growth and development using one or more of these mechanisms and may use different ones at various times during the life cycle of the plant. In addition to these plant-growth-promoting traits, endophytic bacteria must also be compatible with host plants and able to colonize the tissues of the host plants without being recognized as pathogens (Rosenbleuth and Martinez-Romero, 2006). The survival and conservation of endophytic communities of bacteria can also be affected by the type of plant propagation methods used. These bacteria can develop, become distributed throughout the plant as it grows and are then returned to the soil via crop residues. Any plants that are propagated vegetatively have an enduring community of bacterial colonists that are transferred in successive progeny generations. True seeds can also be the source of endophytic bacteria in the developing seedling.

Materials and Methods

Collection of samples

In the present study, Black grapes and Avacado were selected as they are loaded with antioxidant properties and are less probed in terms of endophytic bacteria. The samples were collected from the regional fruit outlet and transported in sterile polythene bags for further processing.

Surface sterilization

The samples were rinsed with autoclaved distilled water, disinfected with Hydrogen peroxide for 2 minutes. Then were rinsed for

5 minutes with 70% Ethanol followed by 3% Hypochlorite + Tween 20 (0.1%) and finally rinsed with autoclaved distilled water.

Processing of Samples and Isolation

Two methods were employed in the present study in order to isolate endophytic bacteria from the fruits. First method involved cutting samples were into 2 halves; and each half was impregnated on Nutrient Agar plates and incubated at room temperature for 24h. The second method involved macerating the surface sterilized samples using a sterile pestle and mortar. 1 g of the macerated sample was serially diluted and the dilutions were plated on Nutrient Agar media and incubated at room temperature for 24h. After overnight incubation, isolated colonies were selected and used further.

Morphological Characterization of endophytic bacterial isolates

The selected strains were morphologically characterized in order to determine the morphology of the bacterial cells upon observable characteristics such as cell shape, colony color and texture. This was determined by the classical gram staining method as described by (Cappuccino and Sherman, 2002)**.**

Biochemical characterization of the Bacterial Isolates

The selected endophytic bacterial strains were biochemically characterized by Indole test, Methyl Red test, Voges Proskauer Test, Catalase test and Citrate utilization as per standard method (Cappuccino and Sherman, 1992).

Screening of endophytic bacteria for PGP traits

The selected bacterial strains were screened for PGP traits using various assays such as Siderophore production, HCN production, Ammonia Production and IAA production.

Siderophore Production

Siderophore production was detected by CAS assay. This assay was done qualitatively and is based on competition for iron between a ferric complex of chrome azurol S (CAS), an indicator dye, and a siderophore produced by the microorganism (Schwyn and Neilands, 1987). Each endophytic isolate was streaked on the surface of CAS medium and incubated at room temperature for 1 to 3 days. Siderophore production was indicated by orange halos around the colonies after the incubation.

HCN Production

Screening of endophytic bacterial isolates for HCN production was done according to (Lorck, 1948). The selected isolates were grown in Nutrient Agar supplemented with glycine (4.4g/l). A Whatman filter paper No.1 soaked in 0.5% (w/v) picric acid solution was placed to the underside of the Petri dish lids. To avoid the escape of the gas, the plates were sealed with parafilm and incubated at room temperature for 5-7 days and the production of HCN was determined by the change in color of filter paper from yellow to red-brown.

Ammonia Production

Endophytic isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water separately and incubated for 48 -72 h at 36 ± 2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

IAA Production

IAA production was determined according to (Brick *et al.*, 1991). The endophytic bacteria were grown in LB broth supplemented with L-tryptophan (1µg/ml) for 72 hours. Overnight cultures were centrifuged at 10,000g for 10 minutes and 1 ml of the supernatant was allowed to react with 2 ml of Salkowsky reagent, incubated for 20 minutes at room temperature (appearance of brownish pink color indicates the presence of IAA) before the absorbance was measured at 530nm. The absorbance of the samples obtained was plotted against a standard to determine the concentration of IAA produced.

Analysis of Enzyme Activity

The endophytic bacterial strains were screened for various enzyme activities such as Lipase activity, Protease activity and Catalase activity.

Lipase Activity

The lipase activity of the selected endophytic bacterial isolates was determined by supplementing the Nutrient Agar media with 0.01% $CaCl_2.H_2O$, followed by adding sterilized Tween 80 to the media to give a final concentration of 1%. The media was poured into the Petri plates, and presence of opaque halo zone around the colonies was considered as positive (Sierra, 1957).

Protease Activity

Proteolytic activity of the cultures was studied in a medium containing skimmed milk. Zone of precipitation of paracasein around the colonies in the next 48 hours were taken as evidence of Proteolytic activity (Vieira, 1999).

Catalase Activity

Catalase activity of the bacterial isolates was determined by adding H_2O_2 over the freshly grown endophytic bacterial cultures in NA plates. The presence of oxygen bubbles gave the evidence of catalase activity of the bacterial isolates (Cappuccino and Sherman, 1992).

Results and Discussion

Isolation of endophytic bacteria

A total of 6 endophytic bacteria were isolated from avacado and black grapes. Among the 6 isolates, 3 isolates namely G1 (N), G2 (N) and G1 (O) were from black grapes and other 3 isolates namely SA3, SA4 and A1 were from avocado.

Morphological Characterization

The selected endophytic bacterial strains were morphologically characterized by Gram staining (Figure 1). It was observed that all the isolates from black grapes were gram positive short rods, whereas two isolates from avocado were found to be gram positive cocco bacilli.

Screening of endophytic bacteria for PGP traits

Among the PGP traits screened, it was observed that all the six isolates were positive for Ammonia Production. IAA production was observed in all the strains and further quantification was performed. Among the 6 isolates, strain G2 (N), G1 (O), SA3, SA4 and A1 were positive for siderophore production. Likewise HCN production was observed only by strain G1 (N) and G2 (N). (Table 1)

Quantification of IAA production

Absorbance of the sample obtained was plotted against a standard to determine the concentration of IAA produced by the isolates (Graph.1). Among the 6 isolates maximum production of IAA was exhibited by strain SA 3 (Table 2) (Figure 2).

Analysis of Enzyme Activity

The selected endophytic bacterial strains were screened for enzyme activity (Figure 3), the results of which are tabulated in Table 3.

All plants are hosts to one or more endophytic microorganisms. However the endophyte plant interaction is one of the least studied biochemical systems in nature. Endophytes include the fungi, bacteria, and actinomycetes that primarily reside in the tissues beneath the epidermal cell layers and the host tissues are transiently symptomless and inconspicuous (Stone *et al.,* 2000). There is ample evidence that many endophytic bacteria have beneficial effects on plants (Hallmann *et al.,* 1997).

Growth promotion of plants may be achieved by bacterial production of plant growth regulators such as auxins, cytokinins and gibberellins; nitrogen or other nutrients may be provided by biological nitrogen fixation or mobilized as is the case for phosphorus; moreover, endophytes may confer plant protection against pathogens by induction of plant defense mechanisms, pathogen-antagonistic substances or through competition for colonization sites and nutrients. The present work is carried out to study the diversity of endophytes of fruits such as Avacado and Black Grapes which are known to have high antioxidant property.

The isolates selected in the present study were chosen for their dominance as well as uniqueness or differences with others in colony morphology. Six isolates were selected from Avacado and Black Grapes and were named according to the host plant. All the bacterial isolates were found to be Gram positive. Earlier workers have reported a predominance of Gram negative bacteria in the tissues of various plants (Sudhir allu *et al.,* 2014). However, Toldi. O *et al.,* (2009) reported an equal presence of Gram negative and Gram positive bacteria in strawberry. The bacterial isolates in the present study were found to be either Bacillus short rods or cocco-bacilli. According to Jacobs *et al.,* (1985) the most common taxa of endophytic bacteria recovered include *Bacillus*, *Enterobacter*, *Erwinia*, *Pseudomonas* and *Flavobacterium*. McInroy and Kloepper (1995) reported that the endophytic bacterial diversity spanned over 40 genera, with predominance of *Pseudomonads* and *Bacillus*. Hallmann *et al.,* (1997) have reported that the former Pseudomonas group (*Pseudomonas, Burkholderia*) and Enterobacteriacea (*Enterobacter, Klebsiella*) are the common taxa found in tomato, potato, cotton, soybean, rice and maize. These taxa were predominantly found in the present study.

The selected bacterial isolates were tested for PGP traits. All the six strains were found to produce IAA. IAA, a member belonging to the group of phytohormones, is generally considered to be the most important native auxin (Strzelczyk and Pokojska, 1984). Further quantification of IAA produced was performed. It was observed that maximum production of IAA was exhibited by strain SA 3 (54.83 µg/ml). IAA production by endophytes isolated from sweet potato which could influence host growth in fertile soil has been reported by Zareen Khan *et al.,* 2005. In the present study among the six

isolates, four isolates exhibited siderophore activity. Several reposts from the past have confirmed that siderophore producing bacteria significantly help in the uptake of various metals such as Fe, Zn and Cu by plants (Carrillo- Castenada *et al.,* 2003; Egamberdiyeva, 2007; Gururani *et al.,* 2012). HCN production was observed only by 2 strains among the 6 strains and Ammonia production was observed in all the strains which were found to be in parallel with those seen by Sudhir Allu *et al.,* 2014. The endophytic bacterial isolates were further studied for their hydrolytic enzyme activity for 3 enzymes: Lipase, Catalase and Protease. Except for A1 all the isolates were found to be Lipase test while catalase was being produced by all the endophytic bacterial isolates. Though, protease assay was positive for strains G1 (N) and G1 (O) only. Buchenauer (1998) also stated that lytic enzymes secreted by bacteria are suspected to play an important role in suppression of pathogens. Further molecular characterization and their effects on plant growth under pot and field conditions would help us to understand the plant – microbe interaction in detail.

Figure.1 Gram staining of the selected isolates

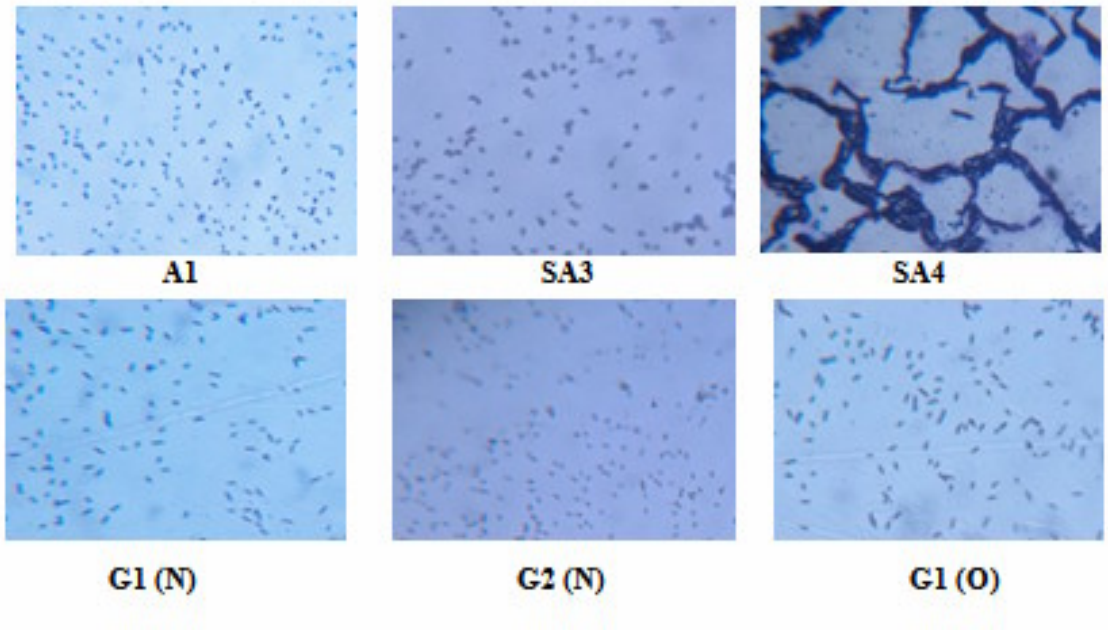


Table.1 PGP Traits

TEST	Siderophore Production	Ammonia Production	HCN Production	IAA production
G1 (N)	Negative	Positive	Positive	Positive
G2 (N)	Positive	Positive	Positive	Positive
G1 (O)	Negative	Positive	Negative	Positive
SA3	Positive	Positive	Negative	Positive
SA4	Positive	Positive	Negative	Positive
A1	Positive	Positive	Negative	Positive

Table.2 IAA concentration

Strain No.	Concentration (µg/ml)
G1 (N)	33.35
G2 (N)	30.26
G1 (O)	24.51
SA 3	54.83
SA 4	34.75
A1	49.97

Figure.2 Quantification of IAA production

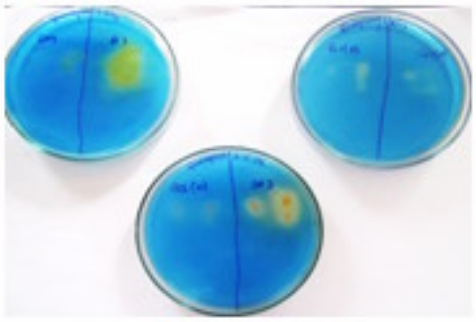


Figure 3a: IAA Assay

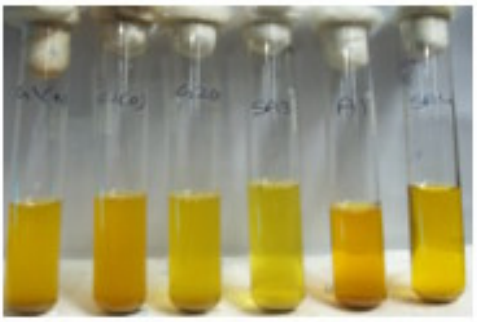


Figure 3b: Siderophore Assay

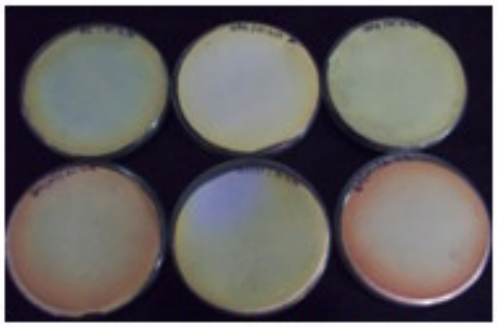


Figure 3c: HCN Production

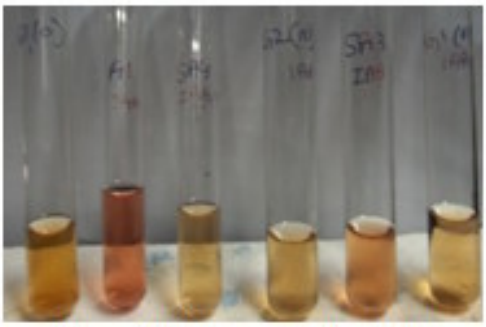


Figure 3d: Ammonia Production

Table.3 Analysis of Enzyme Activity

Enzyme assay	Lipase	Catalase	Protease
G1 (N)	Positive	Positive	Positive
G2 (N)	Positive	Positive	Negative
G1 (O)	Positive	Positive	Positive
SA3	Positive	Positive	Negative
SA4	Positive	Positive	Negative
A1	Negative	Positive	Negative

Figure.3 Analysis of Enzyme Activity

Fig.4a: Lipase Assay

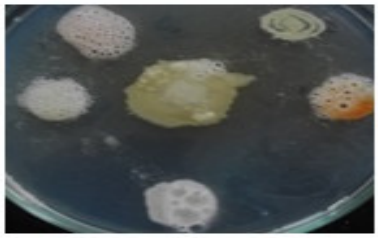


Fig.4b: Catalase Test

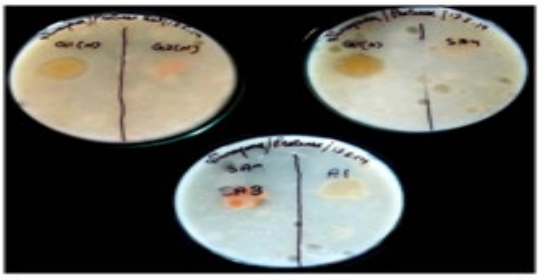
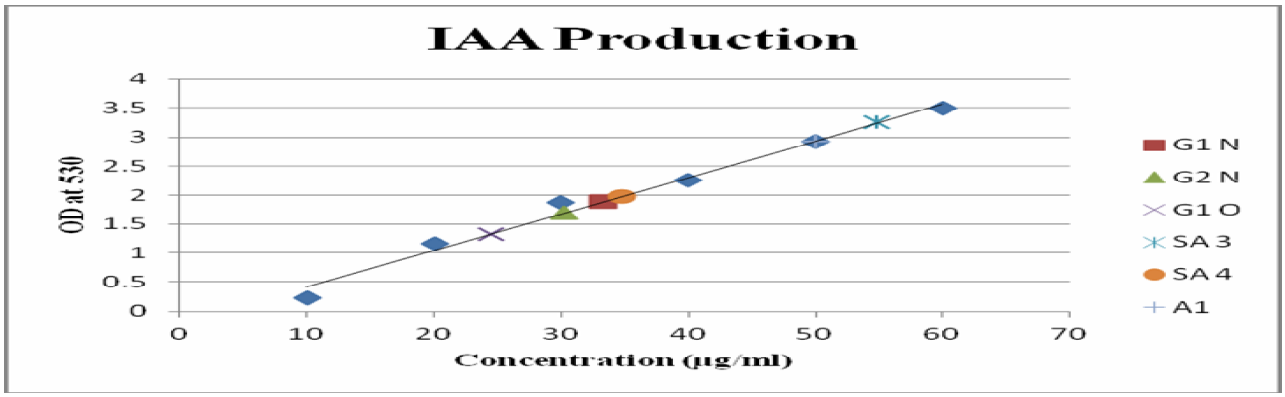


Figure 4c: Protease Activity

Graph.1 IAA concentration of the selected strains



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