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ORIGINAL ARTICLE **OPEN ACCESS**

Characterization of Rhizobacteria from Saline Hydromorphic Soil of Kerala

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ABSTRACT

Rice is the major staple crop grown throughout the world. Nitrogen is the most frequent limiting nutrient for rice production. The production of rice currently depends on the large-scale use of chemical fertilizers, which poses an environmental hazard for rice producing areas. The work has start by describing the many steps that leads to PGPR which are considered as efficient biofertilizers for sustainable agriculture thereby improving crop yields. However in this case soil sample of the rhizosphere of different rice varieties from the saline hydromorphic soil rhizobacteria were isolated and identified. Rhizosphere of Kuthiru variety are named as KS1, KS2, KS3, KS4, KS5, KS6, KS7, KS8, KS9, KS10,KS11 ,KS12,KS13 and the source of Rhizosphere of Jaiva variety are noted asJS1,JS2,JS3,JS4.and Rhizosphere of Ezhome 2 is named as ES1. Whereas, the morphological studies of isolated bacterial colonies were examined by gram'sreactiontodifferentiatebacteriaGrampositiveornegativeonthebasisof standard staining protocol. The study also focuses on stress tolerances test. Effect of pH on bacterial isolates was examined by the nutrient broth having different pH (pH 2, pH 4, pH 6). The plates were observed for the presence of their growth and the salinity concentration of NaCl also shows the isolated bacterial growth. The isolates were screened for phosphate solubilization, IAA production, bio control activity such as HCN (Hydrogencyanide) production and antagonistic activity. From our study KS11 showed production of HCN andthatcould be one of the reasons for its effectiveness against different pathogen such as Rhizoctonia oryzae and Fusarium sp. The result of the study showed that many of the isolates showed plant growth promoting activity. Despite their distinct our investigations have focused on the proper management and application of these bacteria will enhance plant growth and it will be more economical and environmental friendly.

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INRODUCTION

Agriculture's global production rate is significantly below the approximated food requirement of the world's growing population, and the discrepancy will broaden over time. Environmental and climatic conditions, farming techniques, and management practices all have an impact on agro-ecosystems. Plants growing in salt-affected soils suffer from both hyperosmotic and hyperionic effects due to a higher concentration of sodium chloride (NaCl). Such constraints cause decreased water uptake, altered ion and mineral absorption rates, increased production of reactive oxygen species, causing cell membrane disorganisation, and decreased metabolic activities [1]. Halophytes adapt to saline conditions by changing their physiological activities, maintaining their water balance through osmotic adjustments, producing compatible solutes, and altering their antioxidant system [2]. Some plants cope with salinity stress by producing osmolytes, specifically glycine betaine, proline, soluble sugars, and proteins [3].

Rice (*Oryza sativa* L.) is major staple crop grown throughout the world. India has the world's largest area of 44.0 million ha under rice cultivation and is the second largest producer (106.29 million tones - 2014) after China[4]. Nitrogen is the most frequent limiting nutrient for rice production. Consequently, rice production currently depends on the large-scale use of chemical fertilizers, which pose an environmental hazard for rice producing areas. Rice cropping area are characterized by flat topography and hydromorphic soils with deficient natural drainage characteristics. These areas present constraints to agriculture and are mainly used for wetland rice cropping and livestock rearing. In India, rice is cultivated around the year across varied seasons in diverse ecologies. These ecosystems are classified into 5 majortypes: irrigated, uplands, rainfed lowlands, deep water and coastal wetlands (saline soils). Coastal wetlands are very special environments, characterized by soil permanently or seasonally saturated by salt or brakish water. They host microorganisms and plant able to adapt to anoxic conditions.

Rice farming is carried out in a peculiar way in the Kaipad, purely in a nature always relying on them on soon and these atides. Rice cultivation is practiced here during the first season in the low to medium saline phase of production cycle during June to October after the onset of monsoon showers. These areas are subjected to periodic floods in the monsoon and prevalence of high salinity during the summer season. The entry of saltwater from these a during the summer months leads to the salinization of these soil Rice is a major crop suited for the wetland ecosystems and proper emphasis has to be laid to improve and expand rice production in these unexplored new areas[5].

Salinity is one of the major abiotic stress factors limiting plant growth and productivity in arid and semi-arid regions throughout the world [6]. The soil salinity varies with the season. The traditional cultivars are the most tolerant to abiotic stresses. '*Kuthiru*','*Orkayama'*is a traditional land races widely grown in kaipad rice tracts. In recent years, Kerala agricultural university developed saline tolerant high yielding rice varieties for Kaipad ecosystem viz., *Ezhome1, Ezhome 2, Ezhome 3* and *Ezhome 4* [7].

Some wet land soils are characterized by specific problems such as salinity, high sodium content, low pH or poor physical properties following drainage. Normally the p^H of the soil is ranges from 3 to 6.8 [8]. The soils have an acidic soil reaction and high level of soluble salts. There are many reasons for hampering of plant production under salinity. Generally, salinity affects plant growth in three ways such as osmotic effects, ion imbalance, and oxidative stress [9]. The beneficial organism that promote the plant growth is referred as plant growth promoting rhizobacteria (PGPR). PGPR are known to improve plant growth in many ways when compared to synthetic fertilizers, insecticides, and pesticides. In last few decades, a large array of bacteria including species of *Pseudomonas*, A*zospirillum, Azotobacter, Klebsiella, Enterobacter, Alkaligens, Arthrobacter, Bacillus* and *Serratia,* have been isolated and reported to enhance plant growth [10]. All of them are able to exert beneficial effects on plant growth. Phosphorous is a major growth limiting nutrient, and unlikenitrogen, thereis no large atmospheric source that can be made biologically available [11]. P fertilizer throughout the world is around 10-25% and concentration of bioavailable P in soil is very low reaching the level of 1.0 mg/kg soil. [12].

Crop yield improvement in saline soils necessitates a multifaceted approach that includes salt-tolerant varieties or chemical neutralizers, but there is an urgent need for eco-friendly sustainable approaches. Plant growth-promoting rhizobacteria (PGPR) are rhizobacteria that have the potential to improve plant growth [13]. PGPR have the potential to improve plant growth through a variety of mechanisms, including improved plant growth, phytohormone production, and stress alleviation[14].

MATERIAL AND METHOD

The present study on the "Characterization of rhizobacteria from saline hydromorphic soil of Kerala."was conducted during December 2019–March 2020 at the department of Agriculture Microbiology, College of Agriculture, Padannakkad, Kasaragod, Kerala. The soil samples along with roots were collected from the rhizosphere of different rice varieties such as "*Kuthiru*", "*Ezhome 2*" and "*Jaiva*" from kaipad tract, characterized by hydromorphic soil, in Ezhome village, Kannur District of Kerala.

Isolation of Rhizobacteria was done by dilution plate technique with 10g of rhizosphere soil. The 1ml of diluted soil suspension from different dilutions were poured and sterile nutrient medium were added. (pour plate method). The plates were incubated at room temperature for 2-3 days. After incubation bacterial colonies were examined and isolated in sterile nutrient agar and the purified cultures were stored under refrigerated condition for subsequent studies. Morphological studies were done on the basis of shape, elevation, texture, margin, colour, size of colonies of bacteria grown on a specific medium with optimal temperature and time. Identified the isolated bacteria on the basis of standard staining protocol. Effect of pH on bacterial isolates were examined by the nutrient broth having different pH (pH 2, pH 4, pH 6) and the bacterial isolates were inoculated onto Nutrient agar medium supplemented with different concentration of NaCl (2.5, 5.0 and 7.5%) for Salinity tolerance test and both sets of plates were incubated at 37^0C and 28^0C accordingly . for 5 days. and bacterial growth was observed at every 24 hours.

To detect indole acetic acid production, bacterial cultures were inoculated into 10 ml of nutrient broth containing 0.1% tryptophan and Incubated at 28°C for 48 hours.with negative control. After incubation these cultures were centrifuged at 10000 rpm for 15 min. Each 1 ml of the supernatant was mixed with 2 ml of Salkowski reagent (0.5M 2% FeCl3,35%perchloricacid) an done drop of orthophosphoric acid added followed by Incubated at dark condition for 30min. Development of pink color indicates IAA production. IAA concentration was measured spectroscopically at 530nm and quantitated by using a standard curve.

To detect phosphate solubilization, the bacterial isolates were grown on Pikovskaya's medium (PVK) containing 0.5% Ca_3 (PO_4) 2 as P source. The plates were incubated at 28^0C for 7 days. The clear halozone surrounding the bacterial colony indicate phosphate solubilization. The diameter of the halo of solubilization was calculated by subtracting the colony diameter from the total diameter. The phosphate

solubilization index (PSI) can be calculated. Replicates were maintained. Statistical analysis was done by using WASP 2.0.

$$\text{Phosphate solubilization index(PSI)} = \frac{\text{Diameter of halozone} - \text{Diameter of colony}}{\text{Diameter of colony}}$$

For the estimate HCN production, bacterial isolates were streaked on nutrient agar medium amended with 4.4g/l glycine. A filter paper soaked in 2% sodium carbonate in 0.5% picric acid solution was placed over the agar. The plates were sealed and incubated at 28^0C for 1-2 days (Bakker&Schippers,1987). Development of orange to red colour indicate HCN production.HCN production potential of the antagonists was measured as Nil-positive as per the scoring based on the colour variation from neutral, brown, brownish orange and Orange. The antagonistic activity of bacterial isolates was evaluated by dual culture technique (Schoeman & Dickinson, 1999) against Rice plant pathogen such as *Rhizoctonia oryzae* and *Fusarium* sp*.* The bacterial isolates were streaked both ends of the petri plate in the potato dextrose agar (PDA) medium, 3 cm away from the edge of the plate prior to the pathogen inoculation. Then 5mm sized culture discs of pathogen were cut out from the pure culture was placed on the center of the petri plate. The plates were incubated at room temperature for 3 -7 days. Three replications were maintained for each isolate. Petri plate inoculated with pathogen alone served as control. Observation on growth of pathogen were recorded at regular interval till full growth of pathogen was attained in control plates. Percentage of inhibition of pathogen over control was calculated by adopting the formula [15], and Statistical analysis was done by using WASP2.0

$$I\,(\%) = \frac{C-T}{C} \times 100$$

I=Percentage of inhibition
C= Growth of pathogen in control
T=Growth of pathogen in treatment

RESULTS AND DISCUSSION

The rhizobacteria were obtained by dilution plate technique from the rhizosphere of three varieties of rice plant such as *Kuthiru, Ezhome 2* and *jaiva* from Kaipad rice tract. (Fig 1). From that 18 cultures were selected and were maintained in the Department of Agriculture college Padannakkad. Among 18 cultures, 13 different isolates were obtained from rhizosphere of *Kuthiru* variety (Fig2**)**, 4 isolates were obtained from rhizosphere of *Jaiva*variety (Fig3) and only one isolate was obtained from *Ezhome 2* variety(Fig 4).The selected isolates were screened for plant growth activities. Table 1and 2 shows selected bacterial isolates from different source.

Fig.1The rhizosphere samples used for study.a) *Kuthiru*,b) *Ezhome2*,c) *Jaiva*.

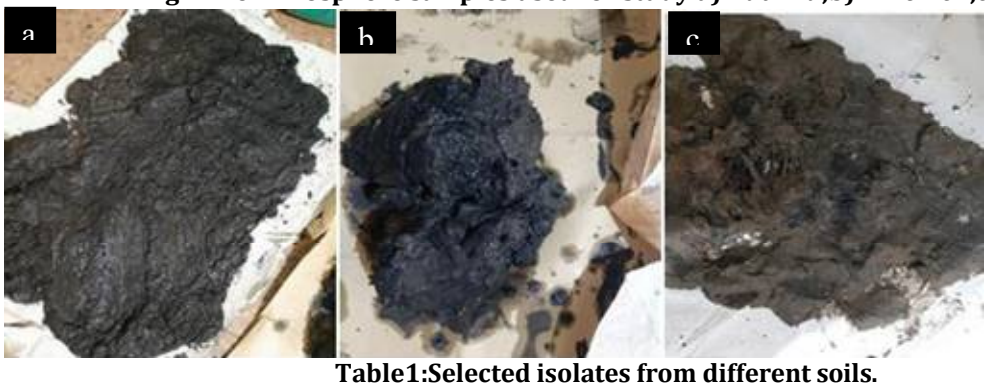

Table1:Selected isolates from different soils.

Sources	Isolates
Rhizosphere of *Kuthiru* variety	KS1, KS2, KS3, KS4, KS5, KS6, KS7, KS8, KS9, KS10, KS11, KS12, KS 13.
Rhizosphere of *jaiva* variety	JS1, JS2, JS3, JS4.
Rhizosphere of *Ezhome 2* variety	ES1.

Table2.Colony morphology and gram's reaction of selected isolates

Isolates	Colony morphology	Gram's reaction	Cell shape
KS1	Circular, translucent, flat, entire, cream colored	+	Rod
KS2	Circular, opaque, flat, entire, cream colored	–	Cocci
KS3	Circular, opaque, flat, entire, slightly orange	–	Cocci
KS4	Circular, opaque, raised, entire, yellowish	+	Rod
KS5	Circular, opaque, raised, entire, cream colored	–	Cocci
KS6	Circular, opaque, raised, entire, cream colored	–	Rod
KS7	Circular, opaque, flat, entire, cream colored	–	Rod
KS8	Circular, opaque, flat, entire, cream colored	+	Rod
KS9	Circular opaque, raised, entire, cream colored	+	Cocci
KS10	Circular opaque, flat, entire, cream colored	–	Cocci
KS11	Irregular, translucent, flat, undulate yellowish	+	Cocci
KS12	Circular, opaque, flat, entire yellowish	–	Cocci
KS13	Circular, opaque, flat, entire, cream colored	+	Cocci
JS1	Circular, opaque, flat, entire, cream colored	+	Cocci
JS2	Circular, translucent, flat, entire, cream colored	–	Rod
JS3	Circular, opaque, raised, entire, orange colored	–	Cocci
JS4	Circular, opaque, flat, entire, whitish colored	+	Rod
ES1	Circular, opaque, flat, entire, cream colored	–	Rod

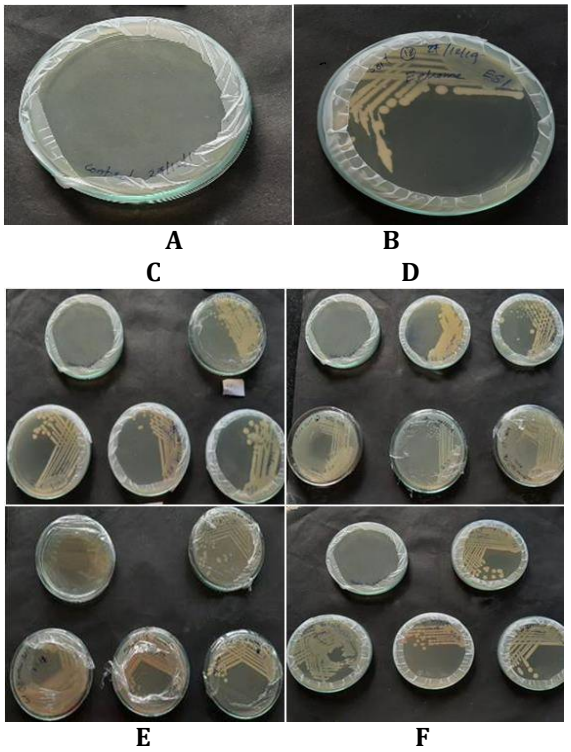

Fig 2.Bacterial isolates from different rhizosphere soils. a,b and c are isolates from *Kuthiru* rhizosphere; d) isolates from *Jaiva* rhizosphere; e) Control; f) isolate from *Ezhome 2* rhizosphere.

The bacterial growth was observed in nutrient agar plate from different pH for effect of pH on the growth of bacterial isolates. Among 18 isolates 8 isolates are acidophiles, (at low pH 2).

All isolates were grown in nutrient medium containing 2.5% NaCl. Among 18, seven isolates were grown in 5% NaCl containing nutrient agar medium. These 7 isolates weren't grown in 7.5%.Halophiles are categorized as slight, moderate, extreme halophiles, by the extent of their halotolerance. Slight halophile prefers 0.3-0.8M(1.7-4.8%)NaCl,Moderatehalophiles0.8-3.4 M (4.7-20%) NaCl, and extreme halophiles 3.4–5.1 M (20-30%) NaCl. Based on this classification, the 7 isolates are moderate halophiles.

Table4.Growth of isolates on different concentration of NaCl in nutrient agar media.

Isolates	2.5%NaCl	5% NaCl	7.5%NaCl
KS1	+++	-	-
KS2	+++	-	-
KS3	+++	-	-
KS4	+++	+	-
KS5	+++	+	-
KS6	+++	++	-
KS7	++	+	-
KS8	++	+	-
KS9	++	+	-
KS10	++	-	-
KS11	++	-	-
KS12	+++	-	-
KS13	+++	-	-
JS1	+++	-	-
JS2	++	+	-
JS3	+++	-	-
JS4	++	-	-
ES1	+++	-	-

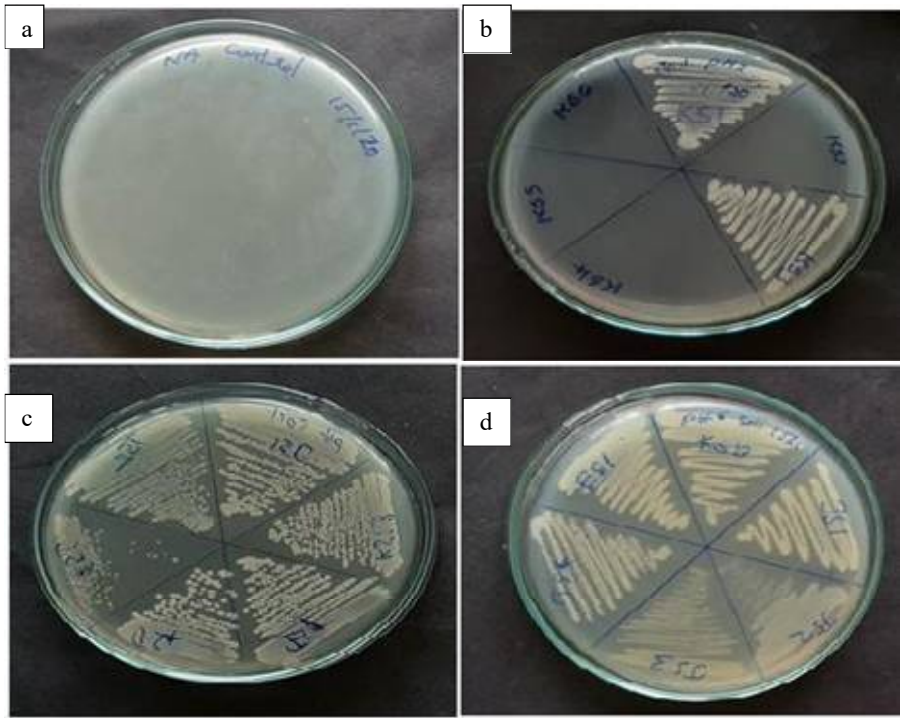

Fig 3.Colony growth of selected isolates grown on agar media.a) Control,b) isolates from pH 2 broth, c) isolates from pH 4 broth, d) isolates from pH 6.

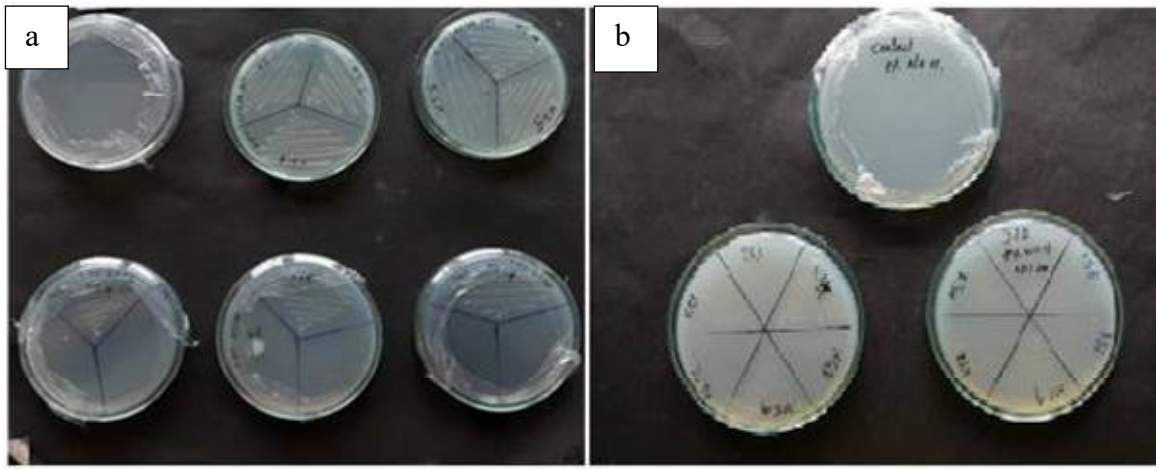

Fig 4.Growth of isolates on different concentration of NaCl.(a) growth of isolates on nutrient agar with 2.5%NaCl;(b) growth of isolates on nutrient agar with 5% NaCl

The isolates were screened for plant growth promoting activities like phosphate solubilization, IAA production, bio control activity such as HCN production, and antagonistic activity.

All the isolates were tested for indole acetic acid production in nutrient media amended with 0.1% tryptophan. From 18 bacterial isolates 7 isolates showed IAA production in the range of 1.2µg/mlto7.4µg/ml (Table5). The highest indole acetic acid production was recorded by KS4 (7.4µg/ml) and least was KS11(1.2µg/ml).The production of indole acetic acid varied with the isolates.

All the isolates were tested for Phosphate solubilization on Pikovskaya's medium. The growth of all isolates was observed after 7 days of incubation. Phosphate solubilization indicated by the formation of clear halozone around the bacterial colony in Pikovskaya's medium.Amongthe18 isolates, 11 isolates showed clear zone around the colony on Pikovskaya's medium containing insoluble mineral phosphate such as tricalcium phosphate (Fig 5) and presented in the Table 6. The highest phosphate solubilization shown by KS8 with 1.92 PSI and the lowest phosphate solubilization was KS4 with 1.0 PSI.

Table 5.Indole acetic acid production by isolates.

Isolates	Concentration of IAA (µg/ml)
KS1	0
KS2	0
KS3	0
KS4	7.45
KS5	0
KS6	1.57
KS7	0
KS8	0
KS9	2.19
KS10	0
KS11	1.22
KS12	0
KS13	0
JS1	3.50
JS2	0
JS3	0
JS4	2.19
ES1	3.76

Table 6.Phosphate solubilization index of bacterial isolates.

Isolates	Phosphate solubilization index
KS1	1.4^{cd}
KS2	1.607^{b}
KS3	0^{h}
KS4	1.087^{g}
KS5	1.163^{fg}
KS6	1.193^{fg}
KS7	1.467^{c}
KS8	1.92^{a}
KS9	1.433^{cd}
KS10	0^{h}
KS11	0^{h}
KS12	1.327^{de}
KS13	0^{h}
JS1	1.24^{ef}
JS2	1.4^{cd}
JS3	0^{h}
JS4	0^{h}
ES1	0^{h}
CV	9.182
CD (0.1)	0.173
CD (0.5)	0.129

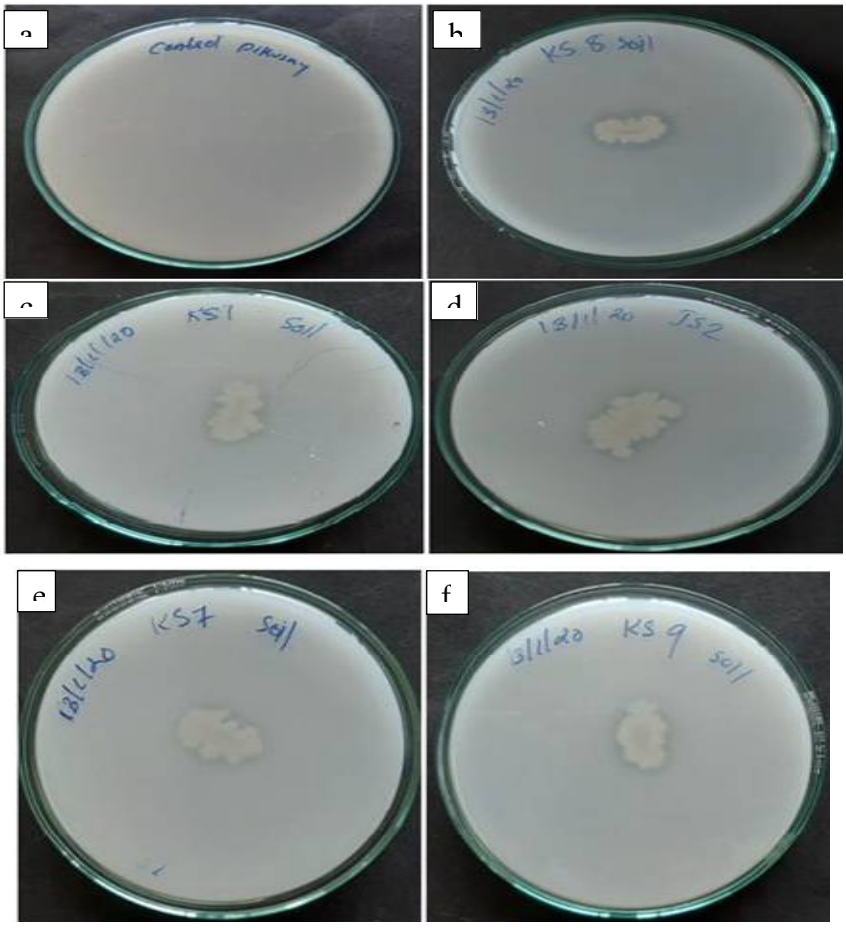

Fig 5.Phosphate solubilizing activity of bacterial isolates.a) Control,b,c,d,e and f are phosphate solubilizing isolates such as KS8, KS1, JS2, KS7, KS9 respectively.

HCN production by bacterial isolates: HCN is volatile compound produced by organism as biocontrol activity.HCN production was indicated by the development of orange to red colour. Only one isolate showed brownish to orange color. It indicates moderate production of HCN by isolates. Based on the

colour change, HCN production can be measured. Completely orange colour indicates positive. Among 18 isolates, only KS11 shows color change (Fig 6).

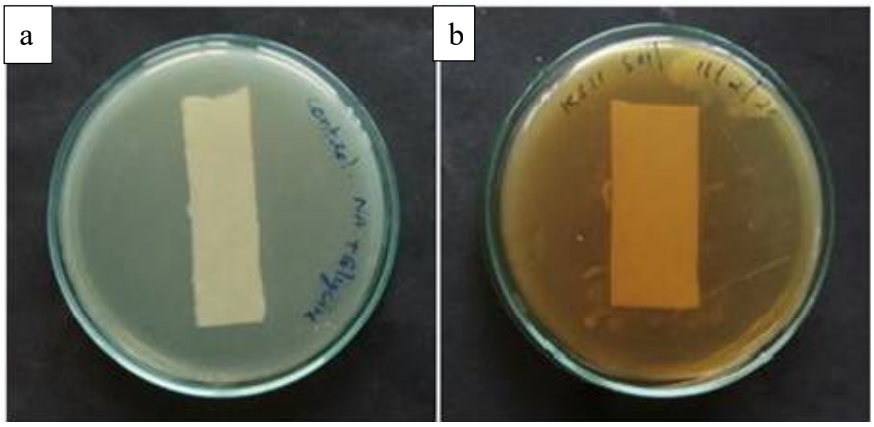

Fig 6.HCN production by isolate.a) Control plate consists filter paper soaked in picric acid, placed over the nutrient agar containing glycine, b) Brownish to orange color developed by KS11 isolate.

ANTAGONISTIC ACTIVITY OF BACTERIAL ISOLATES

Antifungal activity of bacterial isolates was tested against *Rhizoctonia oryzae*, and *Fusarium* sp. All the isolates were screened for their ability to produce antifungal substances by dual culture technique against pathogen on potato dextrose agar medium. Antifungal activity of bacterial isolates was tested against *Rhizoctonia oryzae***,** on PDA by dual culture technique. Among them 9 isolates were show antagonistic activity (Fig 7). The results were presented in Table 7 and Fig 11. The bacterial isolate KS11 displayed the highest percentage of inhibition (58.8%), whereas KS13 and JS2 displayed the lowest percentage of inhibition (39.2 and 39.6% respectively).

Antifungal activity of bacterial isolates was tested against ***Fusarium* sp,** on PDA by dual culture technique. Among them 10 isolates were show antagonistic activity (Fig 13). The results were presented in Table 8 and Fig 12. The bacterial isolate KS11 and KS5 displayed the highest percentage of inhibition (70.7% and 70.06% respectively), whereas KS7 and JS2 displayed the lowest percentage of inhibition (41.8% and 39.2% respectively). KS4, KS5, KS6 and KS11 were showed inhibition after 10 days of incubation indicate that these isolates can inhibit the pathogen on sustained manner.

Table7.Effect of isolates on *in vitro* mycelial growth of *Rhizoctonia oryzae*

Isolates	Percentage of inhibition (I)
KS1	46.25^{b}
KS2	0^{e}
KS3	0^{e}
KS4	46.25^{b}
KS5	49.2^{b}
KS6	0^{e}
KS7	45.5^{bc}
KS8	40.72^{d}
KS9	42.2^{cd}
KS10	0^{e}
KS11	58.83^{a}
KS12	0^{e}
KS13	39.2^{d}
JS1	0^{e}
JS2	39.6^{d}
JS3	0^{e}
JS4	0^{e}
ES1	0^{e}
CV	10.227

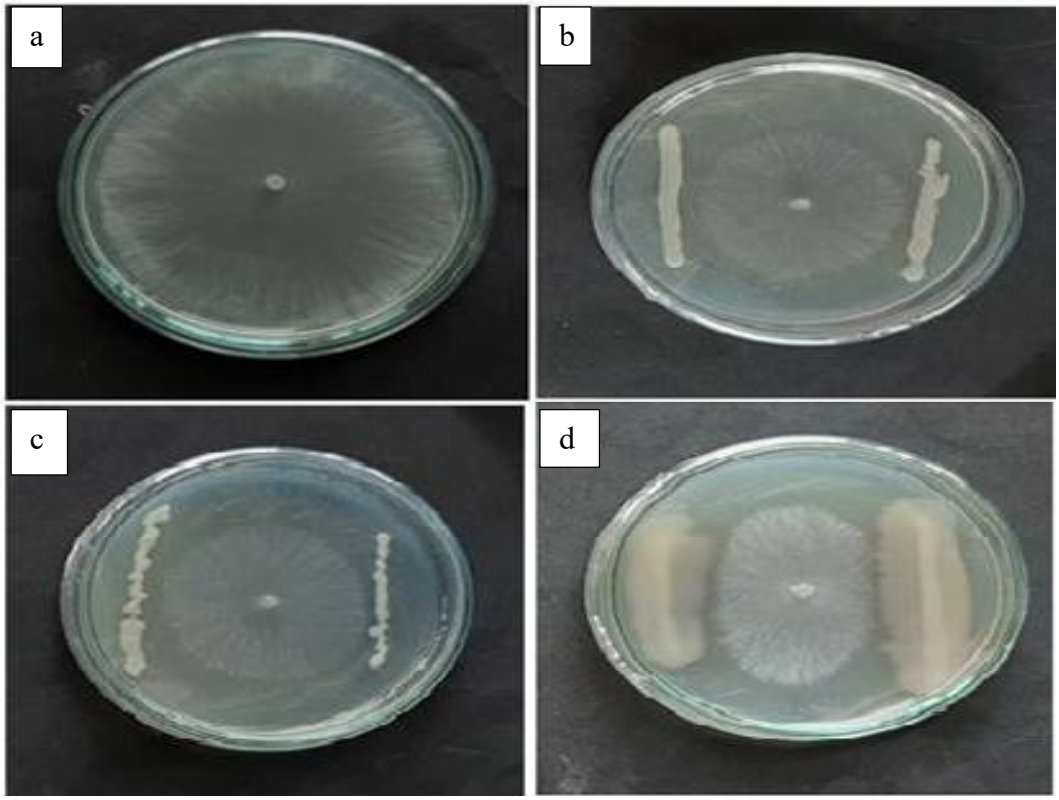

Fig10. Antagonistic effect of bacterial isolates against *Rhizoctonia oryzae.In vitro* dual culture technique. a) Control, b) KS7, c) KS1, d) KS11.

Table 8.Effect of isolates on *in vitro* mycelia growth of *Fusarium* sp.

Isolates	Percentage of inhibition (I)	
	After 7 days incubation	After 10 days incubation
KS1	42.93^{e}	0^{e}
KS2	0^{g}	0^{e}
KS3	0^{g}	0^{e}
KS4	53.3^{c}	39.2^{d}
KS5	70.06^{a}	68.46^{a}
KS6	57.73^{b}	45.5^{c}
KS7	41.8^{ef}	0^{e}
KS8	42.2^{e}	0^{e}
KS9	43.3^{e}	0^{e}
KS10	46.23^{d}	0^{e}
KS11	70.7^{a}	65.5^{b}
KS12	0^{g}	0^{e}
KS13	0^{g}	0^{e}
JS1	0^{g}	0^{e}
JS2	39.23^{f}	0^{e}4
JS3	0^{g}	0^{e}
JS4	0^{g}	0^{e}
ES1	0^{g}	0^{e}
CV	6.240	10.698
CD (0.1)	3.906	2.88
CD (0.5)	2.913	2.152

CONCLUSION

The present study aimed to characterize rhizobacteria from saline hydromorphic soils. Bacteria were isolated from different rhizosphere soil of *Kuthiru, Jaiva*, and *Ezhome 2* rice varieties from Kaipad tract. A

total of 18 isolates were selected for screening of plant growth promotion. Some isolates were found to be acidophilic and halophilic in nature. Among 18 cultures, KS4 showed phosphate solubilization, IAA production, antagonistic activity and it was halophilic in nature. KS4 produced high amount of indole acetic acid than other isolates. IAA is a phytohormone and considered as native auxin. The property of producing indole acetic acid is considered as an effective tool for screening beneficial microorganisms as there have been report suggesting that IAA producing bacteria having beneficial effect on plant growth.
KS11 is an effective antagonistic bacterium against pathogen such as *Rhizoctonia oryzae* and *Fusarium* sp. It may be due to the production of antimicrobial substances such as chitinolytic enzymes, HCN production, antibiotics , siderophore and nutrient competition. KS11 displayed the highest percentage of inhibition. From our study KS11 showed production of HCN and that could be one of the reasons for its effectiveness against different pathogen such as *Rhizoctonia oryzae* and *Fusarium* sp.
The result of the study showed that many of the isolates showed plant growth promoting activity. Among these KS4 and KS11 were shown to be more promising in terms of IAA production and antagonistic activity.KS4 and KS11 are plant growth promoting rhizobacteria.The proper management and application of these bacteria will enhance plant growth and it will be more economical and environmental friendly.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest

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