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Assessing the Plant Growth Promoting Activity of Phylloplane Associated Plant Bacteria of Rice

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A B S T R A C T

Rice (*Oryza sativa*) is the most important staple food crop in India. It ranks second in worldwide production. An anatomical and physiological character of surface of the rice leaf renders a sustainable environment for diversity of phylloplane microorganisms that showed antagonism against rice pathogens. The objective of this present study was to identify and characterize the bacterial population in the phylloplane of rice at different critical stages. Through leaf imprinting techniques bacterial colonies were isolated. Among the various stages of rice, milky stage recorded higher number of bacteria compared to other stages of rice. Based on morphological characterization the bacterial isolates were grouped and purified. Gram staining was performed and the isolates were observed under foldscope a frugal microscope. Biochemical characterization was being done for the bacterial isolates and the results were recorded. IAA is a key hormone for the plant growth hence the IAA production ability of the bacterial isolates were screened. Results revealed that all the bacterial isolates produced IAA in greater quantities.

Introduction

Rice is one of the important primary cereals produced worldwide. Its cultivation has its own impact as the consumers are more and it has global, social and economic importance. The phyllosphere is a term used in microbiology to refer to the total above-ground portions of plants as habitat for microorganisms.

Phyllosphere environment is commonly occupied by diverse microbes such as bacteria, filamentous fungi, yeast, actinomycetes, where bacteria are the most predominant (Lindow and Leveau 2002). The phyllosphere microbes plays an important role in decomposing natural substances as saprophytes, remediating of remnant pesticides and air pollutants, inducing plant health and development as biofertilizer, phytostimulator, and biopesticides against plant pathogens (Muller and Ruppel 2014). A variety of bacteria, yeasts and filamentous fungi have been isolated from the phyllosphere of several plant species.

Microbes residing in the phyllosphere can have various life styles and modes of interaction with the host, being neutral residents, latent pathogens, or plant-health and growth promoters (Kai Wang *et al.*, 2016). The bacteria inhabiting the phylloplane are exposed to adverse environmental conditions and can be a good source of diverse bacterial isolates having plant growth promotion potential (Kishore *et al.*, 2005).

Materials and Methods

Collection of samples

The rice leaf samples were collected at different stages from the field in and around Killikulam and vallanadu regions.

Isolation and purification of bacterial isolates

Leaf samples were collected and surface sterilized using 70% ethanol and washed three times with sterile water. To isolate bacterial population on the adaxial and abaxial leaf surface, both the sides of leaf were imprinted on nutrient agar media. Individual leaf of size 5 cm each was placed on agar media and the imprint was done by gently pressing the leaf sample using a sterile glass rod (Yadav *et al.*, 2010). The plates were incubated for 24hrs at 30°C. Based on the morphological characterization individual colonies were selected and purified in the Nutrient agar medium .The pure culture of bacteria were maintained in nutrient agar slants at 4°c for further studies.

Morphological and biochemical characterization of the bacterial isolates

Colony and cell morphology

Biochemical characterization

Biochemical characterization test such as indole production, Methyl red test, Vogus-Proskauer test, Citrate utilization test, Amylase test, Catalase test, Cellulase test, Gram staining was performed according to the procedure given by Thayer and Murray (1977).

Indole production test

Indole production test was done to test whether the organism can oxidize tryphtophan that results in the formation of indole, pyruvic acid and ammonia.

Bacterial isolates were inoculated in tryptophan broth and incubated for two days. After incubation Kovack's reagent was added and the results were recorded.

Methyl red

Bacterial isolates were inoculated in the broth and incubated for three days. After incubation Methyl red indicator was added and observed the changes in color of methyl red. Results were recorded. This test can detect the ability of the isolates to oxidize glucose with the production of high concentration of acid.

Voges proskauer test

Bacterial isolates were inoculated in the MR-VP broth and incubated for two days. After incubation 12 drops of VP reagent -1 (Napthol solution) and 2-3 drops of VP reagent- II (40% KOH) was added and observed for change in red colour that was recorded to be a positive reaction.

Citrate utilization test

Bacterial isolates were streaked on simmon's citrate agar and incubated for three days. After incubation the colour change was observed and development of green to blue color in medium indicates positive. The results were noted for comparison.

Enzyme activity

Amylase test (Buzzini and Martini, 2002)

Bacterial isolates were streaked on starch agar medium and incubated for 48 h at 30±1°C. After incubation petriplates were flooded with Lugol's iodine solution for 30 sec and drained.

Formation of yellow zone around the colonies against dark blue background, indicates the positive starch hydrolysis. Results were recorded.

Catalase test (Nutaratat *et al.*, 2014)

The bacterial isolates were streaked on nutrient agar plates and incubated for 48 h at 30±1°C. After incubation few drops of 3% hydrogen peroxide (H2O2) was added to the grown culture and observed for appearance of effervescence which indicates positive for catalase test. Production of effervescence indicates presence of oxygen.

Cellulase (Buzzini and Martini, 2002)

The bacterial isolates were streaked on the CMC agar medium and incubated for 2 days. After incubation plates were flooded with 1% congo red and observed for zone formation around the colony. Results were tabulated.

Estimation of IAA production

Culture filtrate to a quantity of 8ml was taken and 2ml of Salkowski reagent was added and incubated at room temperature in dark for 30 minutes for the development of pink colour.

The optical density was read at 530nm. The results were read from standard graph and expressed as µg/L. The IAA production was determined using the Salkowski method (Rahman *et al.,* 2010).

Results and Discussion

At different growth stages of rice leaf samples were collected and the bacterial diversity was analyzed using leaf imprinting technique in nutrient agar medium. Bacterial population was enumerated and based on morphology isolates were grouped and purified. After purification the isolates were characterized (Table 1; Plate 2).

The results revealed that milky stage have more number of bacterial colonies compared to other critical stages. Moreover, this stage has more pigmented bacterial colonies (345 cfu/m^2) followed by panicle initiation stage (155 cfu/m^2) and least number of bacteria was observed at nursery stage (115 cfu/m^2) (Plate 5). The hike in the bacterial population especially in the milky stage might be due to meet out the nutritional and physiological requirements when compared to other stages. Gram negative bacterial population was predominant, together with a few pigmented bacteria was observed by Venkatachalam *et al.,* (2016).

Characterization of bacterial isolates

The bacterial isolates were further studied for their morphological and biochemical characteristics.

Morphological characterization

Dorko *et al.,* (2000) reported that *Cryptococcus neoformans* are white, grey in colour, smooth in texture and had raised colony. The work performed on morphological and physiological characterizations of *Saccharomyces cerevisiae* strains exhibited rough and smooth colonies (Reis *et al.,* 2013). In this study**,** all the bacterial isolates were observed for their morphological characters such as colony shape, pigment, form, margin and elevation.

The color of the colonies were dark brown, yellow, white dull white, orange, dark yellow and were mostly had round margin, irregular form and also most of the isolates were round shaped (Table1: Plate 2).

Gram staining

Gram staining is a common technique used to differentiate two large groups of bacteria based on the different cell wall constituents. The gram stain procedure distinguishes Gram positive and Gram negative groups. Among the 10 bacterial isolates, 8 isolates identified to be gram positive because the isolates had shown violet colour in staining. The bacterial cells were observed through foldscope, a frugal microscope. (Table:1, Plate1). Similarly Rohomania *et al.,* (2015) performed the gram staining and reported the 12 gram negative and 12 gram positive bacteria isolated from Fresh and Salted Hilsa.

Biochemical characterization and enzyme activity

In this study all the bacterial isolates showed positive results in various biochemical tests such as citrate utilization, indole production (Table 2) For MR test and VP test only 8 isolates showed the positive results (Table 2).

Both Amylase and catalase activity showed positive but only in cellulase all the isolates showed the negative results (Table 2, Plate 3). The results revealed that the bacterial isolates obtained from the phyllosphere of rice have the ability to utilize the citrate and also have the ability to produce indole. Similar results were obtained by Jeyashri *et al.,* (2019) who reported that most of the phyllosphere yeast isolates from rice showed positive results in biochemical characterization and enzyme activity.

Table.1 Colony Morphology and gram staining of the phyllosphere bacterial isolates from rice

Bacterial isolates	Gram's stain	Shape	Pigment	Form	Margin	Elevation
B1	+ve	Rod	Dark brown	Irregular	Round	Raised
B2	+ve	Rod	Yellow	Irregular	Round	Flat
B3	+ve	Rod	Dark yellow	Irregular	Round	Raised
B4	-ve	Rod	White	Irregular	smooth	Convex
B5	+ve	Rod	Orange	Irregular	Serrated edges	Flat
B6	+ve	Rod	White	Irregular	Round	Flat
B7	+ve	Rod	White	Irregular	Round	Flat
B8	-ve	Rod	White	Irregular	Round	Convex
B9	+ve	Rod	Dull White	Irregular	Serrated edges	Raised
B10	+ve	Rod	Brown	Irregular	Round	Raised

Table.2 Biochemical and enzyme activity of phyllosphere bacterial isolates from rice

Bacterial isolates	Cellulase	Amalyse	Citrate Utilization	MR	VP	Catalase	Indole Production
B1	-ve	+ve	+ve	+ve	+ve	+ve	+ve
B2	-ve	+ve	+ve	-ve	-ve	+ve	+ve
B3	-ve	+ve	+ve	+ve	+ve	+ve	+ve
B4	-ve	+ve	+ve	+ve	+ve	+ve	+ve
B5	-ve	+ve	+ve	+ve	+ve	+ve	+ve
B6	-ve	+ve	+ve	+ve	-ve	+ve	+ve
B7	-ve	+ve	+ve	+ve	+ve	+ve	+ve
B8	-ve	+ve	+ve	+ve	+ve	+ve	+ve
B9	-ve	+ve	+ve	-ve	-ve	+ve	+ve
B10	-ve	+ve	+ve	+ve	+ve	+ve	+ve

Plate.1 Foldscope – Frugal Microscope

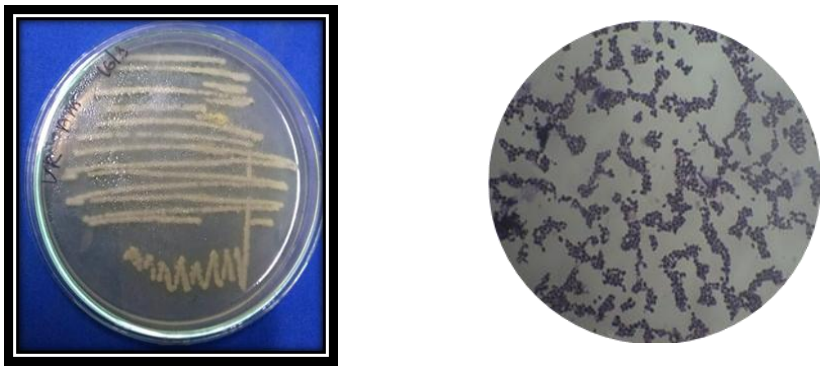

Plate.2 Bacterial isolate and stained cells under foldscope

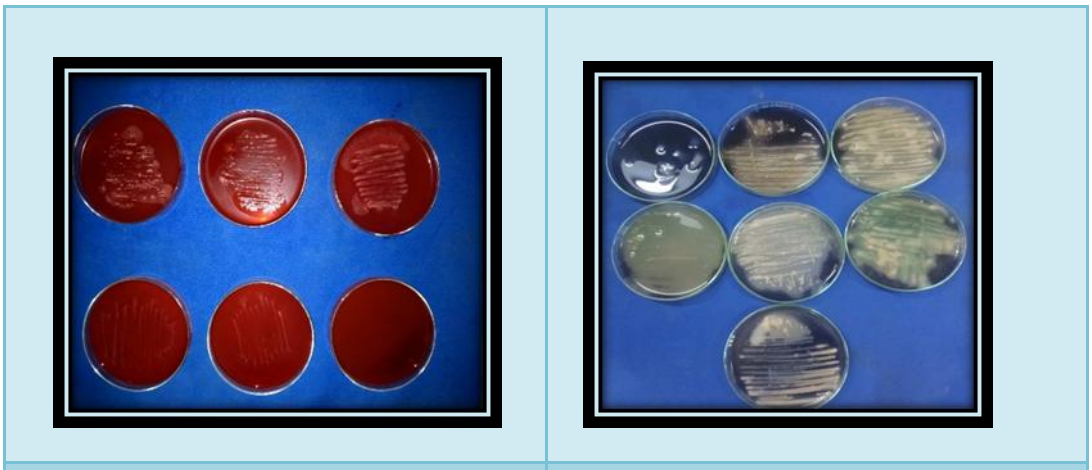

Plate.3 Determination of Enzyme Activity

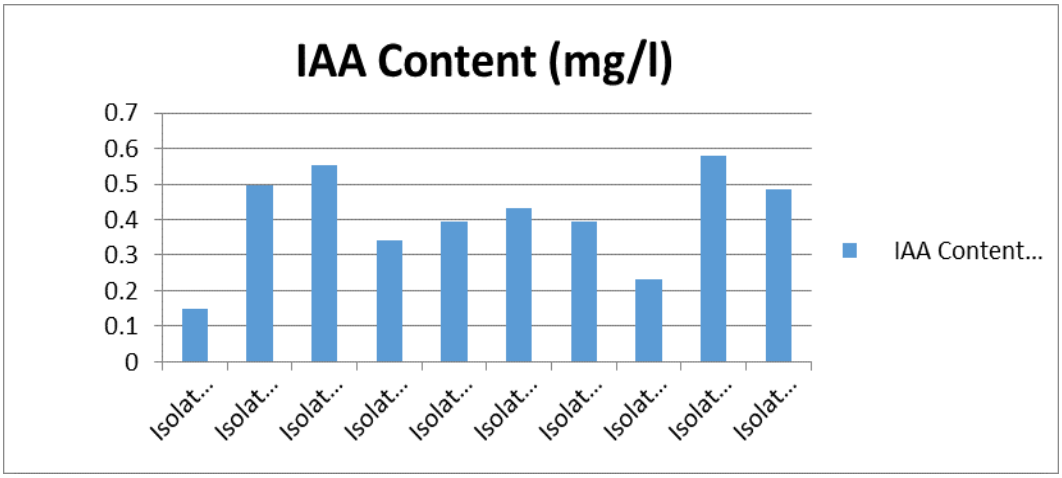

Plate.4 IAA production by phyllosphere bacterial isolates of rice

Different stages of rice				
	115 cfu/m^2	130 cfu/m^2	155 cfu/m^2	345 cfu/m^2

Plates.5 Assessing the phyllosphere bacterial population at different stages of rice

IAA activity

IAA activity was analyzed for the bacterial isolates and among the isolates, isolate B9 showed higher production of IAA followed by the isolates B3, B2 and B10 (Plate 4). Similar results were observed by Arun Kumar *et al.,* (2019) who also reported that IAA production was higher in the drought tolerant isolate obtained from phyllosphere of rice. Mohite, 2013 isolated bacteria from rhizosphere soil and evaluated the isolates for the production of IAA and found many of the isolated produced huge quantities of IAA. Jeyashri *et al.,* (2019) reported that *Rhodotorula paludigena* isolated from the phyllosphere of rice produced higher quantities of IAA (77.18µg ml¯1) in the presence of 0.1% tryptophan.

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