Exploration of The Biocontrol Potential of Halophilic Bacteria Against Sclerotium Oryzae and Rhizoctonia Solani

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Abstract

There is a pressing need for biocontrol agents to control soil borne plant pathogens through biocontrol agents. The focus of this research was on the interactions of halophilic bacterial isolates with the phytopathogenic fungi such as *Sclerotium oryzae* and *R. solanii*. The biocontrol ability of fifty bacterial strains isolated from saline soils of the Koppal (Gangavathi) and Raichur districts was assessed *in vitro*. Eight isolates, HB-10, HB-17, HB-28, HB-30, HB-39, HB-48, HB-49 and HB-50 showed strong antifungal activity against *S. oryzae* and *R. solani* in dual culture method. These prospective isolates were identified as *Bacillus cereus* (HB-48 and HB-49), *Bacillus albus* (HB-17), *Bacillus safensis* (HB-28), *Staphylococcus xylosus* (HB-39), *Lysinibacillus sphaericus* (HB-50) and *Pseudomonas stutzeri* (HB-10 and HB-30) based on 16S rRNA analysis. We found a link between isolates producing HCN, siderophore and hydrolytic enzymes and the biocontrol function of isolates. The isolate *B. albus* (HB-17), produced both catechol and hydroxamate type of siderophores, confirmed in Arrow's and Csaky's tests. All isolates were tested for drug resistance and were shown to be immune to penicillin, ofloxacin and vancomycin. In addition, these isolates were investigated at three NaCl concentrations (3, 6 and 10 % w/v) for their plant growth promoting attributes *viz.*, phosphorous, and zinc solubilization and potassium release. The solubilization zones of zinc carbonate, zinc oxide and phosphorous were within the range of 5.20 to 9.0 mm, 6.06 to 13.20 mm, 6.30 to 9.70 mm respectively, at 6 % NaCl concentration.

Introduction

Rice (*Oryza sativa*), an important staple crop in India, inclined to several biotic and abiotic stresses (Richa et al. 2016; Maclean et al. 2002). As per the recent reports, annual global yield losses caused by biotic and abiotic stress are about 5 % and 20 to 30 % respectively (Savary et al. 2019; Song and Goodman, 2001). Abiotic stresses like excessive or insufficient water, high salinity, heavy metals including Ultra Violet (UV) rays impinges life cycle of the plant. Plants manage them through cuticular alterations, unsaturated fatty acids synthesis, membrane modulators, oxylipin proteins, reactive species scavengers, molecular chaperones and compatible solutes (Fich et al. 2016). Rice is susceptible to more than seventy diseases caused by fungi, bacteria, viruses, and nematodes (Manandhar et al. 1998), among those blast and sheath blight are considered as most destructive (Wopereis et al. 2009). Interestingly, minor diseases like stem rot (*Sclerotium oryzae*) and sheath blight (*Rhizoctonia solani*) have been forecasted as major diseases for the near future (Cothier and Nicol 1999). Rice sheath blight is considered a destructive disease due to annual yield losses of between 5.9-69 % of the total annual yield of the crop (VenkatRao et al. 1990; Tan et al. 2011; Yellareddygar et al. 2014; Dodds and Rathjen 2010). *R. solani* also infects wheat, soybean, sugar beet, barley, corn, canola and potato (Hane et al. 2014). Regardless of the extreme damage caused by *R. solani* and *S. oryzae*, conventional breeding had limited efficacy in introducing genetic resistance to *R. solani* and *S. oryzae* in any crop species (Liu, 2017). Developing the cultivars with resistant genes for sheath blight and stem rot diseases has not been very encouraging due to the lack of high levels of resistance in the available rice germplasm, polygenic nature of the trait of tolerance as well as several unknown complexities associated with resistance phenotype (Bhaskarrao et al. 2019). Currently, non-ecofriendly methods like fungicide sprays and straw burning are being implemented to manage these diseases (Baskarrao et al. 2019; Jackson et al. 1977). Biocontrol agents are environmentally friendly and can effectively control soil borne pathogens, but, identifying the right strain has now been challenging.

Biocontrol agents occupy the pathogen's niche either by natural means or through host environmental control, and can suppress or eliminate most soil borne diseases. Several rhizosphere associated microbes *viz.*, strains of *Bacillus spp.*, *Pseudomonas spp.* and *Trichoderma spp.*, were emerged as biocontrol agents due to their diversity, mode of action, ability to inhabit diverse niches in agro-ecosystems, and capacity to survive in several environmental conditions (Choudhary and Johri 2009; Zhao et al. 2018). However, they have reduced performance at higher salt levels due to their explicable salt sensitivity. Conversely, salt tolerant biocontrol agents have not been explored so far. Halophiles are currently explored for the production of active and stable pigments, enzymes, lipids, and compatible solutes at extreme conditions (used in pharmaceuticals, laundry, food coloring, and as biosurfactants) (Tango and Islam 2002). Several researchers have reported that rhizosphere halophilic microorganisms are rich sources of novel hydrolytic enzymes capable of breaking the cell walls of fungi (Oren 2010). Hypersaline habitats are ubiquitous and can be found in saline lakes, salterns, and saline and hypersaline soils (Oren 2002a, b). In the current investigation, we focused on identifying the potential biocontrol agents from the saline soils of Ganagavathi, the rice bowl of Karnataka.

Material And Methods

Soil sample collection

Composite soil samples collected from Cotton (15.456571, 76.531624), Rice (15.455944, 76.531503) and Red gram (15.456488, 76.530143) crops at a depth of 10 to 12 inches from salinity affected areas of Ganagavathi, Karnataka, India; these were kept in sterile polythene bags at 4 °C until further use. The pH of the soil samples ranged from 8.12 to 9.46 and the electric conductivity from 0.6 to 6.75 dSm⁻¹.

Isolation of bacteria and selection of halophilic nature

Isolation was done on nutrient agar media amended with cycloheximide (50 mg L⁻¹). Concisely, one gram of each of the soil sample was taken into nine ml of sterilized 0.85 % saline blank, then serially diluted up to 10⁻⁷. Then, from the higher dilution (10⁻⁷), 100 µL (0.1 ml) of uniform suspension was used for spreading on nutrient agar plates containing 3 %, 5 % (w/v), and 10 % (w/v) sodium chloride (NaCl), and the plates were incubated in BOD incubator at 28 ± 2 °C for 72 hrs. Lastly, individual colonies observed on 10 % NaCl supplemented plates were sub-cultured with Nutrient Agar. All the isolates were stored at 4 °C until further use.

Physiological characterization of halophilic bacterial isolates

Biochemical tests were performed to identify and confirm the genus and species of selected strains according to the procedure Cappuccino and Sherman, (2013). Pure cultures of isolates were studied for the colony morphology and pigmentation. The cell shape and gram response were also recorded as per the standard procedures given by Barthalomew and Mittewar (1950). Morphological characteristics of each isolate were examined on Nutrient agar supplemented

with 3 % NaCl. Cultural characteristics such as shape, elevation, surface, margin, color, pigmentation, *etc.*, were explicitly recorded. The biochemical tests like hydrogen sulfide production test, indole production test, catalase test, oxidase test, Voges Prausker's test, methyl red test, citrate utilization test, and nitrate reduction test were performed (Shirling and Gottlieb, 1966). The sugar utilization characteristics halophilic bacteria were determined using carbohydrate fermentation test with different carbohydrates *viz.*, Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose, and Sucrose (Coleman et al. 1930).

Extracellular enzyme production

The antagonistic halophilic bacteria were screened qualitatively for the production of three important enzymes namely, chitinase, amylase and gelatinase. The chitinase enzyme assay was performed on SM medium consists of (g L⁻¹): Na₂HPO₄-6; KH₂PO₄- 3; NH₄Cl-1; NaCl-0.5; yeast extract-0.05; agar-15 and colloidal chitin 1 % (w/v) as sole carbon source. Colloidal chitin was prepared by dissolving 20 g of chitin powder (Himedia Ltd.) in 200 ml of concentrated HCl and kept at 30 °C for 60 min with vigorous stirring. This solution was slowly added to 2 L of water at 4-10 °C to form precipitation. Further, this solution was diluted with 5 L of Distilled Water (DW) and suspension was collected by filtration through suction on a coarse filter paper, which was then washed 3 times until the pH of the suspension was about 3.5. After the above treatment, loose colloidal chitin was used as a substrate in the medium as recommended by Hsu and Lockwood (1975). For further use the colloidal chitin was autoclaved at 121 °C for 15 min and stored under refrigerated conditions. The pure cultures of halophilic bacteria were inoculated on SM medium and incubated at 37 °C in the BOD incubator for 5 days (Thamthiankul et al. 2001). The colonies showing clear zones on a creamish background were considered chitinase-positive. Standard methods were adopted for the assay of amylase and gelatinase (Cappuccino and Sherman, 2013).

Antifungal activity of halophilic bacteria

A total of fifty halophilic bacterial isolates were evaluated for their antifungal activity towards plant pathogenic fungi *Sclerotium oryzae* and *Rhizoctonia solani* by dual culture method. The pathogens *Sclerotium oryzae* and *Rhizoctonia solani* used in this study was isolated from infected rice plants according to Gopika et al. (2017) and were maintained as pure cultures in refrigerator at 4 °C. For the test, a loopful of bacterial culture was streaked on the potato dextrose agar plate at one end (1 cm away), which was pre-inoculated with a 3 day old (5 mm mycelial disc) test pathogen at the other end. The pathogen without biocontrol agent served as control. The assay plates were incubated at 28 ± 2 °C for 5 more days and observations were made on inhibition of mycelial growth of the test pathogens (Skidmore and Dickson 1976). After incubation, the zone of inhibition and colony growth was measured and the percent inhibition of pathogen was calculated based on the below formulae

$$\text{Percent Inhibition (PI)} = \frac{\text{Growth of pathogen in control (mm)} - \text{the growth of the pathogen in treatment (mm)}}{\text{Growth of pathogen in control (mm)}} \times 100$$

Plant growth promoting attributes of halophilic bacteria

Halophilic bacterial isolates were assayed for tricalcium phosphate solubilization test, zinc solubilization test, potassium releasing test, siderophore production test, IAA production test and HCN production test. For determining phosphate solubilization, isolates were inoculated on Pikovskaya agar medium supplemented with 3, 6 and 10 % NaCl at 37 °C for 3-12 days for determining halo zone near colony (Pikovskaya, 1948). The zinc solubilization test was performed on TRIS minimal media supplemented with 3, 6, and 10 % NaCl, and inoculated plates were incubated at 37 °C for 3-12 days. Later, the halo zone around the colony was recorded (Simine et al., 1998). The potassium releasing test was performed on Aleksandrov medium supplemented with 3, 6 and 10 % NaCl. The halophilic bacteria were inoculated on the Aleksandrov medium containing potassium alumino silicate and incubated for 3-12 days at 28 ± 2 °C. This test was performed under three different levels of salt concentrations *viz.*, 3, 6, and 10 %.

For siderophore assay, Chromo Azurol S method (Schwyn and Neilands, 1987) was adopted due to its high sensitivity and reliability. Cells were harvested from the broth using centrifugation (3000 ×g, about 10 to 30 min), after careful removal of supernatant the pellet was immediately stored at -20 °C until further use. Cell pellets were diluted using 100 µl of double distil water, from this about 10 µl of culture was inoculated on CAS media and plates were incubated at 37 °C for 24 hrs in the BOD incubator. Observations were made after three days of inoculation with changes in medium color from blue to reddish yellow to determine siderophore production. Siderophore quantitative assay was carried out based on the CAS shuttle assay given by Payne (1994). The culture extract (0.5 ml) was mixed with 0.5 ml of CAS reagent. The color obtained was measured using the spectrophotometer at 630 nm after 20 min of incubation. The blanks were prepared using an uninoculated broth medium. Siderophore content in the extract was calculated using the following formula:

$$\% \text{ of Siderophore unit} = \frac{As-Ar}{As} \times 100$$

Where,

As = Absorbance of the sample at 630 nm (CAS reagent) and

Ar = Absorbance of the reference

Arnow's and the Csaky's methods (for catecholates and hydroxamates respectively), were employed to identify the type of siderophore produced by halophilic bacteria (Payne, 1994). In Arnow method, yellow color changes to orange red color under alkaline conditions (Arnow, 1937). The Csaky's test, presence of deep pink color confirms the presence of hydroximates (Csaky 1948; Martins et al., 2018). As blank, 1.0 mL of deionized water used in both assays.

For IAA production test, fresh cultures of 24 hrs old were inoculated into starch casein broth with 1 µg ml⁻¹ of L-tryptophan and grown for 5 days at 28 ± 2 °C in BOD incubator. Later, the broth was centrifuged at 10000 rpm for 8-10 min, and the supernatant was collected separately and allowed to react with 1 ml of

Salkowski reagent for 30 min. Lastly, development of pink to red color was considered as IAA positive. For the HCN test, the isolates were grown on Bennet agar amended with 3 % NaCl (w/v). A disc of Whatman filter paper No.1 of the diameter equal to the Petri plate size (resized in case of larger than Petri plate), impregnated with alkaline picric acid solution (0.5 % picric acid (w/v) in 1 % sodium carbonate) and placed underneath the petri plate under aseptic conditions. The control plate did not receive the inoculum. The plates were incubated-upside down at $28 \pm 2^{\circ}\text{C}$ for 48-72 hrs. A change in color from yellow to light brown, moderate or strong reddish brown was taken as an indication of HCN production (Castric and Castric 1983).

Genetic characterization of halophilic bacterial isolates

Genomic DNA extraction from bacterial cultures were carried out as per the protocol described by Sambrook et al. (1989). DNA purification was checked based on A260/A280 ratio, samples with 1.8 or above values were used for sequencing. Pure DNA samples were amplified in the PCR using universal primer pair 27F (AGAGTTTGA TCCTGGCTCAG) and 1492R (CGGTTACCTTGTTAC GACTT). Sequence of amplified DNA fragments (16S rRNA) was determined using the Sanger method. The resulting sequences were evaluated by compared with the submitted sequences in NCBI databases. Clustal W program was used to align the multiple sequences and generated phylogenetic tree using neighbour hood joining method.

In vitro antibiotic susceptibility test

Mueller-Hinton agar was evenly seeded throughout the plate with 1 ml of pure culture that was diluted at a standard concentration (approximately, 1 to 2×10^8 cfu ml⁻¹). Commercially available octo-disk containing Cephalothin (CEP-30 mcg), Clindamycin (CD-2 mcg), Co-Trimoxazole (COT-25 mcg), Erythromycin (E-15 mcg), Gentamicin (Gen-10 mcg), Ofloxacin (OF-1 mcg), Penicillin-G (P-10 units), and Vancomycin (VA-30 mcg) (source: Hi-media) pre-impregnated with a standard concentration of a particular antibiotic, then evenly dispensed and lightly pressed onto the agar surface. The test antibiotic immediately begins to diffuse outward from the disks, creating a gradient of antibiotic concentration in the agar such that the highest concentration can be found closest to the disk while the lowest concentration is found further away from the disk. After overnight incubation, bacterial growth around each disc was observed. If the test isolate is susceptible to a particular antibiotic, a clear area of "no growth" may be observed around that particular disk. The zone around the antibiotic disk showing no growth is referred to as the zone of inhibition since this approximates the minimum antibiotic concentration sufficient to prevent the growth of the test isolate. This zone is then measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediately susceptible, or resistant (Franco et al. 2018).

Results

Isolation of halophilic bacteria

In this study, fifty halophilic bacterial isolates were isolated from the saline soils. They have salt tolerance range of 1- 17 % NaCl, and classified them as halotolerant (1-6 % NaCl w/v) and halophilic (6-15 % NaCl w/v) and extremophiles (15-30 % NaCl w/v) based on growth in nutrient broth supplemented with different NaCl concentrations. Out of these fifty bacterial isolates, forty-one isolates classified as halotolerant and nine as halophilic based on *in vitro* assay.

Physiological properties of halophilic bacterial isolates

In this study, six morphological types of bacteria were observed under compound microscope (Olympus) at 1000 x magnification *viz.*, rods, small rods, long rods, coccus, streptococcus, and staphylococcus. In gram staining, twenty eight isolates were gram positive and twenty two were gram negative. The pigmentation in halophilic bacteria varied from creamy white to milky white, red and yellow (Figure 1). Based on growth pattern in the broth, the isolates were classified as aerobic, facultative anaerobe and obligate anaerobes for pellicle formatting, throughout and bottom growing isolates. In biochemical tests, the halophilic bacterial isolates showed diversified results and displayed in Table 1. Thirty-six isolates showed positive for nitrate reduction test, fourteen isolates were positive for MR (Methylene Red) test, ten isolates positive for indole production, eleven isolates positive for citrate utilization test, thirty-seven isolates positive for starch hydrolysis test, ten isolates positive for H₂S production test, all the isolates positive for the cytochrome c oxidase and catalase tests. In carbohydrate utilization test, forty-one isolates positive for lactose test, thirty-three isolates positive for mannitol, ten isolates positive for rhamnose, thirty isolates positive for sucrose, twenty-seven isolates positive for sorbitol, thirty-nine isolates positive for arabinose, sixteen isolates positive for adonitol and glucose utilizing bacteria from saline soils (Table 2).

Production of extracellular enzymes

The chitinase activity was limited to few natural isolates; in the present investigation, a total of fourteen halophilic bacteria found with chitinase activity. Based on colloidal chitin degradation, *Bacillus sp.* (HB-25) was recognized as best solubilizer with halo zone of 12.1 mm in plate, followed by *Staphylococcus sp.* with 9.33 mm and *Pseudomonas stutzeri* (HB-30) with 3 mm solubilization zone. In gelatin liquefaction test, forty-four isolates liquefied the gelatin, thirty seven isolates positive for amylase production.

Selection of antagonistic halophilic bacterial isolates

The antifungal activity of halophilic bacteria was examined on potato dextrose agar using dual culture method. Out of the fifty halophilic bacterial isolates screened for their antifungal activity against *Sclerotium oryzae* ten isolates inhibited the mycelial growth (Figure 2), forming a clear halo zone. The isolate, *Pseudomonas stutzeri* (HB-10) inhibited the *S. oryzae* up to 34.4 %, and *Staphylococcus xylosus* (HB-39) up to 33.33 %. Similarly, the radial growth of *Rhizoctonia solani*, inhibited by eight halophilic bacterial isolates under *in vitro* conditions. The halophilic bacterial isolate, *Bacillus cereus* HB-48 inhibited the radial growth of *R. solani* up to 38.9 % (Figure 3) and *Lysinibacillus sphaericus* (HB-50) up to 33.3 %. In this study, radial growth of *R. solani* inhibited more than *S. oryzae*. Using this approach, eight isolates were selected for further study based on criteria such as biocontrol activity and extracellular enzyme activity.

Plant growth promoting attributes

The ability of halophilic bacteria to solubilize and release phosphorous, zinc and potassium at different concentrations of NaCl (3, 6 and 10 % w/v) was studied in greater detail based on plate assay. In potassium releasing test, out of eight halophilic bacterial isolates selected, two isolates namely, *P. stutzeri* (HB-10) and *Bacillus cereus* (HB-48) showed halo zones at 6 and 10 % NaCl (w/v). Clear halo zones gradually increased over a period in all the isolates. Insoluble tri-calcium phosphate solubilized by five isolates, viz., *P. stutzeri* (HB-10), *B. albus* (HB-17), *B. safensis* (HB-28), *P. stutzeri* (HB-30), *L. sphaericus* (HB-50) at 3, 6, and 10 % salt concentration. The isolates showing solubilization zones > 5 mm were classified as halophilic phosphate solubilizing bacteria. *P. stutzeri* (HB-30) demonstrated the highest solubilization at 3 % salt (11.20 mm), while three halophilic bacteria had no sign of solubilization. In the presence of 6 and 10 % NaCl (w/v), the phosphate solubilization was much lower.

The zinc solubilization ability of halophilic bacterial isolates was evaluated at different salt concentrations (3, 6 and 10 % w/v) based on the diameter of halo zone. Zinc solubilization varied with the isolate and salt concentration. Among all, four isolates showed positive for zinc carbonate solubilization and six isolates positive for zinc oxide solubilization. Maximum zone of 10.03 and 13.20 mm was observed for *Pseudomonas stutzeri* (HB-30) in ZnCO_3 and ZnO amended medium respectively (Table 3). The zone of solubilization was competitively high in ZnO amended medium as compared to ZnCO_3 at 3 and 6 % NaCl (w/v) concentrations. The zone of solubilization increased up to 6 % NaCl thereafter decreased. The halophilic bacterial isolates which showed positive for zinc, phosphorous and potassium solubilization were subjected to inoculation of bromothymol blue near solubilization zones for evaluation of their effect on pH. Decreased in pH was evaluated based on the changes in color from blue to yellow near halo zones, all the positive isolates showed yellow color formation.

Siderophores production was evaluated on CAS agar medium and the type of siderophores were identified based on Arnow's and Csaky's tests. Strain HB-17 had a positive result for both Arnow's and Csaky's test and showed highest siderophore producing capacity with a siderophore unit of 49.34 %. The three isolates (HB-28, HB-30 and HB-39) studied had a strong reaction to Csaky's test, which suggest that they produce hydroxamate type of siderophores. Isolates showed varied yellow halo zone around the colony (Figure 4). Hydrogen cyanide (HCN) was produced by *Pseudomonas stutzeri* (HB-10), *Bacillus safensis* (HB-28) and *Bacillus cereus* (HB-48). Isolates were also checked with respect to their IAA production qualitatively and quantitatively. Among eight isolates, six isolates positive for IAA in quantities ranging from 27.56-58.76 $\mu\text{g ml}^{-1}$. Strains HB-30 and HB-49 had the highest IAA production ability, yielding 58.76 and 46.83 $\mu\text{g ml}^{-1}$ respectively.

Molecular characterization

The 16S rRNA sequence analysis was performed to eight isolates for elucidating the taxonomic position of isolated prospective halophilic bacterial strains. The strain HB-10 and HB-30 highly homologous (99.9 %) to *P. stutzeri*, two isolates were closely related to *Bacillus cereus* (HB-48 and HB-49), one isolate similar to *Bacillus albus* (HB-17), one isolate displayed a highest similarity (99.9 %) with *Bacillus safensis* (HB-28), one isolate had a close similarity with *Staphylococcus xylosus* (HB-39), and one to *Lysinibacillus sphaericus* (HB-50). The resulted sequences were also deposited in Gen Bank with the accession numbers MN098847.1, MN098870.1, MN122435.1, MN098871.1, and MN121550.1 and remaining three isolates are under process of getting the accession numbers.

Screening of halophilic bacteria for their antibiotic susceptibility

In vitro evaluation of antibiotic susceptibility using different antibiotics viz., Cephalothin (CEP-30 mcg), Clindamycin (CD-2 mcg), Cotrimoxazole (COT-25 mcg), Erythromycin (E-15 mcg), Gentamicin (Gen-10 mcg), Ofloxacin (OF-1 mcg), Penicillin-G (P-10 units), and Vancomycin (VA-30 mcg) showed varied responses. Isolates responded very differently to the Cephalothin (30 mcg), which is a β -lactam group antibiotic active against gram-positive and some gram-negative bacteria. The response of the isolates was recorded as viz., *Pseudomonas sp.* (Gram negative), *Pseudomonas sp.* HB-11 (Gram negative), *Pseudomonas sp.* HB-12 (Gram negative), and *Bacillus sp.* HB-20 (Gram positive) were completely inhibited whereas some isolates namely, *Bacillus sp.* HB-8 (Gram positive), *Pseudomonas sp.* HB-13 (Gram negative), *Staphylococcus sp.* HB-14 (Gram positive), *Pseudomonas sp.* HB-35 (Gram negative), *Bacillus sp.* HB-36 (Gram positive), and *Lysinibacillus sphaericus* HB-50 (Gram positive) were completely resistant.

Clindamycin (2 mcg) belong to lincosamide group interferes with protein synthesis by binding with 50S part of ribosome. It is mostly active against anaerobes, gram positive cocci and less effective against enterobacteriaceae family members. Clindamycin showed complete inhibition of *Bacillus sp.* HB-6 (Gram positive, rod), *Pseudomonas sp.* (Gram negative, rod), *Pseudomonas sp.* HB-12 (Gram negative, cocci), and *Bacillus sp.* HB-20 (Gram positive, rod) isolates and some isolates showed complete resistance viz., *Pseudomonas sp.* HB-13 (Gram negative, rod), *Bacillus sp.* HB-41 (Gram negative, rod), and *Neisseria sp.* HB-43 (Gram negative, coccus). Rest of the isolates showed intermediate to low susceptibility for clindamycin. Cotrimoxazole belongs to sulfonamide group, contains two components sulfamethoxazole and trimethoprim. In combination it acts as a bactericidal, sulfamethoxazole inhibits the formation of dihydrofolic acid and trimethoprim inhibits the dihydrofolate reductase. In the present investigation, cotrimoxazole showed complete inhibition of some isolates viz., *Pseudomonas stutzeri* (HB-10), *Bacillus sp.* (HB-20), and *Bacillus sp.* (HB-23), rest of all isolates have low to moderate susceptibility. Isolates, *Pseudomonas stutzeri* (HB-10), *Bacillus sp.* (HB-20) and *Bacillus cereus* (HB-48) were completely susceptible to the Erythromycin, whereas remaining isolates showed moderate susceptibility. Few isolates viz., *Pseudomonas stutzeri* (HB-10), *Bacillus sp.* (HB-20), and *Pseudomonas sp.* (HB-26) showed complete susceptibility to the Gentamicin, a broad spectrum antibiotic. Conversely, a maximum number of isolates showed susceptibility to the Ofloxacin viz., HB-6, *Pseudomonas stutzeri* (HB-10), *Staphylococcus sp.* (HB-15), *Bacillus sp.* (HB-20), *Bacillus sp.* (HB-21), *Pseudomonas sp.* (HB-26), *Moraxella sp.* (HB-27), *Pseudomonas sp.* (HB-35), *Bacillus sp.* (HB-37), *Staphylococcus sp.* (HB-38), and *Staphylococcus xylosus* (HB-39). Almost all the isolates were moderate to highly susceptible to ofloxacin. In contrary to the above results most of the isolates were resistant to Penicillin except few isolates namely, *Pseudomonas sp.* (HB-7), *Pseudomonas stutzeri* (HB-10), *Bacillus sp.* (HB-20), *Clostridium sp.* (HB-34), and *Lysinibacillus sphaericus* (HB-50). Vancomycin has less effect on

bacteria which is evident from the appraised data. Isolates, *Pseudomonas stutzeri* (HB-10), *Bacillus sp.* (HB-20), and *Clostridium sp.* (HB-34) showed maximum susceptibility, rest were poorly inhibited.

Reproducibility of results

All the experiments were repeated, and performed independently at least three times. Data is presented as mean values.

Discussion

It was our aim to isolate and screen halophilic bacteria with biocontrol potential, since these isolates have rhizosphere competency and adaptability. Halophilic bacteria are well adapted to saline soils, but also bring about plant growth and solubilize macro and micronutrients in saline soils (Nagaraju et al. 2020). The halophilic bacterial isolates in our study exhibited antifungal activity through the release of siderophores, chitinase enzyme production and HCN production. In addition, they also possess the plant growth promoting attributes like, phosphorous and zinc solubilization, IAA production. This emphasized that, they may be provide a better growth of plants and inhibit the pathogens. In spite of their promising results, research in this pivotal area has been largely ignored and the information available is at best fragmented.

In the present investigation, a total of fifty halophilic bacteria were isolated from saline soils of Ganagavathi. The isolated bacteria had successfully grown up to 15 % NaCl (w/v) concentration and a strong halotrophic nature was observed. One of the halophilic bacterial isolate, *P. stutzeri* (HB-10) exhibited the strong antifungal activity against *S. oryzae* and *R. solani*. The strong biocontrol activity of halophilic bacterial isolates against plant pathogenic fungi was attributed to the production of siderophores, HCN, and mycolytic enzymes (Figure 5). It has been already established in several reports that microorganisms with biocontrol attributes are very helpful in controlling pathogens which causes menace to plants (Shrivatsava and Kumar, 2015). We reported the production of siderophore by four halophilic bacterial isolates, of these isolate *B. albus* (HB-17) positive for both Arnow's and Csaky's test, which indicates the production of both catecholate and hydroxamate type of siderophores respectively. The production of two different types of siderophores by *Bacillus albus*, agreed with the studies of Dave et al. (2006). Several studies highlighted the production of siderophores by halophilic bacteria viz., a salt tolerant (12 %) *Serratia marcescens* KH1R KM035849 produced siderophores (Vora et al. 2014).

In vitro evaluation showed, one strain of *P. stutzeri* (HB-30) showed a chitin solubilization of 3.06 mm, which might be responsible for the antagonistic activity against plant pathogens. Based on the biocontrol ability under *in vitro* conditions, eight halophilic bacterial isolates chosen for further study. These consisted of six rod shaped halophilic bacterial isolates and two cocci shaped isolates. Of the bacterial isolates, six were gram positive, five were amylase positive, and all are positive for oxidase, catalase, and gelatin liquefaction. All the isolates readily used glucose and lactose and least preferred Rhamnose. After studying the morphological, biochemical and genetic characteristics (16S rRNA analysis), two strains were identified as *P. stutzeri* (HB-10 and HB-30), one as *Bacillus safensis* (HB- 28), two as *B. cereus* (HB-48 and HB-49), one as *B. albus* (HB-17), one as *Staphylococcus xylosus* (HB-39) and one as *L. sphaericus* (HB-50). These bacteria are known to inhabit the saline soils (Berrada et al. 2012). Various species of *Pseudomonas sp.* and *Bacillus sp.* have extensively been studied for their biocontrol activity in neutral soils (Reetha et al. 2014 and Saravanan et al. 2003).

Several researchers highlighted that isolates possessing the biocontrol capabilities can solubilize zinc, phosphorous, potassium and release, IAA (Yandigeri et al. 2012; Khamna et al. 2009). For example, the commercial biocontrol agents like *Pseudomonas sp.* and *Bacillus sp.* have been reported with zinc, phosphorous and potassium solubilization and IAA, siderophore production (Tank and Saraf, 2009; Cray et al. 2015). However, few reports are available for the biocontrol ability and mineral solubilization of halophilic bacterial strains such as *P. stutzeri*, *B. safensis*, *B. cereus* and *L. sphaericus* on *S. oryzae* and *R. solani*. Our strains along with the biocontrol ability exhibited PGP traits such as phosphate and zinc solubilization and potassium release at 3, 6 and 10 % NaCl (w/v) concentration. The strain of *P. stutzeri* (HB-30) was solubilized zinc oxide and zinc carbonate 13.20 and 10.03 at 6 % NaCl (w/v) concentration. For all the isolates, 6 % NaCl (w/v) was found optimal for growth and solubilization of ZnCO₃, ZnO, potassium alumino silicate and tricalcium phosphate. There are few reports on zinc solubilization at different salt concentrations. For example, Ramesh et al. (2014) reported, *Bacillus aryabhattai* solubilized zinc up to 10 mm on solid media at 4 % NaCl concentration. In the present study we also reported the drop in pH of media near halo zone. These findings are in accordance with the works of Ramesh et al. (2014) and Saravanan et al. (2003). Production of organic acids by halophilic bacterial isolates has been reported to be a cause of zinc, phosphorous and potassium solubilization and release (Fasim et al. 2002). The results of this research explicitly shows that increase in salt concentration increased the solubilization of zinc, phosphorous and potassium. These findings are in agreement with the earlier findings of Shahab and Ahmed, (2008).

Resistance to commonly used antibiotics was observed in halophilic bacterial isolates. Out of the fifty halophilic bacteria screened, six isolates showed resistance to Penicillin-G (P-10 units). Similarly, Shawish and Tarabees (2017) reported Penicillin G resistance in *Bacillus cereus* and susceptibility to oxacillin, clindamycin, vancomycin, erythromycin, gentamicin, ciprofloxacin, and ceftriaxone. Antibiotic resistance of halophiles was contributed by the salinity of the media, at optimum salinity moderate halophiles generally tolerate high concentrations of most antimicrobial agents (Nieto et al. 1993). These halophilic bacterial isolates may possess the potential to be applied as biocontrol as well as improving the crop production in salinity affected areas.

Conclusions

In conclusion, the hunt for new biocontrol agents extends from neutral soils to saline soils and also stresses that the possible reservoirs of halophilic biocontrol agents are saline environments. The *in vitro* assessment carried out in the current investigation showed that both biocontrol and plant growth encouraging attributes of halophilic bacteria. For the treatment of soil borne plant pathogenic fungi, these halophilic bacterial strains may be used as prospective biocontrol agents. For these reasons, promotion and utility of halophilic bacteria would greatly help farming community.

Declarations

Ethics approval and consent to participate – not applicable

Consent of publication – not applicable

Competing interests -not applicable

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Authors contribution

Mahadevaswamy, R.C. Gundappagol, Nagaraj M Naik and K. Narayana Rao, designed the experiment and helped in manuscript preparation. Yalvarthi nagaraju performed the experiment. Mahadevaswamy and Nagaraju wrote the manuscript. All the authors read and approved the final manuscript.

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Tables

Table 1. Biochemical properties of halophilic bacteria: Salt tolerance, shape of bacteria, growth pattern, MR-VP test results, Indole production, starch hydrolysis, gelatine liquefaction, catalase and oxidase test results

S.No.	Characteristics	<i>Pseudomonas stutzeri</i> (HB-10)	<i>Bacillus albus</i> (HB-17)	<i>Bacillus safensis</i> (HB-28)	<i>Pseudomonas stutzeri</i> (HB-30)	<i>Stahylococcus xylosus</i> (HB-39)	<i>Bacillus cereus</i> (HB-48)	<i>Bacillus cereus</i> (HB-49)	<i>Lysinibacillus sphaericus</i> (HB-50)
1	Maximum NaCl tolerance	15	6	11	10	9	11	11	8
2	Accession number	MN098847.1	MN098871.1	MN121550.1	-	-	MN098870.1	MN122435.1	-
3	Optimum NaCl	11	4	5	5	2	6	6	3
4	Biochemical properties								
a	Shape	Small rods	Rods	Rods	Small rods	Coccus	Rods	coccus	Rods
b	Growth pattern	Pellicle	Pellicle	Bottom	Pellicle	Bottom	Throughout	Pellicle	Pellicle
c	Gram staining	Gram Negative	Gram Positive	Gram positive	Gram Negative	Gram positive	Gram positive	Gram Positive	Gram Positive
d	Colony colour	Dull white	Brick red	White	White to yellow	Slight yellow	White	White	White
e	MR test	-	+	-	-	+	+	-	-
f	VP test	-	-	-	-	-	-	-	-
g	Indole production	-	-	-	-	-	-	+	-
h	Citrate utilization	-	-	-	-	-	-	-	-
i	Starch hydrolysis	-	+	+	-	+	+	-	+
j	H ₂ S production	-	-	-	-	-	-	-	+
k	Cytochrome c oxidation	+	+	+	+	+	+	+	+
l	Catalase	+	+	+	+	+	+	+	+
m	Gelatine liquefaction	+	+	+	+	+	+	+	+
n	Nitrate reduction	-	+	+	+	+	-	+	+
o	Acid and gas production	+	-	+	+	+	+	+	+

Table 2. Carbohydrates fermentation by halophilic bacterial isolates

S.No.	Characteristics	<i>Pseudomonas stutzeri</i> (HB-10)	<i>Bacillus albus</i> (HB-17)	<i>Bacillus safensis</i> (HB-28)	<i>Pseudomonas stutzeri</i> (HB-30)	<i>Stahylococcus xylosus</i> (HB-39)	<i>Bacillus cereus</i> (HB-48)	<i>Bacillus cereus</i> (HB-49)	<i>Lysinibacillus sphaericus</i> (HB-50)
1	Glucose	+	+	+	+	+	+	+	+
2	Adonitol	-	+	-	-	+	-	-	-
3	Arabinose	+	+	+	+	+	-	+	+
4	Lactose	+	+	+	+	+	+	+	+
5	Sorbitol	+	+	+	-	+	-	+	+
6	Mannitol	+	+	-	+	+	-	+	+
7	Rhamnose	-	+	-	-	-	-	-	-
8	sucrose	-	+	+	-	-	+	-	+
9	Chitinase								
	Solubilization zone (mm)	0.00	0.00	0.00	3.06	0.00	0.00	0.00	0.00
	Solubilization efficiency (%)	0.00	0.00	0.00	153.33	0.00	0.00	0.00	0.00
10	Amylase	-	+	+	-	+	+	-	+

Table 3. Plant growth promoting characteristics: Isolates screened for siderophore production, IAA production, Nitrogen fixation, zinc carbonate, zinc oxide, tricalcium phosphate solubilization, potassium release at different concentrations of NaCl (w/v)

S.No.	Characteristics	<i>Pseudomonas stutzeri</i> (HB-10)	<i>Bacillus albus</i> (HB-17)	<i>Bacillus safensis</i> (HB-28)	<i>Pseudomonas stutzeri</i> (HB-30)	<i>Stahylococcus xylosus</i> (HB-39)	<i>Bacillus cereus</i> (HB-48)	<i>Bacillus cereus</i> (HB-49)	<i>Lysinibacillus sphaericus</i> (HB-50)
1	Siderophore production								
	Qualitative	-	+	+	+	+	-	-	-
	Quantitative (%)	0.00	49.34	36.77	42.99	31.61	0.00	0.00	0.00
	Arnow's method	-	+	-	-	-	-	-	-
	Csaky's method	-	+	+	+	+	-	-	-
2	IAA production								
	Qualitative	+	-	+	+	+	-	+	+
	Quantitative (µg/ml)	44.56	0.00	31.80	58.76	27.56	0.00	46.83	31.56
3	Nitrogen fixation	-	-	-	-	-	-	-	-
4	Potassium release								
	0 % NaCl	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3 % NaCl (w/v)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	6 % NaCl (w/v)	11.70 ± 0.14	0.00	0.00	0.00	0.00	14.83 ± 0.08	0.00	0.00
	10 % NaCl (w/v)	9.06 ± 0.13	0.00	0.00	0.00	0.00	9.76 ± 0.08	0.00	0.00
5	Zinc carbonate (ZnCO ₃) solubilization								
	0 % NaCl	7.80 ± 0.05	7.70 ± 0.10	0.00	0.00	0.00	0.00	0.00	0.00
	3 % NaCl (w/v)	8.26 ± 0.20	0.00	0.00	10.03 ± 0.08	0.00	0.00	0.00	0.00
	6 % NaCl (w/v)	6.90 ± 0.10	9.00 ± 0.05	5.50 ± 0.05	5.20 ± 0.05	0.00	0.00	0.00	0.00
	10 % NaCl (w/v)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	Zinc oxide (ZnO) solubilization								
	0 % NaCl	3.23 ± 0.03	4.26 ± 0.06	3.26 ± 0.08	0.00	0.00	0.00	6.33 ± 0.06	6.26 ± 0.06
	3 % NaCl (w/v)	5.40 ± 0.11	7.33 ± 0.08	6.53 ± 0.03	11.90 ± 0.08	0.00	7.63 ± 0.03	0.00	5.20 ± 0.11
	6 % NaCl (w/v)	8.10 ± 0.11	0.00	0.00	13.20 ± 0.17	0.00	0.00	6.53 ± 0.03	6.06 ± 0.08
	10 % NaCl (w/v)	0.00	0.00	5.90 ± 0.05	0.00	0.00	0.00	0.00	5.36 ± 0.06
7	Phosphorous solubilization								
	0 % NaCl	7.63 ± 0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	3 % NaCl (w/v)	7.56 ± 0.12	11.00 ± 0.21	0.00	11.20 ± 0.11	0.00	0.00	0.00	0.00
	6 % NaCl (w/v)	6.30 ± 0.05	7.43 ± 0.08	8.03 ± 0.12	9.70 ± 0.11	0.00	0.00	0.00	6.86 ± 0.06
	10 % NaCl (w/v)	9.26 ± 0.06	0.00	9.66 ± 0.03	0.00	0.00	0.00	0.00	0.00
8	HCN production	+	-	+	-	-	+	-	-

Figures

Figure 1

Halophilic bacteria colony morphology: variation in colony morphology and colony colour was observed among the isolates

Figure 2

Mycelial growth inhibition by halophilic bacteria: Control plate with full growth of *Sclerotium oryzae* and right-side plate showing inhibition zone (HB-49) by halophilic bacterial isolate

Figure 3

Inhibition of *Rhizoctonia solani*: The mycelial growth of *R. solani* was inhibited by halophilic bacteria, the colony is surrounded by clear halo zone.

Figure 4

Siderophore production by halophilic bacterial isolates: Halophilic bacterial isolates produced yellow halo zone around the colony

Figure 5

Chord Diagram: Isolate with siderophore production and chitinase activity are potential inhibitors of sclerotium oryzae and Rhizoctonia solani