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Rhizosphere Bacteria with the Potential of Forming Biofilm and Plant Growth Promotion Under Salt Stress

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ABSTRACT: *Bacteria develop microbial communities as biofilm under different environmental stress factors like salinity, temperature, pH and antimicrobial agents and help in the adherence of bacteria to different surfaces. The growth of microorganisms is inhibited in the presence of salinity. An objective of the present research work was to check the growth response and biofilm behavior of indigenous bacteria isolated from plant rhizosphere in the presence of salt. Bacterial strains were named as TAK, TAF and SY. Biofilm formation response was also observed at different molar concentration (0M, 1M, 2M and 3M) of NaCl following test tube assay after 24, 48 and 72 hours of culture incubation. Results showed that TAK has the best biofilm forming ability on abiotic surface as compared to isolate TAF and SY, however, isolate SY showed growth and form biofilm under saline conditions. Bacterial plant growth promoting response was also determined on the basis of improvement in seed germination, shoot length and root length. In general, bacterial biofilm was best at 1M to 3M NaCl stress and 72 hours of culture incubation . Inoculation with SY improved (shoot length 3 % and root length 10.41 %) at 100 mM when compared to inoculated seedlings at 0 mM NaCl stress. It was concluded that among all three isolates, isolate SY used for broad perspective to increase soil fertility. While working to strive for the most promising isolates involved in plant growth promotion, the indigenous isolates showed the promising ability to improve germination of seeds in saline soil while helping the seeds to grow in salt stress conditions.*

Key word: *Biofilm, abiotic surface and NaCl, rhizospheric bacteria, saline condition*

INTRODUCTION:

Bacteria are unicellular prokaryotes that live in planktonic as well as in biofilm. They adhere to the different surfaces in nature under stress conditions (Ansari & Ahmad, 2018) and live as three-dimensional clusters of biofilms (Vickery et al., 2013) which consist of extracellular DNA (eDNA), polysaccharides and protein (Pommi et al., 2013). They produce extra

polymeric substances in biofilm that gives stability under harsh environment and protect from antibiotic action (Boyle *et al.,* 2013). Secondly, the biofilm not only provide protection but also ensure the availability of necessary nutrients by communicating through cell to cell via signaling molecules (Blackledge et al., 2013). Physiochemical properties of surface and cell surface properties of bacteria influence the bacterial biofilm formation (Rzhepishevska et al., 2013). Such bacterial communities when get associated with plant roots seems promising for growth promotion of plant. They improve the plant growth by their own metabolism or directly by improving the plant metabolism Pérez-Montaño et al., (2014) and can be used as biofertiliser in future as reported by Afzal et al., (2015)

Ionic constraints and osmotic stress conditions are the leading biochemical and physiological processes which reduces plant growth in salinity (Abd-Allah et al., 2015a). Colonization of microbes under stress significantly improves the physiological processes and plant tolerance to abiotic stresses and particularly nutrient acquisition (Egamberdieva et al., 2016). Presence of salts can improve the bacterial biofilm formation ability as reported by Philips et al., (2017) because sodium chloride favors the development of biofilm formation.

The present research work is focused on the study of biofilm behavior and plant growth promotion of rhizosphere bacteria towards yield increment of crops in rhizosphere under saline soil.

MATERIALS AND METHODS

Bacterial Isolation, Morphological and Biochemical characterization of bacterial isolates

Bacterial isolates were obtained from plant rhizosphere by serially diluting (10^{-8}) rhizosphere soil and finally spreading (50 µL) on L-agar plates supplemented with 1M sodium chloride and incubated at 37ºC for 24 hours (Gerhardt et al., 1994). The colonies were further purified by sub culturing on 6 % sodium chloride containing L-agar plates. All the isolates were primarily characterized on the basis of culture charactertistics and biochemical behavior as described by Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Biofilm formation:
Qualitative analysis of biofilm formation

Qualitative analysis of biofilm was done by preparing Congo red supplemented brain heart infusion broth (BHI) supplemented with different combinations of glucose (2 %) and 1.5M NaCl following previous modification by (Hassan et al., 2011). The strains were streaked on plates and incubated for 24 hours at 37°C. The plates were recorded as black colonies for positive and red colonies for negative results.

Quantificational analysis of biofilm formation

Bacterial biofilm was quantified (in terms of tightly bound cells) following procedure of Qurashi et al., (2012) at optimum pH (7), temperature (37°C), agitation and different salt concentrations. For this the inoculum (100 µL) from fresh culture (cell

densities adjusted to OD 600nm 0.3) of each isolate was added in sterile L-broth (10 mL) with different molar concentrations (0, 0.5, 1, 1.5, 2 and 2.5) of NaCl and incubated at non-shaking conditions. For determining optimum pH, each salt supplemented media was adjusted at different pH values (pH 6, pH 7, pH 8) at incubation temperature of 37ºC. Similar experiments were performed for different incubation temperatures i.e., 4ºC, 37ºC and 42ºC at pH 7.0 (Fig. 2).

To study the effect of shaking on bacterial strains, cultures on optimized culture conditions of pH or temperature were incubated for 120 hours (optimized) under shaking (160 rpm-shaker-Lab tech Daittanlabtech Co. Ltd) using borosilicate test tubes. Results of biofilm (tightly bound cells) were reported as a normalized value (OD 570 nm / OD 600 nm) following Qurashi et al., (2012).

Plant Growth experiments:

To check the growth promoting potential of these bacteria, plant microbe interaction experiments were performed as described previously by (Qurashi and Sabri, 2012). Healthy and sterilized seeds (using 0.1 % $HgCl_2$ solution for 10 minutes) of *Triticum aestivum* Var. (from Punjab seed corporation, Lahore, Pakistan) were used. Then seeds (control and inoculated) were sown in plastic pots for 15 days, and pots containing autoclaved and dried garden soil (120 g per pot) was supplemented with different concentrations of NaCl (0, and 100 mM) per gram weight of soil. Pots were placed in the department of Biology, Lahore Garrison University under laboratory control conditions. After 15 days of growth, seedlings were harvested. The plant growth was observed in terms of seed germination, root length (cm), shoot length and total soluble protein content (µg per gram fresh weight) (Afrasayab et al., 2010) and total soluble sugar contents (mg per gram fresh weight) following Dubois et al., (1956).

Statistical analysis:

In this study all experiments was performed in replicates and data was statistically analysed. Analysis of difference between the means was tested using the least significant difference test ($P<0.05$) (Steel and Torrie, 1981) as shown in each figure.

RESULTS

Bacterial characterization:

Bacterial isolates were taken from plants root. Initially ten isolates were isolated from which three isolates was selected for further study and named as SY, TAF and TAK. Study of colony morphology reveals that both isolates SY and TAK were white in color whereas TAF was yellow. The colony morphology of all isolates showed circular shape, pinpoint size, flat elevation and smooth margins. Colonies of TAF were non mucoidy and TAK were mucoidy whereas SY had buttery consistency. Staining behavior of three isolates was done through simple staining, gram staining, capsule staining and spore staining. Results showed that isolate TAF was cocci and gram negative, TAK was bacilli and gram positive whereas SY was rod shaped gram negative bacteria. In case of capsule staining TAF and TAK strains are capsulated, spore staining results reveals that SY and TAK was spore forming bacteria whereas TAF was non-spore former. Biochemical

analysis of bacterial isolates showed positive results for catalase and methyl red test in all three isolates while motility behavior, Sudan-III results, Voges prosker and DNase test showed negative result. Isolate SY showed negative results for indole test and oxidase test while TAF showed positive results for both isolates. In case of isolate TAK, negative results were recorded for oxidase and positive results for Indole test (Table.1)

Table 1: Colony morphology, staining behavior and biochemical analysis of bacterial isolates

Bacterial isolates	texture	Colony shape	colour	size	Margin
TAF	Non mucoidy	circular	yellow	pinpoint	smooth
TAK	mucoidy	circular	white	pinpoint	smooth
SY	Buttery consistency	circular	white	pinpoint	smooth

Staining behavior

Bacterial isolate	Simple staining	Gram staining	Capsule staining	Spore staining
TAF	cocci	negative	capsulated	Non spore former
TAK	bacilli	positive	capsulated	Spore former
SY	Rod shaped	negative	Non capsulated	Spore former

Biochemical test

isolates	Methyl red test	Indole test	Catalase test	Oxidase test	DNase test	Voges Proskeur test	Motility test	Sudan test
TAF	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
TAK	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative
SY	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative

Biofilm Formation:

Analysis for biofilm formation was done for all isolates in the presence as well as in the absence of salt stress following test tube assay. The biofilm formation varied from all three isolates under various salt concentartion (0M, 1M, 2M and 3M). The maximum biofilm formation of SY isolate was observed at 2M concentration of NaCl at 48 hours and minimum biofilm formation at the same conentration after 72 hours as shown in figure 1. In general biofilm of isolates was started to develope after 24 hours at (0-3 M) NaCl stress.

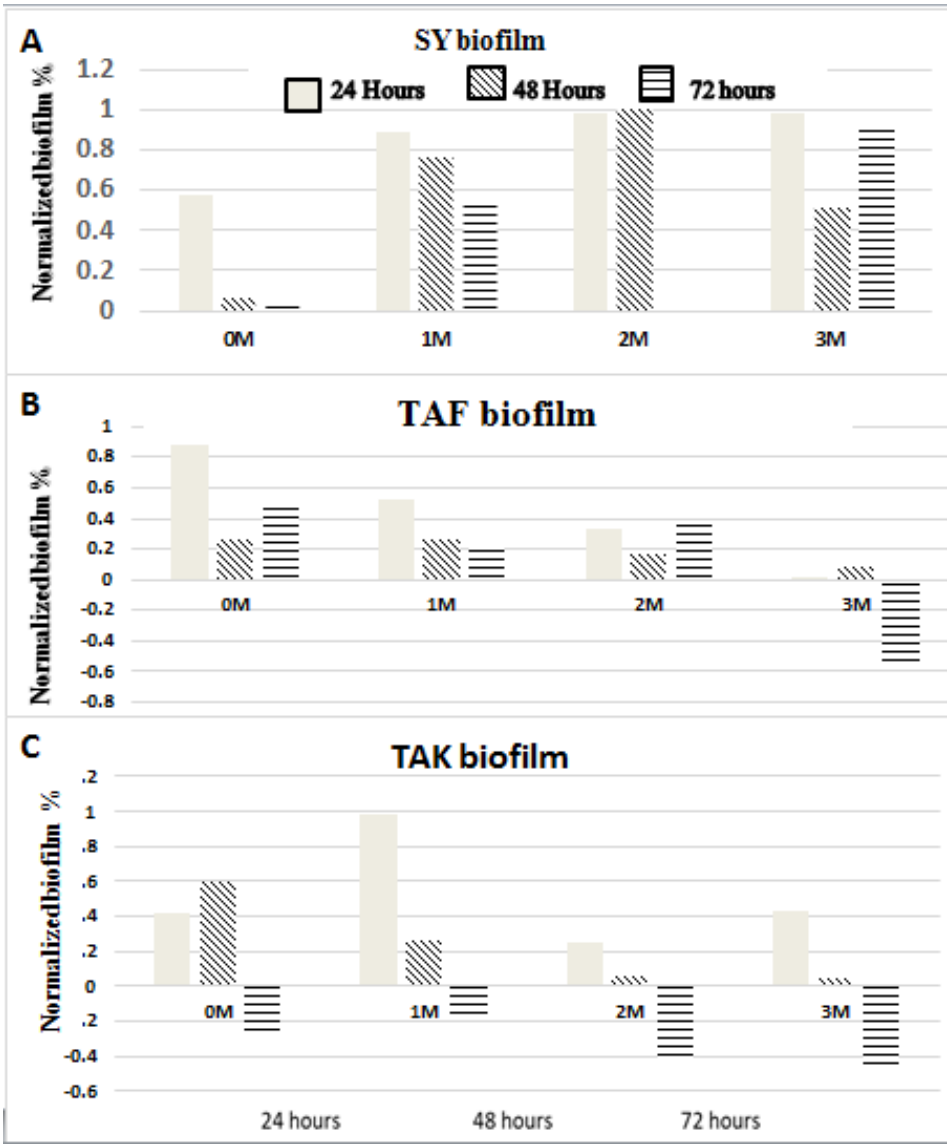

Fig 1: Effect of different concentration (0M, 1M, 2M and 3M) of NaCl on bacterial biofilm formation after 24, 48 and 72 hours of culture incubation.

Biofilm formation was tested for TAF isolte at various concentration (0M, 1M, 2M and 3M) of salt and the best biofilm formation response was observed at 0M concentration after 24 hours or we can say that the bacteria form stronge biofilm in the absence of salt. Whereas, biofilm formation declined with the increase of salanity and it showed sharp decline in the presence of 3M as shown in figure 2. The biofilm formation was decreases as the time of incubation and salt concentration increases .At 0M to 1M NaCl

stress,biofilm was favored after 24 hours of culture incubation ,however at 2M NaCl stress lighter biofil was recorded after 72 hours while at 3M NaCl stress biofilm peaked at 48 hours (Fig. 1). In case of isolate TAK maximum biofilm formation was recorded at 1M NaCl stress of salt after 24 hours . in general ,bacterial biofilm was best after 24 hours at 1M to 3M NaCl stress.With few exception i.e in the absence of salinity highest biofilm was observed at 48 hours.Biofilm was recorded at 72 hours of culture incubation .

Plant Growth experiments:

Seeds of *Triticum aestivum* (Punjab 11) were sown in plastic pots. NaCl was added in the pots to make final concentration at 0 m M and 100 mM in each pot. When inoculated with SY the germination of wheat seed *Triticum aestivum* remained unaffected at 0 mM or 100 mM NaCl concentration in the absence of presence of inoculum. In case of TAF the germination of wheat seed (*Triticum aestivum*) was reduced (25%) in the presence of inoculation as compared to control non inoculated seeds whereas TAK inoculated or non-inoculated seed germinating was 25 % reduced in presence of salinity. Short length was improved (0.57%) in inoculated at seedling 100 mM NaCl stress when compared to 0mM NaCl stress in seedling inoculated with SY. Root length was reduced (20%) at 100 mM stress compare to non –inoculated plants at 0mM NaCl stress but inoculation with SY improved (3%) at 100mM when compared to inoculated seedlings at 0 mM NaCl stress. Reduction (8%) in short length was observe at 100 mM in the absence of inoculums while inoculation improved the shoot length (10.41%) at 100 mM NaCl stress in the case of TAF. Root length was reduced (27 %) at 100 mM stress compared to non-inoculated plants at 0mM NaCl stress but inoculation with TAF also decreased (4.09%) its growth at 100mM when compared to inoculated seedlings at 0mM NaCl stress (Fig. 2)

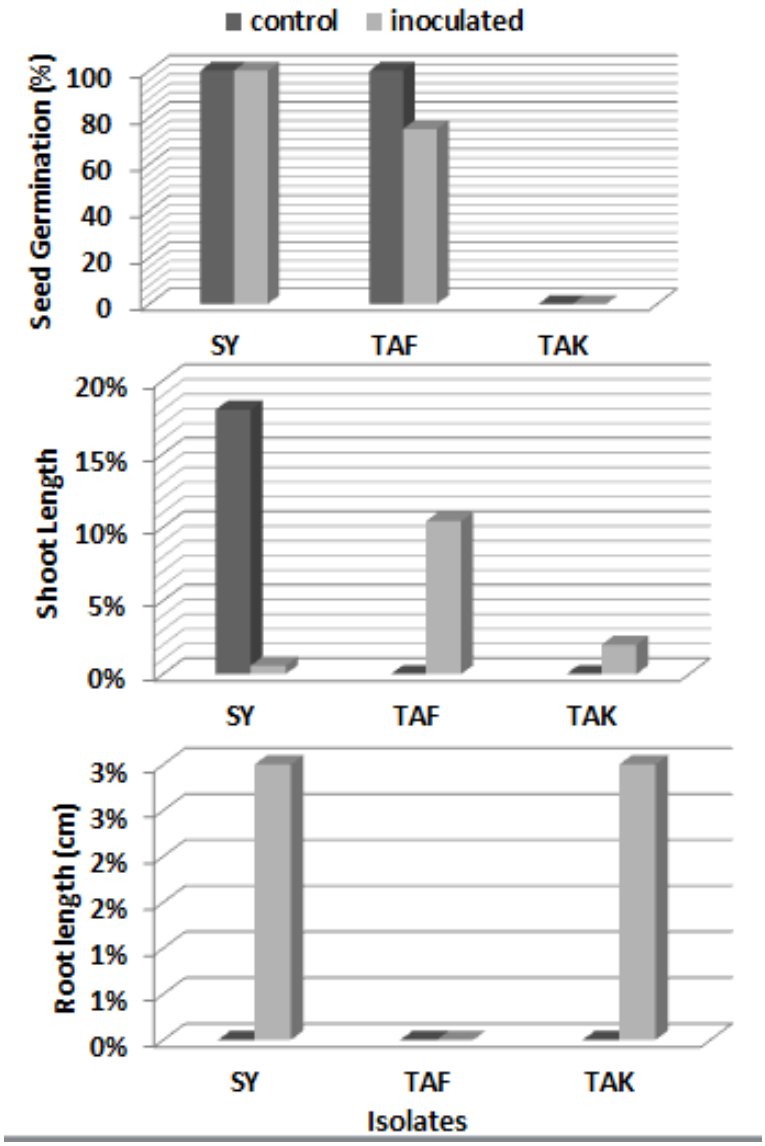

Fig 2: Effect of different concentration (0M, 1M, 2M and 3M) of NaCl on Seed germination, shoot length and root length (cm).

When inoculated with TAK (9 %) reduction in short length and (12 %) reduction in root length was observed at non inoculated seedlings at 100 mM NaCl stress when compared to 0mM NaCl stress .Inoculation improved the short length at 0 mM (4 %) and 100 mM (2 %) when compared to respective non-inoculated control .Short length increment (3 %) at 100 mM salinity was recorded in inoculated as compared to seedling compared to respective control (Fig. 2).

DISCUSSION

Three bacterial strains were isolated from rhizosphere were named as SY, TAF and TAK. Different phenotypic characteristics of bacteria associated with plant roots showed that there is a microbial diversity associated with roots of plants. Recently a study reported the significance of root associated microbial communities and their role in offering protection to the plants under natural stress conditions like disease disaster (Wang and Wang, 2018). The recruitment of microbes at the rhizosphere may be facilitated by the development of biofilm that is commonly reported by Qurashi et al., 2012 and others (Velmourougane et al., 2017). Microbial diversity was associated with bacterial growth and biofilm formation at different concentration (0 M, 1M, 2M and 3M) of NaCl in borosilicate test tubes. Biofilm formation by these bacteria revealed that these isolates possess a high capacity for biofilm formation. The biofilm forming ability of these three isolates varies with the different salt concentration and time duration. Some bacteria grow better in the presence of salt (Philips et al., 2017) while other grows better in the absence of salt as in case of SY isolate it showed best biofilm forming abilities under salt stress at 2M NaCl concentration. Some recent studies revealed that the salinity enhanced the biofilm formation ability of bacteria (Philips et al., 2017). Biofilm formation on plant roots act as a major sink of nutrients in the plant rhizosphere. This favors the crop productivity in agriculture (Singh and Chauhan, 2017).Previous studies also reported the molecular factors involved in the biofilm formation in the wild-type strain PA14 in an abiotic biofilm system (Anderson *et al*., 2014). In plant growth experiment it was observed that 100% seeds germinated under 0m M and 100 mM NaCl concentration whereas shoot length and root length decreased in the presence of salinity. Many reports confirmed the NaCl tolerance of bacteria and plants towards salinity .Without salt stress, bacteria showed 10 % increase in seed germination over respective non-inoculated control (Afrasayab et al., 2010). The previous findings are in line with the results showing that salinity along with bacterial inoculation significantly improved the length parameters as compared to non-inoculated plants of peanuts (*Arachis hypogaea*) (Dey et al., 2004). Similiar findings were observed in plants inoculated with *Brachybacterium saurashtrense*, *Brevibacterium casei* and *Haererohalobacter* which increased the growth of plants in comparison to the control plants (Ullah and Bano 2017). Farqan Orhan et al., 2016 also reported similar results for radish inoculated with *Staphylococcus kloosii* and *Kocuria erythromyxa* and wheat inoculated with *P. rifietoensis* under salt stress. In order to enhance wheat productivity under salt stress, different approaches such as nutrient management fertilizer use and hormonal applications have been used. Bacterial isolates with a potential of forming biofilm showed a potential of plant growth promotion under salt stress and from the present study it was found that isolate SY can tolerate salt stress by forming strongest biofilm in the presence of salt. As the result of plants growth experiment reveals that the 100 % seed germinated in the presence of this particular isolate under 0 mM and 100 mM NaCl concentration. Salinity has been reported to reduce the seed germination phase however, inoculation with plant growth promoting

rhizobacteria have been reported to promising for promoting the growth and development of plants (Foti et al., (2018) The length of root reduction is concomitant with increase in salinity as reported in recent study where the root has been reported as a regulatory organ for solute transportation across the cell (Rahneshan et al., 2018). This means that this isolate can be used for future studies to enhance its traits on molecular level and used as biofertiliser for the germination of seeds in saline environment.

While working on the plant growth promoting Rhizobacterial isolates with a promising potential for plant growth promotion further research work is in progress to check the efficacy of these isolates in natural saline conditions of soil and water.

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