Isolation and characterization of phyllosphere microorganisms in rice through Foldscope – A frugal microscope

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Abstract

Phyllosphere microorganisms in rice ecosystem were analyzed using foldscope. Rice leaf samples were collected at critical stages from different places. At different time intervals leaf samples were collected from different regions. Samples were subjected to leaf imprinting technique in the respective media viz, Nutrient agar medium, Rose Bengal agar medium, Kenknight's agar medium, YPS medium for the isolation of bacteria, fungi, actinomycetes and yeast respectively. After staining samples were seen under foldscope and the images were recorded.

The information obtained through foldscope analysis of various microbes revealed that numerous microorganisms occupy the phyllosphere region and its population varies with time and place from which they have been isolated. Compared to the normal growth stages of crop, critical stages of crop were observed to have multiple colonies and wide varieties of microorganisms. Their population also get enhanced at different stages. Bacterial colonies were found to be high in number followed by yeast, fungi and actinomycetes. The pure cultures of microorganisms were maintained in respective agar slants at 4°C and stored at -20°C in 50 % glycerol for further studies. Based on the biochemical characterization tests the organisms were further analyzed. Bacterial isolates were subjected to grow in different carbon sources and most of the isolates were growing well by using glucose as the carbon source followed by sucrose, fructose and maltose. Indole acetic acid, Methyl red, Voges proskauer and Citrate utilization tests were done for the isolates and the results were recorded for further analysis.

Key words: Phyllosphere, Foldscope, Microorganisms

Introduction

Rice is the staple food crop in many of the countries. Low soil fertility is a serious problem in attaining the expected yield. Many of the nutrients that are present in the soil are in unavailable form. Microorganisms play a major role in making the unavailable form of nutrients into available form. Microorganisms not only present in the soil but also in the phyllosphere region of the crop plants.

The rice plant represents a habitat for diverse microorganisms, those that colonize the aerial parts (phyllosphere), the root surface (rhizoplane) as well as the zone around the root (rhizosphere) (Knief *et al.* 2011). The phyllosphere comprises the aerial parts of plants and is dominated by the leaves. Most studies on the identity of organisms in the phyllosphere have focused on bacteria and, to a lesser extent, fungi (Vorholt, 2012). Potential beneficial interactions of phyllosphere bacteria with rice plants, such as plant growth promotion by bacterial nitrogen fixation or plant hormone production have been studied (Knief *et al.* 2011). The present study was undertaken to know the microbial diversity in the phyllosphere of rice.

Materials and methods

Collection of samples

Rice leaf samples were collected from different locations at different stages. Leaf samples were collected from different fields and kept separately in covers and brought to the laboratory. Leaf samples were kept for further analysis.

Isolation and Characterization of microorganisms from phyllosphere of rice

The microorganisms such as bacteria, fungi, actinomycetes and yeast was isolated on their respective media such as Nutrient agar medium, Rose Bengal agar medium, Kenknight's agar medium, and YPS medium. The rice leaf samples were surface sterilized using 70% Ethanol and washed with sterile water and used for isolation of microorganisms. Surface sterilized leaf samples were imprinted and the plates were incubated at room temperature at 25° C. After specific incubation period the plates were observed for the growth of bacteria, fungi, actinomycetes, lactobacillus and yeast. Morphology of the isolates was recorded based on shape, colour, form, elevation and margin. Individual colonies were selected based on their morphological characteristics and re-cultured on fresh media to purify the particular microorganism. The pure cultures were maintained at 4°C and stored at -20°C in 50 % glycerol for further studies. All the isolates were observed under foldscope for the morphological characters and the photographs were taken. For bacterial cultures gram staining was performed to find gram negative and gram positive bacteria. After staining the cells were observed under foldscope and recorded the images for further analysis.

Similarly fungal isolates were identified based on morphology such as colony colour and pigmentation. Microscopic observation was done for the hyphae and spores of the fungi and viewed under foldscope. Images were taken and kept for further reference.

Actinomycetes and yeast cultures were also observed under foldscope after staining and the pictures were taken and documented for reference.

Biochemical characterization and phosphate solubilization

Biochemical characterization tests such as Indole acetic acid, Methyl red, Voges proskauer and Citrate utilization tests were done for the bacteria, actinomycetes and yeast isolates and the results were

recorded. Isolates were subjected to grow in different carbon sources such as sucrose, fructose, maltose, lactose etc.

The phosphate solubilisation test was done for bacteria, fungi and yeast isolates. Pikovskaya's medium was prepared and incubated with the isolates and then the plates were incubated at 30±1°C for the respective incubation days based on the microorganism. Clear halo zone was observed around the colony.

Results and discussion

Isolation of microorganisms from the phyllosphere of rice

Isolates of bacteria, fungi, actinomycetes and yeast were obtained from respective agar medium. Total of 80 bacterial isolates, 28 fungal isolates, 19 actinomycetes isolates, and 65 yeast isolates were obtained from different locations. Based on morphological characteristics different colonies were taken for further study. Morphology of bacterial isolates were given in Table 1. The bacteria images taken in foldscope were given in Plate 1. Many of the researchers have reported the presence of bacterial species in the leaf samples such as *Bacillus pumilus* in citrus (Marcon *et al.*, 2002), *Stenotrophomonas* species in sweet potato (Vega *et al*., 2005), *Psuedomonas putida* in coffee (Rasche *et al*., 2006) and *methylobacterium* in rice (Kneif *et al*., 2011)

Table 1: Morphological characteristics of bacteria

Bacterial Isolate	Shape	Elevation	Margin	Colour of the colony	Gram staining
B4	Round	Raised	Smooth	White	Positive
B7	Round	Convex	Smooth	Pink	Negative
B13	Round	Raised	Smooth	Yellow	Positive
B18	Round	Convex	Smooth	White	Positive
B19	Round	Raised	Smooth	Brown	Positive

The fungal isolates were morphologically characterized according to the colony surface, reverse and periphery colour. The isolates varied widely in colours and has been tabulated in Table 2 and the images were documented in plate 2. Similar results were obtained by Prabakaran *et al.*, (2011) who observed 10 species of fungi from the phylloplane of medicinal plants.

Fungal Isolate	Surface colour	Reverse colour
F2	Dark green	Yellow
F3	Grey	Yellow
F10	Yellow	Creamy
F12	Pink	Red
F16	Dark green	Brown
F25	White	Creamy

The isolated actinomycetes were tabulated in Table 3. The media were sterilized and poured into surface aseptically and incubated at 27 °C for 7 days. Morphological properties such as colony

characteristics, type of areal hyphae, growth of vegetative hyphae, fragmentation pattern and spore formation were observed in Petri dish.

Table 3: Morphological characteristics of actinomycetes

Actinomycetes Isolate	Colony texture	Appearance	Pigment	Gram reaction
A1	Powdery	Wrinkled	Pink	Positive
A3	Cottony	Umbonate	Grey	Positive
A4	Velvety	Wrinkled	White	Positive
A7	Powdery	Concentric	Green	Positive
A8	Powdery	Concentric	Creamy	Positive

Different colonies of yeast were observed and numbered as 1 to 23. All the twenty three yeast isolates were observed for the morphological characters such as colony size, colour, form, margin, elevation. The colour of the colonies were pink, white, dull white, red, light red and were mostly circular, flat, raised at the centre, (Table 4). The cell shape such as spherical and cell arrangements such as single and cluster were observed. Gram's reaction was positive for all isolates. In Plate 3 the yeast isolates were projected through Foldscope. Hejri *et al.* (2005) isolated and identified yeasts present on the surface of Pistachio leaves and fruits. Sartori *et al.* (2005) isolated endophytic yeasts and filamentous fungi from leaves, flowers and fruit of healthy apple trees Alejandro *et al.* (2007) isolated and investigated the yeast population in fresh Olives. Glushakova *et al.* (2007) isolated anamorphous ascomycete yeasts from the phyllosphere of diverse plants of Moscow.

Table 4: Morphological characteristics of yeasts

Yeast isolates	Size	Pigment	Form	Margin	Elevation
Y1	Moderate	Dark pink	Circular	Sharply defined	Dome shaped elevation
Y2	Large	White	Circular	Sharply defined	Slight elevated
Y4	Small	Light red	Circular	Sharply defined	Flat
Y6	Small	Light red	Circular	Sharply defined	Flat
Y9	Small	Dull white	Circular	Sharply defined	Flat
Y12	Small	Light red	Circular	Sharply defined	Slight elevated
Y20	Pin head	Light red	Circular	Sharply defined	Flat
Y23	Small	White	Circular	Sharply defined	Flat

Characterization of microbial isolates

Bacterial and yeast isolates were grown in different carbon sources viz, sucrose, fructose, maltose and lactose. All the isolates have grown well in all the carbon sources and some of the isolates have shown light growth in the lactose (Table 5).

Table 5: Biochemical characterization of bacterial isolates

Bacterial isolates	Sucrose	Lactose	Fructose	Maltose
B4	++ve	++ve	++ve	++ve
B7	++ve	++ve	++ve	++ve
B13	++ve	-ve	++ve	++ve
B18	++ve	++ve	++ve	++ve
B19	++ve	++ve	++ve	++ve

All the yeast isolates were also subjected to different carbon sources such as glucose, mannitol, sucrose and lactose. The results were tabulated in Table 6.

Table 6: Biochemical characterization of yeast isolates

Yeast isolates	Glucose	Mannitol	Sucrose	Lactose
Y1	++ve	++ve	++ve	++ve
Y2	++ve	++ve	++ve	++ve
Y4	++ve	++ve	++ve	++ve
Y6	++ve	++ve	++ve	+ve
Y9	++ve	++ve	++ve	++ve
Y12	++ve	++ve	++ve	++ve
Y20	++ve	++ve	++ve	++ve
Y23	++ve	++ve	++ve	++ve

Table 7: Biochemical characterization of actinomycetes isolates

Actinomycetes Isolate	Indole test	Citrate utilization test	Starch	Methyl red	Voges Prosekauer
A1	-	+	+	+	-
A3	-	+	+	+	-
A4	+	+	+	+	-
A7	+	+	+	+	-
A8	-	+	+	+	-

Phosphate solubilisation test for bacteria, fungi and yeast

Phosphate solubilisation test was done for the bacteria and fungi isolates and all the isolates were found to be positive but in yeast isolates some isolate found to be negative and some were positive and it was tabulated in Table 8. Pradhan and Sukla (2005) studied fungal strains isolated from agriculture soil, which has potential to solubilize insoluble inorganic phosphates were characterized and two fungal isolates were tested for their tricalcium phosphate (TCP) solubilisation efficiency in both solid and liquid medium. Varsha *et al.* (2007) isolated Phosphate Solubilizing Yeast (PSY) from rhizosphere, non-rhizosphere and fruits from Bhavnagar district. Interestingly, none of the yeast isolate was found to solubilise phosphorus (Swathi, 2015).

Table 8: phosphate solubilisation of microbial isolates

Bacterial isolates	**Phosphate solubilisation**	**Fungal Isolate**	**Phosphate solubilisation**	**Yeast isolates**	**Phosphate solubilisation**
B4	+ve	F2	+ve	Y1	+ve
B7	+ve	F3	+ve	Y2	-ve
B13	+ve	F10	+ve	Y4	+ve
B18	+ve	F12	+ve	Y6	-ve
B19	+ve	F16	+ve	Y9	+ve
		F25		Y12	+ve
				Y20	-ve
				Y23	+ve

Conclusion

Phyllosphere microorganisms from rice leaf were analyzed and the presence of bacteria, fungi, actinomycetes and yeast was found on the leaf surface. The population of bacteria was found to be higher compared to other organisms. Using foldscope images were captured and documented. At critical stages of rice the population of microorganisms were high. Biochemical characterization was carried out and the results have been tabulated. Screening work has to be done to proceed further.

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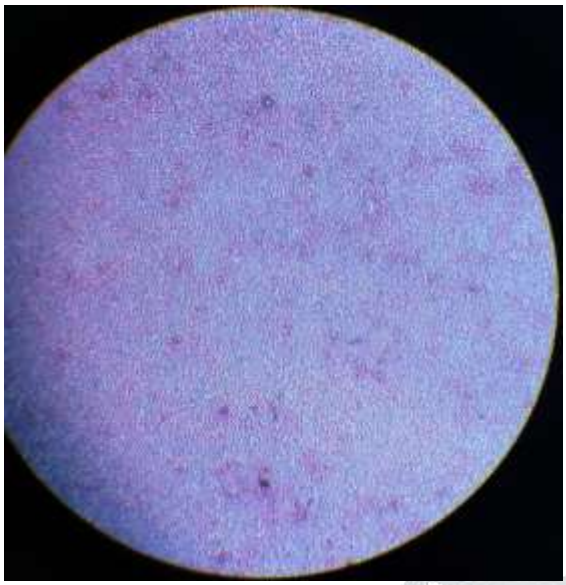
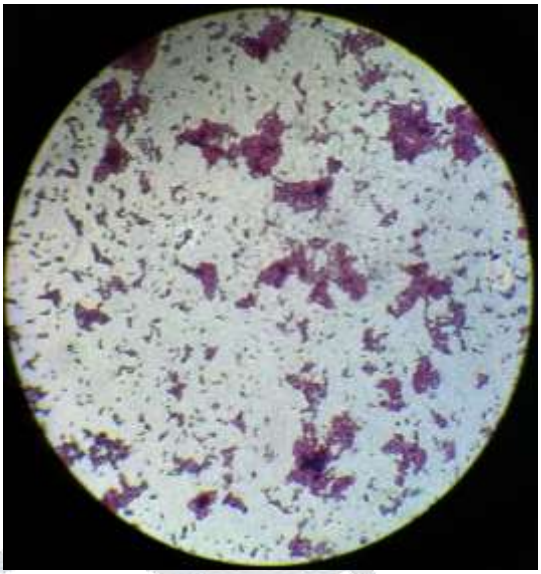
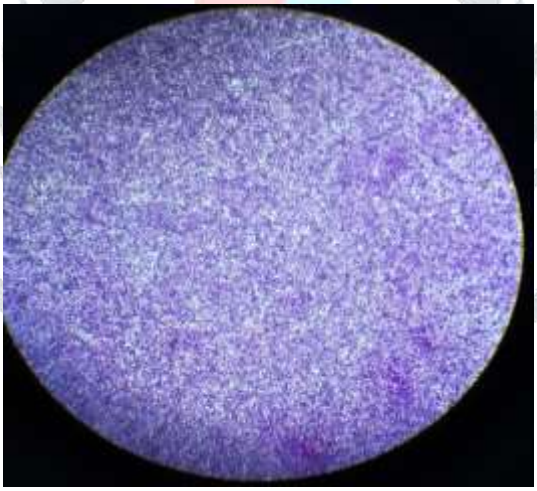
Plate 1: Bacterial isolates in the phyllosphere region observed using foldscope



Bacterial Isolate B4

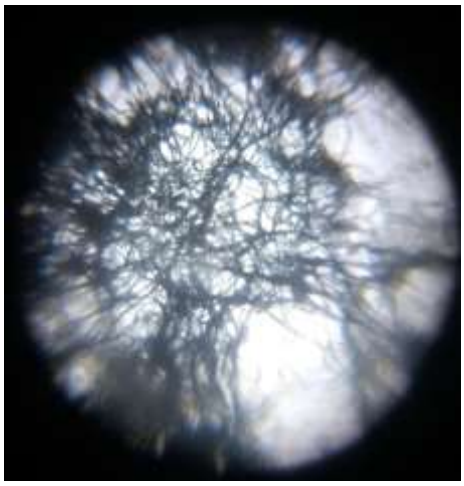


Bacterial Isolate B7



Bacterial Isolate B18

Plate 2: Fungal isolates in the phyllosphere region observed using foldscope

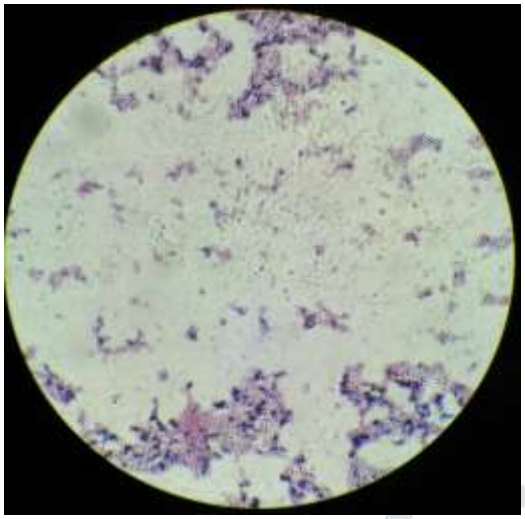


Fungal Isolate F10

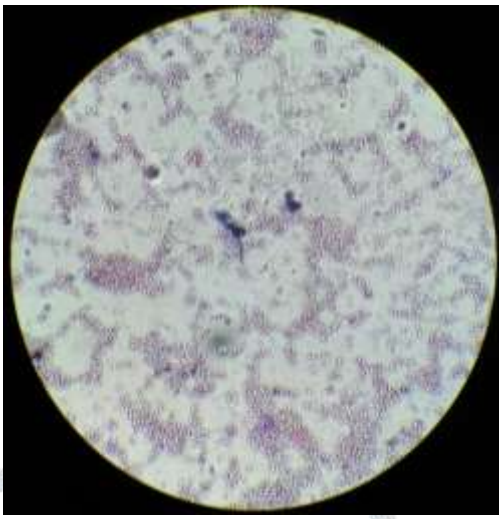
Fungal Isolate F12

Fungal Isolate F25

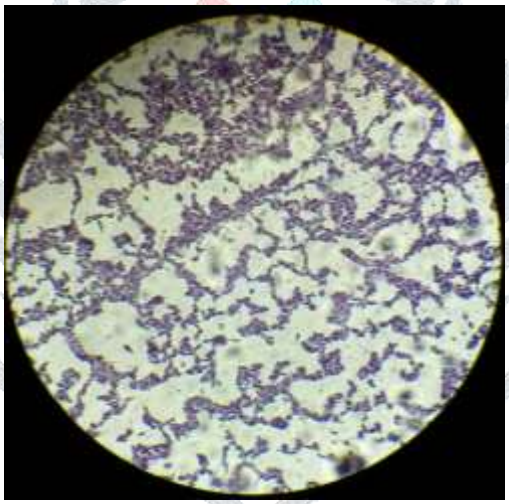
Plate 3: yeast isolates in the phyllosphere region observed using foldscope



Yeast Isolate Y 9



Yeast Isolate Y12



Yeast Isolate Y15