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Establishing and Quantifying the Nitrogen Fixing Potential of the Brinjal Bacterial Isolate

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Abstract
Rhizosphere, phylloplane and caulosphere is the region where a complex community of microbes, mainly bacteria and fungi are present. The microbe plant interaction in these regions can be beneficial, neutral, variable, or deleterious for plant growth. The bacteria that exert beneficial effects on plant development are termed plant growth promoting bacteria. To quantify the amount of nitrogen fixing bacteria from rhizosphere, phylloplane and caulosphere of brinjal (*Solanum melongena* L.). Brinjal (*Solanum melongena* L.) plants of different varieties were collected from seven locations around Bangalore *viz.,* Hessaraghatta, Yelahanka, Kengeri, Madi vala, Hebbal, Tirumalapura and Attibele were also screened for the presence of nitrogen fixing bacteria. Nitrogenase activity was estimated by acetylene reduction assay and analyzed by gas chromatography. The amount of nitrogen fixed brinjal bacterial isolate was quantified by micro Kjeldahl method. The amount of nitrogen fixed by the BBI was equivalent to 23.5 nm of C_2H_2 reduced/tube/hour. The amount of nitrogen fixed by the BBI showed a steady increase up to three days (75 nm of C_2H_2 reduced/tube/hour) after which there was a decline in the amount of nitrogen fixed by the microbe. For the first time the presence of nitrogen fixing bacteria on the rhizosphere and endorhizosphere of brinjal *(Solanum Melongena* L.) cultivars was established.

Keywords
Solanum melongena L. Nitrogen fixing bacteria, Rhizosphere, Phylloplane, Caulosphere,

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INTRODUCTION
Associative bacteria have been isolated from the rhizoplane, phylloplane and stem of many non-leguminous plants. Many studies have dealt with isolation of associative microorganisms from the roots of cereals, vegetable and fruit crops such as sweet potato [1], arecanut, banana, coconut, cashew, citrus, custard apple, grape, guava, jackfruit, litchi, mango, papaya, pomegranate- ate, phalsa, pepper, and strawberry [2], Spartina altemifolia [3], sugarcane [4], barley [5], wheat, maize, sorghum, millet and rice [6-11]. Nitrogen fixing organisms were

isolated from the roots of many non- leguminous crops such as Spiacia oleracea, Brassica chinensis and Brassica rapa by Ahn et al. [12]. The isolated associated bacteria in many of these studies was identified as Azospirillum [13-17]. Lukin observed spatial distribution of associated microorganisms identified as Azospirillium in rhizosphere of barley plants [18]. Holguin et al isolated two nitrogen-fixing bacteria from the rhizosphere of mangrove trees, which were characterized as Azospirillium [19]. Hill et al characterized a nitrogen-fixing bacteria associated with the roots of sweet potato as Azospirillum [1]. The nitrogen fixing potential of associated microorganisms were evaluated in the soil and rhizosphere under controlled conditions. Acetylene reduction activity has been used to estimate the amount of nitrogen fixed by the associated bacteria in many studies. Linderberg assessed the nitrogen fixing ability of Bacillus polymyxa and Azospirillwn using acetylene reduction assay [20]. Christiansen et al determined the acetylene reduction activity of free living and associated Azospirillum brasilense by using N15 dilution technique and calculated the amount of nitrogen fixed to be 0.067 mg per plant of which 3.3% nitrogen was fixed in the root and 1.6% of nitrogen was fixed in the shoot [21]. They proved that the nitrogen fixed by the shoot was of atmospheric origin. Lethbridge et al critically evaluated acetylene reduction test for evaluating the activity of nitrogen fixing bacteria associated with the roots of wheat and barley [22]. Wani discussed this aspect by using a soil core assay to highlight precautions that should be taken during estimating nitrogenase activity [23]. In this present study the qualitative and quantitative studies for the occurrence of nitrogen fixing bacteria from the rhizoplane, phylloplane and stem of brinjal (Solanum melongena L.) plants were undertaken.

MATERIALS AND METHODS

Brinjal (Solanum melongena L.) plants of different varieties were collected from seven locations around Bangalore viz., Hessaraghatta, Yelahanka, Kengeri, Madi vala, Hebbal, Tirumalapura and Attibele were also screened for the presence of associative bacteria. The standard laboratory chemicals were used, and Bacteriological media used were from Himedia. The procedure given by Patriquin and Dobereiner was followed to isolate the associative bacteria from the root, stem, and leaf of brinjal [10,11]. The bacterial growth from the sterile roots was isolated and subcultured in nitrogen free Burk's media and they were multiplied in TYMB media [24]. Pure cultures were maintained in stab cultures containing nitrogen free Burk's media. Both sterile and unsterile root, leaf, and stem bits of brinjal *(Solanum melongena* L.) plants were used for the initial screening of associative bacteria. The dominant colonies of bacteria present in the rhizoplane, phylloplane and stem were isolated and subcultured. The pure cultures of the bacterial isolate were screened for nitrogen fixing potential.

Establishing the nitrogen fixing potential of the brinjal bacterial isolate (BBI)

The nitrogen fixing potential of the dominantly occurring brinjal bacterial isolate was established by the acetylene reduction assay using a gas chromatograph. The rhizoplane bacterial isolate was grown in 100ml of nitrogen free liquid Burk's media of pH 6.7 at 37° C for 48 hours. The cultures were harvested and suspended in 100ml sterile distilled water. The tube containing the suspension in distilled water was put in a shaker for 30 minutes for uniform suspension. Serial dilutions were done and 1ml of the diluted culture having 1.6 x 1010 bacterial cells was inoculated into tubes containing 10ml of liquid Burk's media and incubated for 3, 6 and 15 days at 37° C. Tubes with heat killed bacteria served as a control. Nitrogenase activity was estimated by acetylene reduction assay following Turner and Gibson [25]. 1ml of 10% acetylene was introduced into the test tubes containing 10 ml of the culture of the BBL. The tubes were incubated for one hour at 37 °C. After incubation, 1ml aliquots of the gas sample from each tube were analyzed by gas chromatography.

The gas chromatograph (Hewlett Packard 5890 series-11) had a hydrogen flame ionization detector (FID) having a stainless-steel column 3m long and 1mm in diameter. The steel column was filled with Porapak N. The column temperature was 60° C, with carrier gas flow rate of 50µL/minute. The air and hydrogen gas flow rate were 300mL/min. The retention time was recorded as peaks. The amount of acetylene reduced was expressed in nano moles of C_2H_2 ml^{-1} of gas phase in 10ml of liquid culture having a bacterial population of 6 x 104 per 0.1ml of the culture. Commercial acetylene was used for this experiment. Twelve samples were analyzed, wherein each set had three replicates. The amount of acetylene reduced by the BBI at different intervals of time was calculated using standard ethylene gas.

Quantifying the amount of nitrogen fixed by the BBI

To quantify the amount of nitrogen fixed by the BBI, 0.1ml of pure culture was inoculated into 0ml of liquid Burk's media in a 50ml conical flask and incubated at 37°C for 48 hours. The culture was harvested by centrifugation and the harvested cells were suspended in 100 ml of sterile distilled water. 1ml of the above suspension was introduced into culture broth and incubated at 37°C for a week after which the tubes were centrifuged and the weight of

the harvested bacteria was made equal to 1mg in each tube. To each of the test tubes, 1ml of digestion reagent was added. The tubes were then placed on hot water bath for 3-4 hours. The volume of the tube containing the digested bacteria was made up to 100ml to give a solution strength of 5% H2S04. Two reagents used in this micro Kjeldahl technique were termed as reagent A and reagent B. Reagent A consisted of 5g/L phenol and 0.25g/L of sodium nitroprusside and reagent B contained 0.25g/L of sodium hydroxide and 2. l g/L of sodium hypochlorite. One part of reagent A and one part of reagent B were diluted with 4 parts of water. 5ml of the diluted reagent were added to 5 ml of digested sample and agitated. The colour was developed after 30 minutes at room temperature and read spectrophotometrically at 625nm. Each set of estimates included at least two standards and a reagent blank. Pre-dried anhydrous KNO3 was used as standard nitrogen. Standard nitrogen was prepared by dissolving 14.2mg of KNO3/L of distilled water. One ml of the solution was equivalent to 1mg ml^{-1} of nitrogen. Dilutions of this solution were prepared ranging from 0.05 mg of nitrogen to 6.0 mg of nitrogen. To each of these dilutions reagents were added and the colour was read at 625nm using spectrophotometer. A standard graph was prepared, and amount of nitrogen fixed by the bacteria at different intervals of time was determined.

RESULTS

The amount of nitrogen fixed by the BBI was equivalent to 23.5 nm of C 2H2 reduced/tube/hour. The amount of nitrogen fixed by the BBI showed a steady increase up to three days (75 nm of C2H2 reduced/tube/hour) after which there was a decline in the amount of nitrogen fixed by the microbe. The amount of nitrogen fixed over the period of a week is depicted in Fig. 1.

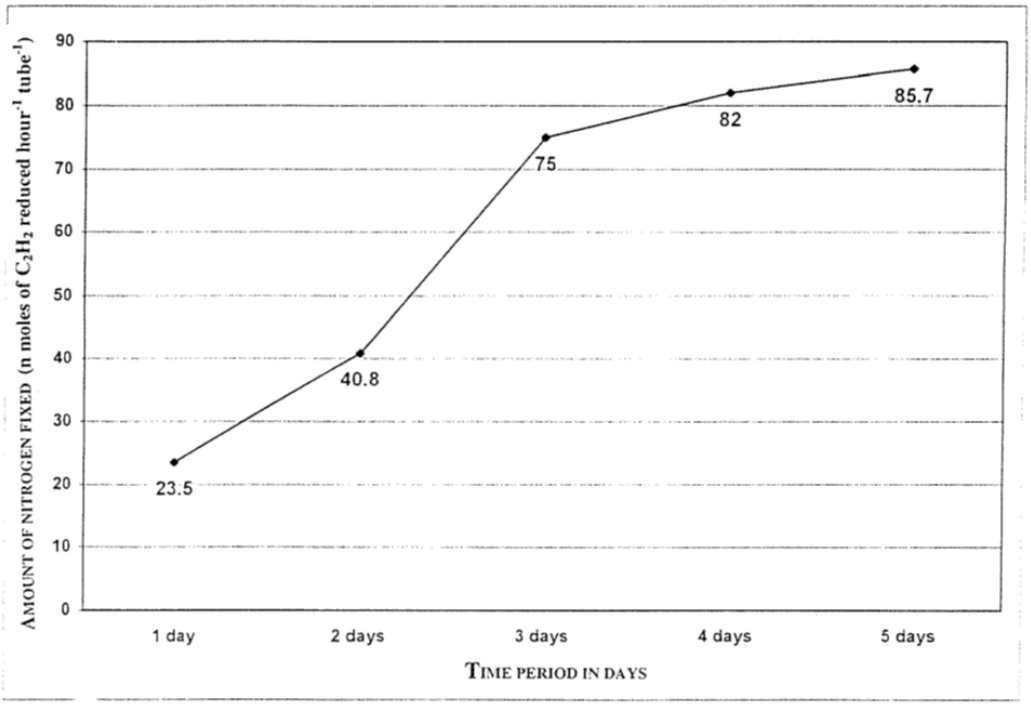

Figure 1: Amount of nitrogen fixed by the brinjal bacterial isolate in nitrogen free Burk's medium

DISCUSSION

Growth promoting bacte1ia have been isolated in vegetables like tomato, cabbage, spinach, winged bean, capsicum and sweet potato. Bashan and Holguin, and Bashan et al observed aggregates of bacteria on the surface and endosphere of root hair, root cap and elongation zones of tomato using scanning rhizobacteria [26,27]. Similar isolations have been reported from roots of cereals, grasses and plantation crops [7,28-30].

The bacterial isolate in this study could efficiently fix nitrogen as revealed by acetylene reduction bioassay. The bacterial isolate could reduce 23.5 nm of acetylene/tube/hour. In a similar study Gamo et al reported 28 nm/tube/hour of acetylene reduced in roots of eggplant [31]. Rao established nitrogen fixing capacity of many plantation and orchard crops by acetylene reduction bioassay [7]. Sukhada recorded 891.6 nm of acetylene reduced/hour/g of dry weight of tomato. While in Chinese cabbage and

spinach it was 139 moles of acetylene reduced/tube/hour and 197.7 nm of acetylene reduced/tube/hour respectively [14]. Hill et al; Bouton, Day et al and Dobereiner and Day demonstrated the nitrogen fixing potential of associated bacteria of sweet potato, pearl millet and grasses in similar studies [1,32-34]. Though the rate of nitrogen fixation in associative bacterial systems is much lower than a legume-Rhizobium system, it is quite significant in the overall nitrogen economy of the plant.

CONCLUSIONS

The present study clearly indicates the potential of efficient nitrogen fixing isolates from rhizosphere, phylloplane and caulosphere of *Solanum melongena* L. The use of nitrogen fixing bacterial isolate as inoculants biofertilizer could be an efficient approach to replace chemical fertilizers and pesticides for sustainable cultivation. Further studies are required involving detailed characterization of molecular and functional properties of these nitrogen fixing bacteria for their applications in the field.

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