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A Halotolerant Bacterium *Staphylococcus haemolyticus* Designated 15%S5-H-2 Strain, Characterization and Identification of Salt-Tolerant Plant Growth-Promoting Bacteria (ST-PGPB): A Study on its Effects on Rice and Black Gram Plant Growth Promotion

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A B S T R A C T

In this study, we have investigated the ability of *Staphylococcus haemolyticus* designated 15%S5-H-2 strain, a multifunctional salt-tolerant plant growth-promoting bacteria (ST-PGPB). The bacteria were isolated from rhizosphere soil from a salt production pond near Coringa Mangrove forest, Kakinada, Andhra Pradesh, INDIA. The bacteria were capable of producing Auxin indole -3 acetic acid (41µg/ml), solubilizing insoluble phosphate, siderophore and ammonia, synthesizing other plant growth-promoting enzymes. It has shown positive results for antibacterial and antifungal activities and salt tolerance up to 25%NaCl (optimum growth at 3-6% NaCl). The growth occurred at temperatures from 18°C–45°C (optimal growth at 35°C). The tested bacteria significantly enhanced the growth of two plants black gram and rice in comparison to control.

Introduction

The FAO and ITPS, (2015) discussed soil salinity as one of the major thread out of 7 threads of the world's soil resources. Soil salinity causes major reductions in cultivated land area, crop production and quality (Yamaguchi, *et al.*, 2005, Shahbaz, *et al.*, 2015). Soil salinization is globally growing. Globally, 25% to 30% of irrigated lands are salt-affected and turned unproductive. The accelerated rate of salinization has also created food insecurity in several countries. Soil salinity not only affects plant quantitatively by reducing the growth and development of plant but also the quality of plant products get compromised. World widely, crop production needs to raise, either

by expanding the arable lands or by amplifying productivity on existing agricultural lands. The expansion of farmland means deforestation and wildlife habitat damage which leads to the biggest threats to the planet's ecosystem. Practising modern agriculture like highly productive crops has been damaging global biodiversity (Lanz, Bruno *et al.*, 2017). A few salt-tolerant genes can improve the productivity of saline soils(Morton *et al.,* 2019). To secure attainable crop yield in saline soil, besides using salt-tolerant varieties and chemical neutralizers, the salt-tolerant plant growth-promoting bacteria (ST-PGPB) can be harnessed for enhancing productivity and improving soil fertility as well. There is extensive literature exist on microbial involvement as Salt tolerant plant growth-promoting bacteria under salt stress. Various genera of salt-tolerant plant growth-promoting bacteria (ST-PGPB) have been isolated from extreme saline soils. The genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Agrobacterium*, *Streptomyces*, *Klebsiella*, and *Ochrobacter* are best reported for improving the productivity of diverse crops under saline conditions (Sharma, *et al.*, 2016, Sarkar *et al.*, 2018).

The aim of the present study was isolation, characterization and identification of salt-tolerant plant growth-promoting bacteria and its effect on rice and black gram plant growth promotion.

Materials and Methods

In search of the most sustainable salt-tolerant plant growth-promoting bacteria (ST-PGPB), a rhizosphere soil sample was collected from a salt production pond near Coringa, Mangrove Forest, Kakinada, Andhra Pradesh, India (16 54'23.1" N82 14'09.2"E). The sample was collected in May (2019). The soil sample was brought into the lab in the sterilized plastic capped bottle in an ice pack and stored in a laboratory at -15°C. For the isolation of bacteria, the method proposed by Vlassak *et al.*, (1992) was followed with slight modification. Briefly, 1g of soil sample was serially diluted to 10^{-2} and 50µl suspension was used as inoculum, spread plate technique was applied on a plate with nutrient agar supplemented with 15% NaCl for the isolation of bacteria. The plates were incubated at 35°C for 24hrs. Composition of 15% NaCl nutrient agar (g/L): Peptone 5g, meat extract 1.5g, yeast extract 1.5g, NaCl 150g, agar 15g. The bacterium was transferred on nutrient agar supplemented with 3% NaCl, purified and preserved in 20% glycerol at -15°C for further detailed studies. Based on rich growth at several subculturing, differentiated morphological, biochemical properties and associated with multiple plant growth-promoting traits, the bacterial strain 15%S5-H-2 was selected for species identification by 16s rRNA sequencing. The bacterial isolate 15%S5-H-2 was assayed for Indole acetic acid activity and other plant growth-promoting properties.

In vitro screening of plant growth-promoting properties

Indole acetic acid and Gibberellic acid assay

The isolate 15%S5-H-2 was tested for production of indole3-acetic acid (IAA) following the methods given by Gordon *et al.* (1951), Loper *et al.* (1986), Umang Bharucha *et al.* (2013). The concentration of IAA was estimated using the standard curve of synthetic Indole 3-acetic acid in the medium in the range of 1-100µg/ml.

The production of Gibberellic acid by isolate 15%S5-H-2 was assayed following the method discussed by Pandya *et al.*, (2014). Briefly, the test isolate was cultured in 25 ml

of nutrient broth supplemented with 3% NaCl for 4 days at 35°C. After incubation, centrifuged the culture at 10000 rpm for 20min. Then adjusted the supernatant pH to 2.5 using 3.75N HCl. The equal volume of ethyl acetate to supernatant was added and followed liquid/liquid (ethyl acetate/$NaHCO_3$) extraction following 10 minutes of vigorous shaking.

The separated organic layer was collected. The process was repeated twice for extraction of gibberellic acid with ethyl acetate and allow evaporation of excess volume of ethyl acetate. The Gibberellic acid produced by test isolate 15%S5-H-2 was read at wavelength 254nm using UV Spectrophotometer.

The concentration of Gibberellic acid was measured by the calibration curve of synthetic Gibberellic acid in absolute alcohol in the range of 100-1000 µg/ml.

Screening of Siderophore production

The screening for siderophore production of test isolate was carried out following the method proposed by Schwyn and Neilands (1987). Briefly, in this assay, the test isolate inoculated on the 3% NaCl supplemented chrome-Azurol sulphonate agar and was incubated at 30^0C for 48 hrs. The siderophore producing bacteria can be identified through a colour change of the blue media into orange.

Screening of phosphate solubilization

The phosphate-solubilizing tendency of test isolate 15%S5-H-2 was screened by following Pikovskaya RI (1948) method.

Ammonia production assay

The bacterial strain 15%S5-H-2 was grown in 10 ml of 3% NaCl supplemented peptone broth at 30^0C and at 120 rpm rotation for 4 days. After incubation, centrifugation was carried out at 5000rpm for 5minutes. The bacterial strain 15%S5-H-2 was tested for ammonia production following the method given by Dweipayan Goswami *et al.,* (2013). The concentration of ammonia was estimated using the standard curve of ammonium sulphate in the range of 50-1000µg/ml.

Screening of Chitinase

Colloidal chitin was prepared from chitin by employing the procedure described by Saima M K *et al.,* (2013). For screening of chitinase production, the procedure followed given by Krithika *et al.,* (2016).

Screening of Amylase, Protease, Carboxylase production

The overnight culture was spot inoculated on 3% NaCl supplemented starch agar, skimmed milk and nutrient agar containing 0.5% carboxymethyl cellulose(CMC). The plates were incubated for 48hrs at 35^0C. After incubation, Starch hydrolysis was tested with iodine solution, cellulose hydrolysis was visualized by flooding with 1% congo red solution for 15min then washed with 6N NaCl to remove remaining congo red. Casein hydrolysis was detected from the observation of a clear zone.

The Solubilization index(SI) was calculated from the diameter of the clear zone (mm) divided by the diameter of the colony(mm)

Salt tolerance

Salt tolerance test was performed on test isolate 15%S5-H-2 by growing bacteria in nutrient agar with different concentration of NaCl from 0 to 20% (w/v) at optimum pH 7±0.2 and temperature 35^0C for 24 hrs(T. Damodaran *et al.,* 2013, Amaresan N *et al.,* 2014)

Temperature tolerance

For determination of temperature tolerance, overnight culture was streak on 3% NaCl supplemented nutrient agar and incubated at 18°C, 25°C, 45°C and 50°C for 24 hrs. Observed growth of isolate after 24hrs.

Antimicrobial sensitivity assay

The test isolate was spot inoculated on 3% NaCl supplemented nutrient agar for 24 hrs at 35^0C. The antimicrobial assay was conducted following the agar overlay method (Cooper K. E. 1963). The test pathogen used in this assay was *Vibrio cholera*.

In vitro screening for antagonistic activity

The overnight test isolate 100µ l transferred into 2ml of 3% NaCl supplemented nutrient broth. Incubated at 120rpm rotation and temperature 35^0C for 48hrs. After incubation, supernatant collected by centrifugation at 5000rpm for 10minutes.

For the antagonistic assay, a fungal lawn of the *Aspergillus niger* was grown on potato dextrose agar at one side of the plate and a well of 6mm diameter was formed at another end, in which 50 µl supernatant of test isolate was added. Incubated at 28 ± 2^0C for 5 days. zone of inhibition was measured in mm.

Biochemical characterization

Biochemical tests were performed for the test organism 15%S5-H-2 following a standard protocol Cappucino JC *et al*., (1992).

16s rRNA sequencing and molecular identification

DNA isolation was carried out by Qiagen QIAamp DNA Mini Kit (Cat No./ID: 51304)

Two Primers used to amplify 16s rRNA genes.

27F AGAGTTTGATCCTGGCTCAG

1492R CGGTTACCTTGTTACGACTT

GeNeiTM PCR Master Mix (2X) SKU: MME22 was used. DNA amplification was carried out in an Applied Biosystems MiniAmp Thermal cycler with Thermocycler setting: An initial denaturation - 94^0C for 2 mins, for 25 cycles 94^0C for 30sec(denaturation), 55^0C for 30sec(annealing), 72^0C for 1min(synthesis) and final elongation step at 72^0C for 6mins and 4^0C hold. Its quality was evaluated on 1.0 % agarose gel, The PCR amplicon was purified to remove contaminants. Using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence of the 16S rRNA gene was generated from forward and reverse sequence data using aligner software(Barcode biosciences, Bangalore, India). The 16S rRNA gene sequence was used to carry out BLAST with the 'nr' database of the NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using the RDP database and the phylogenetic tree was constructed using MEGA X.

In vivo pot experiment: 15%S5-H-2 effect on rice and black gram plant growth promotion under salt stress

To study the effect of 15%S5-H-2 on plant growth promotion, Rice and black gram seeds were surface sterilized with 0.5% sodium hypochlorite solution for 5 minute and 70% alcohol for 2-minute following wash thrice with sterilized distilled water. Seed bacterization was performed for 2 hours. Then

seeds dried and tied in cloth for germination and seedling transferred in the soil and with additional salt concentration 50mM and 100mM. The experiment was conducted in a potting mix of soil and cocopeat in 2:1 ratio. All three soil combinations namely natural soil, additional 50mM soil and additional100mM soil were sterilized by autoclaving at 121°C for 30 min for three consecutive days and 200g sterilized soil was filled in each pot.

The initial pH and electrical conductivity of soil were analyzed by digital pH and EC meter on a 1:2.5 ratio of soil and water respectively. Water sprinkled twice every day for the 7 days in soil condition. Fresh weight, root and shoot lengths were measured in cm.

Statistical analysis

All Statistical data was calculated using Software Microsoft office Excel 2007 and SAS 9.2 version. For Indole acetic acid, Gibberellic acid and ammonia assay, the regression analysis was performed. Mean, standard deviation and standard error of 5 replicates were calculated for all observed shoot and roots lengths of *invivo* pot experiment.

Using Software Sigmaplot 14.5, Column bar diagram was plotted i.e, means of the five replicates ± SE (standard error). The differences within control and 15%S5-H-2 and between control and 15%S5-H-2 was measured by one way ANOVA ($p<0.05$) (Akhilesh Kumar *et al.,* 2021).

Results and Discussion

Plant growth-promoting traits and some biochemical test of 15%S5-H-2

Several plant growth-promoting traits and biochemical tests were performed on the bacterial strain designated as 15%S5-H-2 shown in Table 1.

IAA production assay

The IAA production for the bacterial strain was directly proportional to the concentration of tryptophan and incubation period. The amount of IAA was high in shaking incubation than static incubation. 15%S5-H-2 has produced 41µ g/ml concentration of IAA at 72 hrs incubation period.

Gibberellic acid produced by test isolate 15%S5-H-2 at 96hrs incubation period and temperature 35^0C was 7mg/mL.

Siderophore production and Phosphate solubilization

The siderophore production capability of the bacterial isolate 15%S5-H-2 could increase with the increase of the incubation period. The maximum clear zone was observed on the 4^{th} day of incubation. The clear zone of 3mm radius was produced around isolate 15%S5-H-2 at 2 days of the incubation period. Phosphate solubilizing efficiency of the bacterial strain 15%S5-H-2 could test by solubilization of inorganic phosphate in the medium containing tricalcium phosphate in 3% NaCl supplemented pikovskaya agar. The phosphate-solubilization index of 3mm was observed of 15%S5-H-2.

Ammonia production

The production of ammonium ion increased with an increase in the incubation period and on rotary shaker compared to the static incubator. 15%S5-H-2 produced a 903µg/mL concentration of ammonia at 4^{th} day of incubation.

Chitinase production and Antagonism

The colloidal chitin degradation (the synthesis of chitinase, biocontrol agent especially for

phytopathogenic fungi) efficiency of 15%S5-H-2 was measured as a 6mm zone of clearance around the inoculum. Antagonistic assay of tested bacteria was formed 12mm zone of clearance against fungi *Aspergillus niger*.

Amylase, protease, carboxylase and antimicrobial Screening

15%S5-H-2 capability of synthesizing enzymes amylase, protease and carboxylase have demonstrated in-vitro positive results by observation of zone of clearance 20mm,7mm and 21mm round the tested inoculum respectively.

Salt tolerance

Thou the bacterial strain 15%S5-H-2 was isolated in 15%NaCl supplemented nutrient agar medium. But further based on several subculturing and repeated salinity tolerance tests revealed 15%S5-H-2 could tolerate maximum salinity of 12% NaCl concentration. Its optimum growth was observed at 3-6% NaCl concentration.

Temperature tolerance

15%S5-H-2 has shown growth at a maximum temperature of 45°C for 24 hrs incubation. Complete growth inhibited at a minimum temperature of 15°C and maximum temperature of 50°C. The optimum temperature for the growth of 15%S5-H-2 was investigated as 35°C.

16s rRNA sequencing of bacterial strain 15%S5-H-2

The bacterial strain 15%S5-H-2 was found Staphyl*ococcus haemolyticus*, had shown high similarity (100%) with Top based on nucleotide homology and phylogenetic analysis (Fig 1).

In vivo pot experiment: 15%S5-H-2 effect on rice and black gram plant growth promotion under salt stress

In the study of the effect of bacterial strain 15%S5-H-2 on the black gram and Rice plants growth under salt stress, the bacterial strain 15%S5-H-2 inoculated rice seeds had shown germination at a salinity of 517mM salt stress and the shoot and root length of seedlings were measured higher in inoculated rice seedlings than nonsaline uninoculated control seedlings (Fig.3 A, B). Rice plants were checked for further growth in soil condition with electrical conductivity and salinity (natural soil) 0.4dS/m and 0.3ppt respectively, with additional 50mM salinity to natural soil(electrical conductivity 1.7dS/m) and with additional 100mM salinity to natural soil(electrical conductivity 3.8dS/m). Black gram plants were checked further growth in soil condition with electrical conductivity 1.4dS/m and salinity 0.7ppt, with an additional 50mM and 100mM salinity on natural soil. Besides the growth in natural soil, rice seedlings have shown plant growth in additional salinity of 50mM and 100mM also. The control rice plants and inoculated rice plants both were showed decrease in shoot and root lengths with an increase of salinity (Akhilesh Kumar *et al.,* 2021). Comparatively, inoculated rice plants were showed higher in shoot and root lengths than uninoculated control rice plant at additional 50mM and 100mM of salinity (Table 2, Fig 2, A, B, Fig 3 E).

In the another pot study of black gram plant growth under salt stress, Inoculated black grams seedlings and uninoculated control seedlings have shown plant growth only in natural soil. No growth was observed in additional 50mM and 100mM salinity. In natural soil, inoculated black gram plants were showed higher shoot and root lengths

compared to the uninoculated control plant (Table 2, Fig 2 D, E, Fig 3F, G). In both plants(Black gram and rice), the total fresh weights of inoculated plants were more than uninoculated control plants. The total fresh weights of rice plants decreased with the increase of salinity (Table 3, Fig 2C, E) This was maybe bacterial strain 15%S5-H-2 stimulated root elongation under salt stress but at higher salinity nutrient uptake was prevented and thus fresh total weight was reduced from natural soil to 50mM and from 50mM to further 100mM salinity (Febri doni 2014).

Table.1 The consolidated results of preliminary plant growth-promoting traits and biochemical test were shown in Table 1as follows

Characteristic	15%S5-H-2	Characteristic	15%S5-H-2	Characteristic	15%S5-H-2	Characteristic	15%S5-H-2
PGP traits		**Cell morphology**	Cocci	**Growth at max. % of NaCl**	12%	**Arginine**	+
IAA assay	41 µg/mL						
Gibberellic Acid	7mg/mL	**Colony colour**	White	**Xylose**	+	**Ornithine**	+
Siderophore Clear zone(mm)	3mm	**Spore shape**	Non spore	**Arabinose**	-	**Citrate**	+
P- Solubilizing	3mm	**Catalase**	+	**Galactose**	+	**Asculin**	-
Ammonia	903 µg/mL	**Oxidase**	-	**Maltose**	+	**Urease**	-
Chitinase	6mm	**KOH**	-	**Trehalose**	+	**Nitrate**	+
Amylase	20mm	**Max. temp. for growth**	45^0C	**Sorbitol**	+	**Indole**	-
Protease	7mm	**Min. temp. for growth**	18^0C	**Xylitol**	+	**ONPG**	+
Carboxylase	21mm	**VP**	+	**Cellobiose**	+	**Gelatinase**	-
Antimicrobial	17mm	**MR**	+	**Salicycline**	-	**motility**	-
Antagonism	9mm	**Growth at 0.5%NaCl**	+	**Lysine**	-	Haemolysis on blood agar	9mm

Table.2 *Invivo* experiment on Rice and black gram plant growth and effect of salt-tolerant bacterial strain *Staphylococcus haemolyticus* 15%S5-H-2; Length based comparison between control plant and bacterial inoculated. Rice and black gram plant growth under salt stress

	Rice plant growth								**Black Gram plant growth**	
Plant	Seedlings on the 7th day		Plants on 15th day in the soil		Additional 50mM NaCl salinity to natural soil		Additional 100mM NaCl salinity to natural soil		Plant on the 7th day in the soil	
	Shoot (mm)	Root (mm)	Shoot (cm)	Root (cm)	Shoot	Root	Shoot	Root	Shoot (mm)	Root (mm)
Control	9.8±0.7	15.6±1.3	7.3±0.8	4.8±0.5	2.8±1.1	5±0.9	2.58±0.4	2.14±0.5	5.5±0.5	2±0.42
15%S5-H-2	11±0.84	28±0.58	9±0.59	7.3±0.92	7.3±0.7	4.5±0.7	2.2±0.53	3.3±0.73	8±0.46	3.95±0.66

Fig.1. Molecular Phylogenetic analysis by Maximum Likelihood method

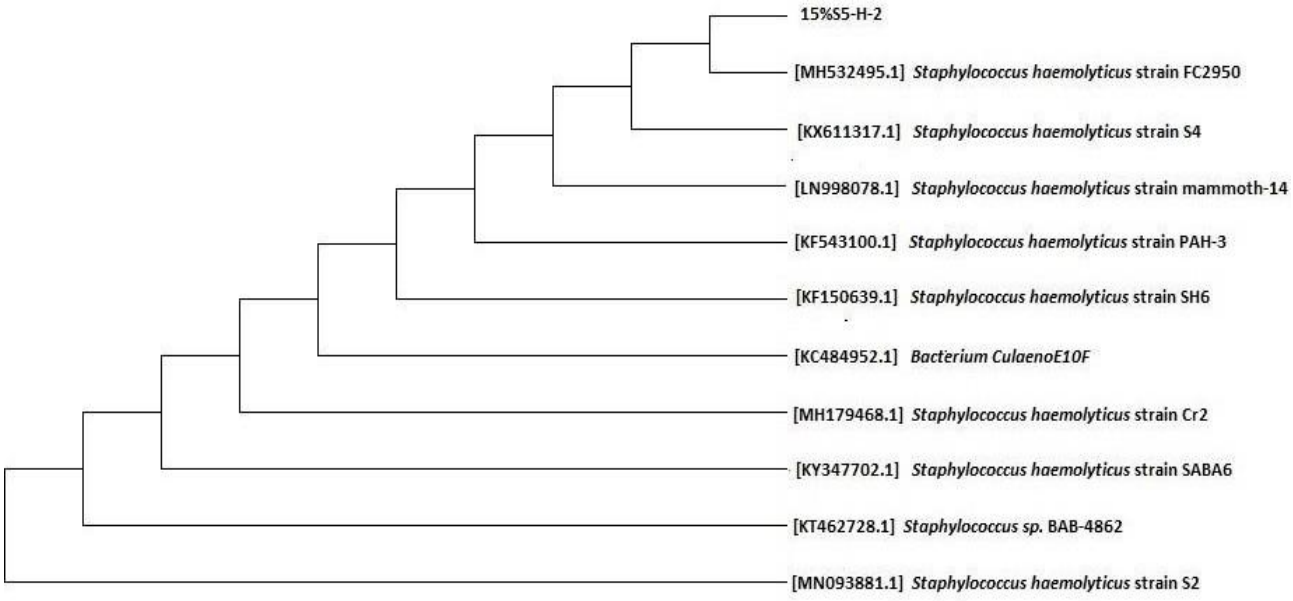
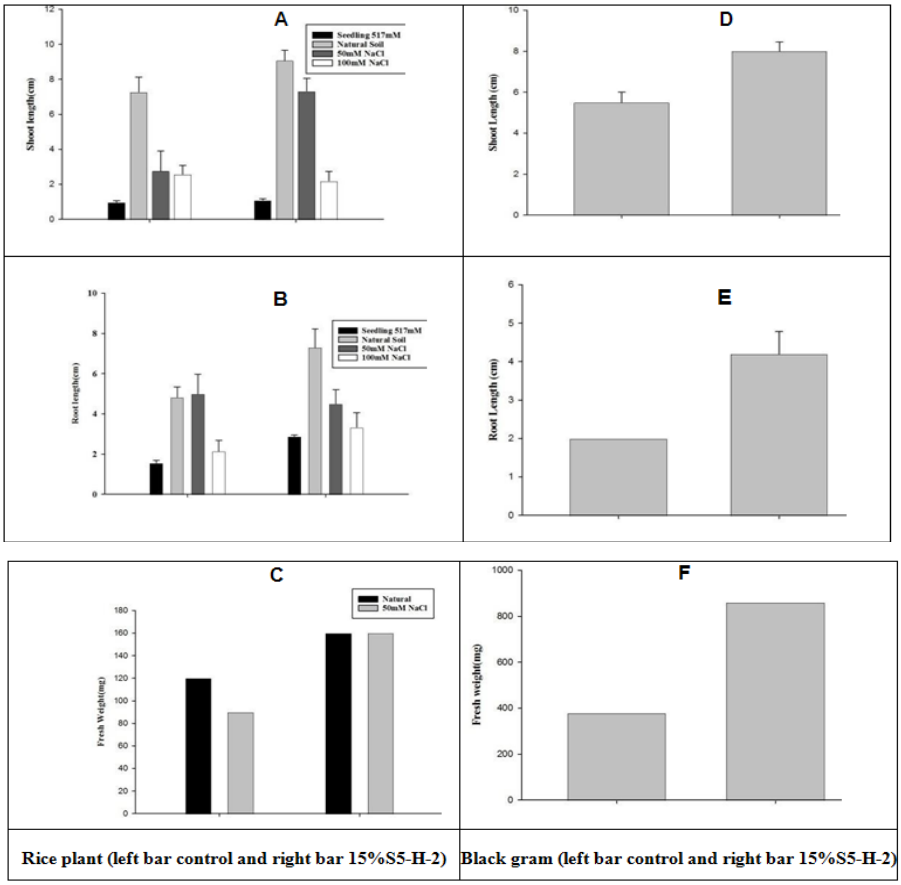


Fig.2 Rice and Black gram plant growth under salinity



Rice plant (left bar control and right bar 15%S5-H-2) | Black gram (left bar control and right bar 15%S5-H-2)

Table.3 Total fresh weight comparison between control plant and treated black gram and rice plant growth promotion under salt stress

	Total fresh wt(mg) on the 7th day	**Total fresh wt(mg) on the 7th day**	**Total fresh weight on the 15th-day in soil condition**		
	Natural soil	Seedling	Natural soil	Addl. 50mM NaCl	Addl.100mM NaCl
Plant	Fresh wt(mg)	Fresh wt (mg)	Fresh wt (mg)	Fresh wt (mg)	Fresh wt (mg)
Control	380	120	120	100	90
15%S5-H-2	860	150	160	150	100

Fig.3 Picture of Rice and Black gram under salinity

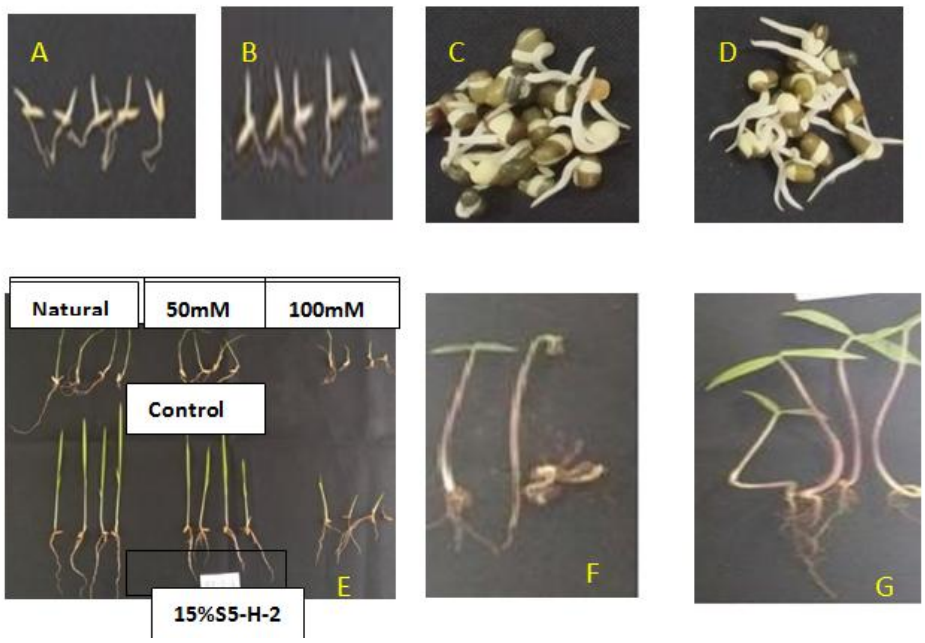
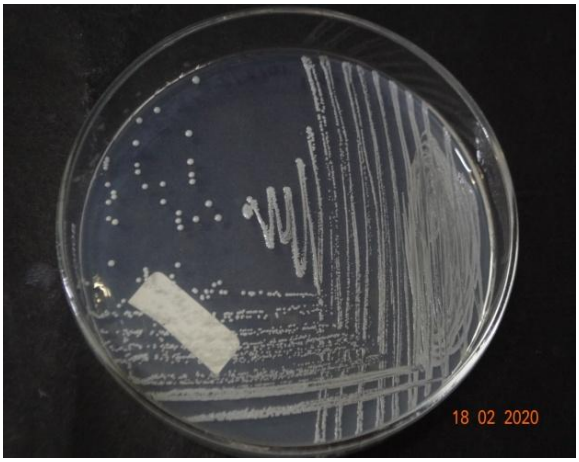


Fig.4 *Staphylococcus haemolyticus* on 3%NaCl nutrient agar



15%S5-H-2 was Gram Positive, White coloured colony, opaque circular coccus, non-motile occurred in single and in the cluster (Fig 8). The strain isolated from a 15% NaCl supplemented nutrient agar plate. The Strain was able to grow at a salt concentration from 0-250g/L. Optimum growth at a salt concentration of 30-60g/L (Amina, *et al.*, 2011). Thus strain is called a halotolerant (Parthiban, *et al.*, 2010). The strain has shown all in-vitro plant growth-promoting properties in a moderate amount. Thus went for further molecular analysis. The molecular analysis highlighted that strain 15%S5-H-2 could be closely related to members of the genus *Staphylococcus*, especially to the species of *Staphylococcus haemolyticus,* with a sequence similarity of 100%.

According to most recent literature, Staphylococcus species have been isolated from various environments, such as human skin, crude oil-contaminated soil and fermented food (Jung-Suk Sung *et al.*, 2020), earthworm guts (Jayanta Kumar Biswasa *et al.*, 2018). *Staphylococcus* species are known as halotolerant bacteria that habitat in seawater (Kakizaki E *et al.*, 2008) In another study, *Staphylococcus saprophyticus* bacteria antimicrobial activity was observed ((Erkaya *et al.*, 2018).

Another most recent study discussed bacteria *Staphylococcus haemolyticus* involvement in the degradation of crude oil and petroleum hydrocarbons. We were studied and identified the bacteria *Staphylococcus haemolyticus*, A halotolerant plant growth-promoting bacteria. The current preliminary findings of in-vitro plant growth-promoting traits of bacteria *staphylococcus haemolyticus* demonstrated a low amount of IAA, siderophore and phosphate solubilization but the intentive amount of enzyme chitinase production. Antagonism, antimicrobial screening indicates its active involvement in plant protection. Despite the low amount of IAA(41µg/ml) it was produced, the strain showed the potential of improving plant growth of both the plants (black gram and rice) in a pot experiment. Improved growth of black gram and rice plants may be the result of a large quantity of Gibberellic acid (7mg/mL) and ammonia (903µg/mL) ions it produced which might encourage seed germination and promoted shoot elongation. Its intense capability of synthesizing exoenzymes like amylase, protease and carboxylase, leads enhanced nutrient uptakes and total fresh weights.

In conclusion a halotolerant plant growth-promoting bacteria S*taphylococcus haemolyticus* isolated from salt production pond near Coringa mangrove forest, Andhra Pradesh, INDIA. It showed the capability of producing most of the plant growth-promoting traits like IAA, siderophore, phosphate and ammonia. In the biochemical test, it has shown positive results for trehalose, xylitol and sorbitol thus the probability it may produce compounds and enzymes related to stress like exopolysaccharides and ACC deaminase are high. Our findings open the scope to study more in this aspect and needs to be verified experimentally.

Conflict of Interest

There is no conflict of interest in research findings and article writing with any person or institution in this connection.

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