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SURVIVABILITY OF *PSEUDOMONAS PUTIDA* RS-198 IN LIQUID FORMULATIONS AND EVALUATION ITS GROWTH-PROMOTING ABILITIES ON COTTON

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ABSTRACT

This study was aimed to screen nutrient carriers, additives, and major chemical fertilizer components for the development liquid formulations of *Pseudomonas putid* Rs-198 bacterial strain. The number of viable colony forming cells in the formulation amended with corn flour, urea, bentonite, sodium carboxymethyl cellulose, and sodium alginate were found to be 1×10^8 CFU/ml after six months at 25±3°C. *D*- and *Z*-values of strain Rs-198 formulated with different substances were significantly higher than that control at 40-60°C. Moreover, the *D*- and *Z*-values of strain Rs-198 incorporated in chemical fertilizer nutrient components was slightly differed at the same temperature however was significantly higher as compared to control. Pot study showed that the inoculation of developed four *Pseudomonas putida* Rs-198 liquid formulations was beneficial in terms of cotton growth under salt stress conditions; as a result, germination rate and biomass were significant increased. These formulations may alleviate salt stress by promoting soluble protein content or decreasing proline and malondialdehyde accumulation in cotton.

Key words: *P. putida* Rs-198, survivability, biofertilizer, inoculation, cotton.

INTRODUCTION

Conventional agriculture is highly dependent on chemical fertilizers inputs in order to maintain high yields, while its usage can have adverse effects on groundwater quality and making these agricultural practices unsustainable in the long term (Sanchez Perez *et al*., 2003). PGPR is a group of root-colonizing bacteria in the rhizosphere of many plant species and they have the ability to maintenance of root health, nutrient uptake and tolerance to environmental stresses to enhance plant growth and commercial yield (Yue *et al*., 2007; Yang *et al*., 2009).

However, the natural roles of rhizosphere microorganisms are being marginalized due to conventional farming practices such as tillage and high inputs of and pesticides (Mader *et al*., 2002). To overcome this problem, a new eco-friendly management strategy has been developed by treating crop seeds and seedlings with plant growth-promoting rhizobacteria (PGPR) (Datta, 2012; Egamberdieva, 2012). Tomato plants treated with *Bacillus subitilis* yielded the highest number of leaves compared to the control (Woitke *et al*., 2004). *Azospirillum lipoferum* can colonization at wheat rhizosphere of non-soil culture and promote wheat growth and biomass accumulation, alleviate the damage caused by salt stress on wheat (Bacilio *et al*., 2004).

Microbial inoculants, in which microorganisms play a key role, can be used in agricultural production to obtain a specific fertilizer effect, because these inoculants contain microbial activity factors that can promote plant growth. Therefore, developing protective inoculants to extend the high population of viable of long-term retention and effectiveness in microbial agents are very important. As Caesar and Burr (1991) reported that, the survival of strains treated with sucrose, with or without bentonite, and formulated in methylcellulose and talc was equal to or greater than that of 24 h old cultures suspended in 20% xanthan gum and talc.

Numerous researchers have demonstrated the positive effects of nutrient carriers and additives on the survivability of bacteria. Tittabutr *et al*. (2007) reported that liquid inoculants formulated with polymeric additives promoted long-term survival of all rhizobial strains. The active ingredient of biofertilizer is sensitive to many environmental factors, temperature is one of the main factors contributing to the quality of the formulation, which reflected in the efficacy and the long shelf life of the biofertilizer (Mejri *et al*., 2013). In addition, a model was developed by Yao *et al*. (2008), who reported that the survival rate of five freeze-dried bacteria species was described in terms of reaction rate constants (*D*) and temperature sensitivity of rate constants (*Z*).

Under stress conditions, bacteria in rhizosphere may enhance the plant growth by different mechanisms such as by optimizing the supply of nutrients, synthesis of phytohormones IAA and ACC, solubilization of inorganic phosphorus, bioaccumulation or leaching of metals and inhibiting the activity of pathogens (Yang *et al*., 2009). Typically a bacterium directly affects the plant growth and development by using one or more of these mechanisms (Gamalero *et al*., 2008). Soluble protein and proline are common osmolytes in higher plants and accumulate in response to stress. Ansary *et al*. (2012) reported that drought stress triggers an increase in leaf

proline in maize plants; this response is further enhanced in plants inoculated with *Pseudomonas fluorescens*, which can promote plant growth. MDA content is considered as one of the determinants that indicate the severity of stress experienced by a plant (Parvanova *et al*., 2004).

Thus, in this study, we investigated the compatibility of Rs-198 with different nutrient carriers, additives, and main chemical fertilizer components. For obtaining a suitable liquid formulation of the Rs-198 liquid bacterial fertilizer, the effect of nutrient carriers, additives, and main chemical fertilizer components on Rs-198 stability at room temperature as well as at different high temperatures were studied. Appropriate *P. putida* Rs-198 liquid formulations were used to investigate cotton seed germination and growth indicators under high concentration of salt stress condition. Soluble protein, proline, and MDA contents were analyzed. The results could be used as a basis of further development of microbial fertilizer liquid formulation.

MATERIALS AND METHODS

Bacterial strain and culture medium: The strain, Rs-198 used in this study was previously isolated from salinization soil in Xinjiang (Yao *et al*., 2010; Peng *et al*., 2013). The Rs-198 were cultured in nutrient broth (NB) liquid medium (5 g beef extract, 10 g peptone, 5 g NaCl, 1000 ml H_2O, pH 7.0-7.2) with shaking at 200 rpm and at 30°C for 48 h. The cells concentration in the broth was determined as 10^{13} CFU/ml by counting the colony-forming units (CFU) present on NA agar plates after serial dilution for overnight incubation at 30°C.

Biocompatibility of Rs-198 with different constitutents liquid formulations: Various nutrient carriers (humic acid 10 g/L, glutamic acid 10 g/L, urea 5 g/L, and corn flour 100 g/L), additives (bentonite 10 g/L, sodium carboxymethyl cellulose (CMC) 20 g/L, sodium alginate 50 g/L, and polyvinyl alcohol 20 g/L), and the main chemical fertilizer components (K_2SO_4 60 g/L, $(NH_4)_2HPO_4$ 10 g/L, $CaCl_2$ 20 g/L, and KCl 20 g/L) were mixed with nutrient agar (NA) solid medium, inoculated with 3% Rs-198 inoculum and incubated for 48 h in an electric incubator at 30°C. This process was performed in triplicate. The degree of compatibility of Rs-198 with the nutrient carriers, additives, and the main chemical fertilizer components were determined following the method of Wu *et al*. (2014).

Survivability of Rs-198 in liquid formulations: Nutrient carriers, additives and chemical fertilizers were investigated to determine the survivability of Rs-198 strain with these substances. The incubated solutions were sterilized at 121°C for 20 min, 10% inoculated and allowed to stand at different temperature (room and 40, 45, 50, 55, 60°C). The survival performance of Rs-198 was investigated at 3, 7, 10, 14, 21, 28, 35, 60, 90, 120, 150, and 180 d. The viability of bacterial population count among various formulations was determined by serial dilutions from formulations plated in triplicate on nutrient agar after 24 h culture (CFU/ml) (Omer, 2010). Sterile distilled water served as a control and was processed similar to the formulations.

Survivability evaluation of Rs-198: Thermal inactivation of microorganisms obeys first-order kinetics (Shull *et al*., 1963). Therefore, for each temperature, the natural logarithm of the survivor ratio was plotted versus the equivalent time durations. The mathematical model for determining the *D*-value of Rs-198 in a fixed concentration of different substances was based on the differential balance of the first order (considering similarities with thermal processes):

$$\log(\frac{N}{N_0}) = -\frac{1}{D}t \quad (1)$$

Where N_0 is the initial number of viable bacteria; N is the number of viable bacteria per milliliter (CFU/ml) at any time, t is the exposure time; D is defined as the constant of decimal reduction, which represents the time necessary to reduce a $\log_{10}$ cycle along the process.

For obtaining the *D*-value, a linear regression was performed among the different exposure time of the microorganism to the substances and the CFU $\log_{10}$ of survivors. Following the procedure, the death constant for Rs-198 in relation to the substances (*Z*-value) was calculated by equation (4):

$$\log(\frac{D_1}{D_2}) = \frac{1}{Z}(C_1 - C_2) \quad (2)$$

Where D_1 and D_2 are the values of decimal reduction for concentrations C_1 and *C2*, respectively. The Z constant represents the alteration in a concentration necessary to produce a reduction in one $\log_{10}$ cycle (90% of reduction) on the death time caused by the substances. For the *Z*-value determination, a linear regression between bacteria concentration (C) and the $\log_{10}$ of the respective *D*-value was calculated.

The temperature ranges from 40 to 60°C, spanning most of the growth range for Rs-198, was selected to assess the effect of high temperatures on inactivation rate.

Greenhouse pot experiment: Pot experiments on the cotton plant were carried out with four formations of *P. putida* Rs-198 liquid bacterial manure under salt condition, such as MF1, MF2, MF3, and MF4 (Table 4). The treatments were made uniform by adding 50 ml of 0.7% NaCl (w/v) solution into the vermiculite in per pot. Ten cottonseeds were sown in each pot filled with 100 g vermiculite (diameter 0.5 mm) and each treatment included eight replicates. Approximately 20 ml of *P. putida* Rs-198 liquid formulations diluted ten times (10^{12} CFU/ml) were injected using syringe to cotton roots once

a week after these plants were planted. The pots were arranged in a randomized design and placed in an artificial climatic box with day/night temperature of 20°C, 400 µmol photons $(m^2s)^{-1}$ of light supplied for 14 h during the day time and humidity was maintained at 60% by irrigated sterile water using injection 4-5 times a week. The number of germination, germination rate was calculated at 10 d after planting. Plants were harvested at maturity (50 d) and cotton growth characteristics, i.e., fresh weight, dry weight, root length, soluble protein, proline, and MDA were recorded.

Growth parameters: 50 days after sowing, five plants from each replicate were randomly harvested, and data on plant growth variables, such as germination, plant height, root length, fresh weight and dry weight, were collected. The plant material for dry weight was dried at 105°C for 10 min and then 75°C for constant weight.

Soluble protein contents: Bradford (1976) method was followed to measure protein contents in green leaves. About 0.2 g fresh leaves from each sample, with 5 ml of distilled water after grinding homogenate, centrifugal at 10000 g for 10 min and the supernatant was collected. A reaction mixture consisting of 1 ml leaf extract and 2 ml of Bradford reagent was incubated at room temperature for 2 min. Absorbance was measured at 595 nm and total soluble protein concentration (µg/ml) was calculated using bovine serum albumin standard curve.

Estimation of proline content: The proline content was determined according the method by Bates *et al*. (1973). Leaf tissue (0.5 g) was crushed to a fine powder in the presence of liquid nitrogen homogenized in 5 ml of 3% aqueous sulphosalicylic acid. The homogenate was boiled at 100°C for 10 min, then filtered and 2 ml of the filtrate was mixed with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube for 30 min at 100°C in water bath. The reaction was terminated by placing the test tubes on ice followed by proline extraction with 4 ml toluene. The chromophore-containing phase was collected and kept at room temperature for 15 min before measuring the absorbance at 520 nm. Proline concentration was determined from a standard curve and calculated on a fresh weight basis (µmol/g FW).

Determination of MDA content: The MDA content of the cotton leaves was calculated following the method described by Jambunathan (2010). 0.5 g leaf tissues was homogenized in 4 ml phosphate buffer solution and then centrifuged at 8000 g for 20 min. Then 1 ml of the supernatant, extracted in 3 ml of 0.1 % trichloroacetic acid (TCA, 20%) and 1 ml of thiobarbituric acid (0.5%) was heated at 95°C boiling 30 min, after cooling were collected by centrifugation (8000 g for 10 min). Finally, the supernatant were measured in 532 nm and 600 nm wavelength absorbance values and in accordance with the following formula to calculate the MDA content.

MDA concentration (mmol g^{-1}) =

$$\frac{(OD_{532} - OD_{600}) \times A \times V \times a}{1.55 \times 10^{-1} \times W} \quad (3)$$

where A is the amount of reaction liquid (5 ml), V is the amount of extract (4 ml), a is the actual amount of extract (1 ml), and W is the weight of materials (g).

Data analysis: Data were analyzed by analysis of variance (ANOVA) using the general linear model version 9.1; SAS institute Inc, Cary, NC, USA. Significance levels were within confidence limits of 0.05 or less.

RESULTS AND DISCUSSION

Biocompatibility of Rs-198 and different substances: Experimental results (Table 1) show that the nutrient carriers, additives, and main chemical fertilizer components have a good compatibility with Rs-198. In the liquid formulations, the bacteria in nutrient carriers, additives, and main chemical fertilizer components were in a relatively stable environment. The nutrition and substance metabolism of bacteria with the external environment was slow when the bacteria were at rest and dormant state. The effect of the nutrient carriers, additives, and the main chemical fertilizer components on Rs-198 stability was further studied.

Survivability of Rs-198 in liquid formulations with different nutrient carriers: Room temperature: As expected, longer storage periods led to lower numbers of viable bacteria in different nutrient carriers after six months of storage (Fig. 1). However, the viability of Rs-198 in different nutrient carriers was not uniform as the storage time elapsed. The number of viable bacteria in humic acid, glutamic acid, and urea significant increased in the first 30 d, followed by a stationary phase. The number of viable bacteria in corn flour slightly increased in the first 45 d, before undergoing a stationary phase. This result is probably because the nutrients in different nutrient carriers were more adequate in the initial bacteria inoculation and is suitable for bacteria growth. Corn flour contains a variety of nutrients, providing more carbon and nitrogen sources for Rs-198 growth, so its bacterial growth phase became longer. But the nutrients were gradually reduced with continued bacterial grow, and not sufficient to maintain bacterial growth requirement or could be due to competition and stationary phase. Therefore, the numbers of viable bacteria in different nutrient carriers decreased. At 180 d, the number of viable bacteria in corn flour and urea were over 1×10^8 CFU/ml, compared with over 1×10^7 CFU/ml in humic acid and glutamic acid. Anandham *et al*. (2007) proposed that clay and rice bran (1: 1) +10% rock phosphates amend with 1% glucose supported the longer shelf life of *Burkholderia* sp. compared to glycerol-amended and

nutrient-unamended pellets. Therefore, Rs-198 has better bacterial survivability in corn flour and urea as nutrient carriers, and its shelf life was prolonged successfully.

Table 1. The compatibility of strain Rs-198 with different nutrition carriers, additives and main components of chemical fertilizers.

Compatibility					
Nutrition carrier		**Additive**		**Fertilizer**	
Humic acid	++	Bentonite	+++	K_2SO_4	++
Glutamic acid	+	CMC	+++	$(NH_4)_2HPO_4$	++
Urea	+++	Alginate	+++	$CaCl_2$	+++
Corn flour	+++	Polyvinylalcohol	++	KCl	++

Note: +++grow well (The numbers of colonies on the plate of different nutrition are one order magnitude smaller than that of the control); ++better growth (The numbers of colonies on the plate of different nutrition are as much as that of the control); +growth (The numbers of colonies on the plate of different nutrition are one order magnitude more than that of the control). All values are means of three independent readings.

Effect of high temperature on *P. putida* Rs-198: Study shows that the *D*-values of Rs-198 in different nutrient carriers continued to decrease as the temperature increased (Table 2), which indicated that the survivability of Rs-198 strain continues to decrease. The *D*-values of Rs-198 in the four additives were higher than those in the four nutrient carriers at different high temperatures. The *D*-values of strain Rs-198 in urea and corn flour were significantly higher than those of Rs-198 in other nutrient carriers. In addition, the *Z*-values of Rs-198 in urea and corn flour were 31.56% and 30.04% higher than that in control, respectively (Table 2). This result indicates that Rs-198 in different temperature-sensitive nutrient carriers is relatively weak, and the effects of temperature are smaller in this condition. This phenomenon illustrate that urea and corn flour in which contents more abundant nutrients than other nutrient carriers play a protective roles promoting bacterial growth and reducing bacterial mortality to a certain extent, thus, has better bacterial survivability.

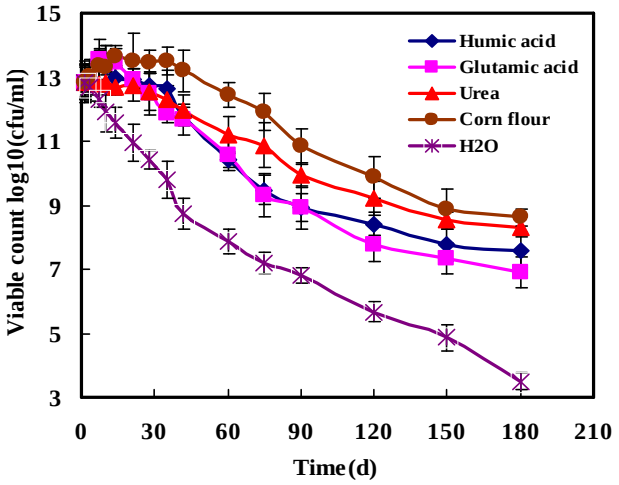

Fig. 1 Viability of Rs-198 in different nutrient carriers incubated at room temperature. Error bars are standard error of means (± SE)

Table 2. *D*-values and temperature sensitivity indicator Z-values of *Pseudomonas putida* Rs-198 incubated with different nutrition carriers.

D-values	Humic acid	Glutamic acid	Urea	Corn flour	Control H_2O
D_{40}	20.24	20.96	21.94	25.84	19.1
D_{45}	16.39	17.48	17.95	19.42	14.7
D_{50}	12.63	14.73	14.77	15.51	11.34
D_{55}	7.26	8.27	8.38	8.38	7.12
D_{60}	6.71	6.72	7.66	7.13	4.35
Z-value	40.98	33.84	45.66	44.67	31.25

D_{40}-D_{60} is the *D*-value at 40°C to 60°C.

Survivability of Rs-198 strain in liquid formulations with different additives:

Room Temperature: Additives and nutrient carriers had the same effect, and the shelf life of Rs-198 was extended significantly after adding additives (Fig. 2). In the additives, the number of viable bacteria was over 1×10^{12}

CFU/ml and remained unchanged within the first 45 d. This phenomenon may be because that these formulations contained appropriate amount of water and nutrients, and after the additives has been inoculated, bacterial growth continued under appropriate conditions. The number of viable bacteria was over 1×10^8 CFU/ml in bentonite, CMC, and sodium alginate at 180 d, compared with over 1×10^7 CFU/ml in polyvinyl alcohol. Bentonite had the highest number of viable bacteria, followed by sodium alginate, probably because bentonite forms a colloid with water after undergoing complete hydration, in addition to good adsorption and fixed bacterial performance, thus, playing a protective role for bacteria. These mechanisms may have contributed to the observed increase in growth parameters (Hao, 2012). During decay, the amount of biomass decreased after 45 d. This population decline on prolonged incubation may be attributed to nutrient depletion and cell autolysis (Anandham, 2007).

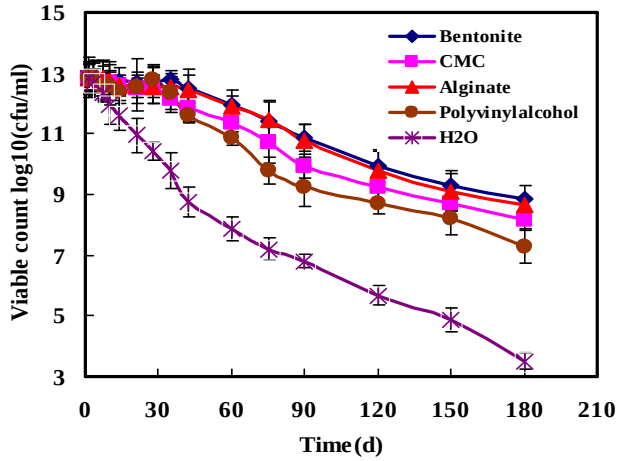

Fig. 2 Viability of Rs-198 in different additives incubated at room temperature. Error bars are standard error of means (± SE).

High Temperature: This result indicated that additives have better survivability with bacteria and preferably protect bacteria against the environment. The *D*-values of Rs-198 in bentonite, sodium alginate, and CMC were significantly higher than that in the control (Table 3). The *D*-values of Rs-198 strain in bentonite were highest, and its D_{40} and D_{60} were 25.92% and 53.94% higher than those in the control. And CMC has D_{40} and D_{60} were 22.86% and 50.39% higher than those in control. In addition, the *Z*-values of Rs-198 in bentonite, CMC, and sodium alginate were higher by 26.96%, 23.75%, and 27.28% than that in the control, respectively (Table 3). This result indicated that temperature-sensitive Rs-198 strain are relatively weak and become smaller in these liquid formulations as a result of temperature. At 180 d, the number of viable bacteria in bentonite, CMC, and sodium alginate were over 1×10^8 CFU/ml, and they were relatively low sensitivity to temperature. Therefore, they were selected as the additives of Rs-198 liquid fertilizer. Similar results were found by Heijnen *et al*. (1992) that the presence of bentonite-created protective microhabitats had a greater influence on rhizobial survival than the physiological state of the cells prior to the introduction into soil. This explained that strain Rs-198 has a good survivability with additives and extent effectively the shelf life of the bacteria. These results are similar with the findings of Omer (2010) in which 1% CMC as binder in formulations of the *Bacillus megatherium* with other enrichment materials, which have a long storage life, acid product delivery and promote the plant growth parameters, were prepared to be used instead of the traditionally used free spore suspension.

Survivability of Rs-198 in the main chemical fertilizer components

Room Temperature: Use of biological methods combined with chemical fertilizer was one of the most effective ways to reduce fertilizer levels below those recommended for optimum yields (Yang, 2009). Therefore, the survivability of Rs-198 strain in the main chemical fertilizer components was studied for the partial or complete replacement of chemical fertilizer with microbial agents. Fig. 3 indicated that the number of viable bacteria continued to decrease by prolonging the storage time in the main chemical fertilizer component $CaCl_2$, $(NH_4)_2HPO_4$, KCl, K_2SO_4, whereas the number of viable bacteria in these components slightly differed. At 180 d storage, the number of viable bacteria was maintained at over 1×10^7 CFU/ml in the main chemical fertilizer components. The results indicated that the survivability of Rs-198 strain in the main chemical fertilizer components is better and can be completely applied with a fertilizer, instead of partially combining with the chemical fertilizer.

High Temperature: The *D*-values of Rs-198 strain slightly differs at the same temperature in the main chemical fertilizer components, but are significantly higher than that in the control (Table 4). KCl has the highest *D*-value of Rs-198, followed by K_2SO_4, $CaCl_2$, and $(NH_4)_2HPO_4$ indicating that Rs-198 strain has good compatibility with the main chemical fertilizer components along with its ability to protect bacteria and reduce bacterial mortality. Those result further illustrates that Rs-198 microbial agent can be applied in combination with the chemical fertilizer, instead of being partially used with the chemical fertilizer.

Table 3. *D*-values and temperature sensitivity indicator Z-values of *Pseudomonas putida* Rs-198 incubated with different additive.

D-value	Bentonite	CMC	Alginate	Polyvinylalcohol	Control H_2O
D_{40}	25.77	24.75	24.88	24.51	19.1
D_{45}	23.53	20.12	21.12	20.16	14.7
D_{50}	17.35	15.37	16.66	14.82	11.34
D_{55}	14.21	12.7	13.6	12.21	7.12
D_{60}	9.44	8.76	8.28	8.12	4.35
Z-value	42.787	40.984	42.971	35.553	31.25

D_{40}-D_{60} is the *D*-value at 40 °C to 60 °C.

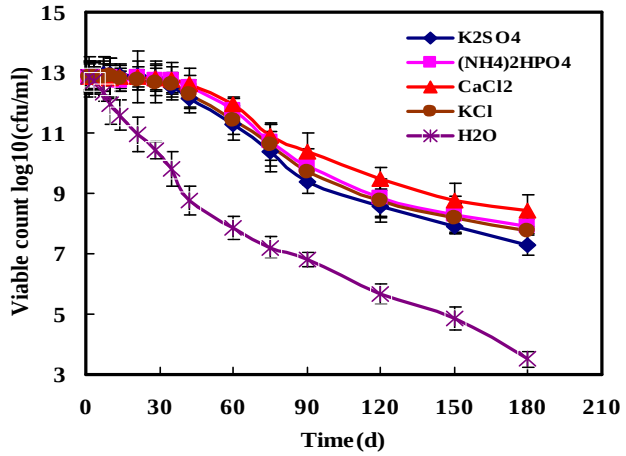

Fig. 3 Viability of Rs-198 in the main components of chemical fertilizers incubated at room temperature. Error bars are standard error of means (± SE).

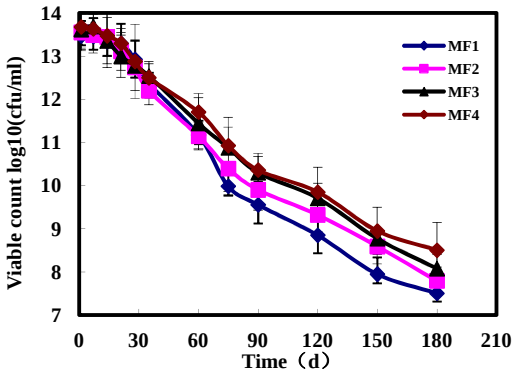

Fig. 4 Viability of Rs-198 in compound microbial fertilizer incubated at room temperature. Error bars are standard error of means (± SE).

The *Z*-values of Rs-198 in the main chemical fertilizer component were significantly higher than that in control (Table 4) indicating that temperature sensitive Rs-198 is relatively weak and affected by the temperature as it became smaller in this condition. Experimental results also showed that the N, P, and K elements in liquid microbial fertilizer were helped to prolong the survival of Rs-198 strain. Several studies are currently testing the hypothesis that PGPR might enable agricultural plants to maintain productivity with reduced fertilizer application, and the preliminary results are promising.

For example, one field study with wheat (*Triticum aestivum* L.) (Shaharoona *et al*., 2008), the yield for plants that were given 75% of the recommended amount of N-P-K fertilizer plus a PGPR strain was equivalent to the yield for plants that were given the full amount of fertilizer but without PGPR. These results are as similar as described by Omer (2010) that a maximum spore percentage of *Bacillus megatherium* was recorded after 96 hours of inoculation into a modified nutrient medium containing a mixture of 500 ppm of $MnSO_4$, $CaCl_2$, $ZnSO_4$ and KCl.

Survivability of Rs-198 in the compound microbial fertilizer: The ingredients of compound microbial fertilizers have been obtained by studying the survivability of Rs-198 in liquid formulations with different nutrient carriers, additives, and chemical fertilizers at room temperature as well as at high temperatures. Through screening of the nutrient carrier, additives, and main chemical fertilizer components, the ingredients of four compound microbial fertilizers were obtained completely (Table 5). At first, the number of viable bacteria was 4.8×10^{13} CFU/ml and maintained at over 3×10^{8} CFU/ml in the compound microbial fertilizer after 180 d storage at room temperature (Fig. 4). The commercial use of bacterial fertilizers requires an inoculum with high bacterial viability retention and is easily transported and applied to seed. Viable bacteria formulation is intended to ensure that adequate bacterial viability is sustained to increase bacterial efficacy as well as facilitate the delivery and handling processes (Omer, 2010). Therefore, this compound microbial fertilizer has a longer storage life and may be used for commercial production.

Table 4. *D*-values and temperature sensitivity indicator Z-values of *Pseudomonas putida* Rs-198 incubated with different chemical fertilizer.

D-value	K_2SO_4	$(NH_4)_2HPO_4$	$CaCl_2$	KCl	Control H_2O
D_{40}	24.1	21.79	23.1	26.53	19.1
D_{45}	19.34	17.92	19.17	20.34	14.7
D_{50}	14.25	13.18	14.14	14.66	11.34
D_{55}	11.52	8.13	8.53	11.55	7.12
D_{60}	7.69	7.07	7.46	7.89	4.35
Z-value	41.152	36.364	37.362	39.216	31.25

D_{40}-D_{60} is the *D*-value at 40°C to 60°C.

Table 5 Components of Rs-198 liquid bacterial formulations (wt.%)

Formulations	Bacterial	Humic acid	Urea	Corn flour	Bentonite	Alginate	K_2SO_4	KCl	$(NH_4)_2HPO_4$
MF1	50%	1%	4%	7%	2%	1%	1%	5%	2%
MF2	60%	2%	3%	6%	2%	1%	2%	4%	3%
MF3	70%	3%	2%	5%	1%	2%	3%	3%	4%
MF4	80%	4%	1%	4%	1%	2%	4%	2%	5%
NMF	0	4%	1%	4%	1%	2%	4%	2%	5%

Table 6. Effects of salt stress condition on germination rate and seedling biomass.

Treatment	germination rate (%)	plant height (cm)	root length (cm)	fresh weight (g)	dry weight (g)
NMF	40.55±1.75	13.48±0.51	5.30±0.25	1.23±0.038	1.12±0.021
MF1	60.44±1.98	15.29±0.45	5.84±0.31	1.62±0.045	1.30±0.018
MF2	65.67±2.03	15.75±0.58	6.22±0.29	1.65±0.031	1.33±0.025
MF3	68.32±1.94	16.53±0.48	7.06±0.31	1.68±0.033	1.36±0.021
MF4	70.34±2.15	17.08±0.51	7.42±0.25	1.72±0.035	1.43±0.027

Error bars are standard error of means (± SE).

Inoculation of *P. putida* Rs-198 liquid bacterial fertilizer on cotton under salt condition

Seedling germination rate of cotton: *P. putida* Rs-198 liquid bacterial fertilizer significantly improved germination rate from 32.91 % to 42.35 % under salt stress compared with the respective non-inoculated controls. MF3 and MF4 treatments also improved germination rate by 40.65 % and 42.35 %, respectively. These results showed that MF3 and MF4 *P. putida* Rs-198 liquid bacterial fertilizer were beneficial for cotton exposed to salt stress, resulting in a significant increase in germination rate compared with the control group (Tables 6). Thus, MF3 and MF4 liquid bacterial fertilizer can be applied as biotechnological tools to increase secondary metabolite production and help understand specific adaptive processes (Cappellari *et al*., 2013).

Biomass of cotton: Results showed that Rs-198 applied to soil could significantly ($P \leq 0.05$) increase cotton growth compared with the control plants (Tables 6). Plants inoculated with Rs-198 showed an 11.84 % to 21.08 % increase in plant height. Furthermore, a 9.25 % to 28.57 % increase in shoot length was observed in plants grown in soil treated with Rs-198 compared with that of the control plants. Plants grown under salt stress condition but not treated with Rs-198 showed approximately 24.07 % to 28.49 % increase in fresh weight and 13.85 % to 21.68 % increase in dry weight compared with cotton plants grown in the presence of Rs-198. A maximum increase in plant height, shoot length, fresh weight, and dry weight was observed because of Rs-198 compared with the respective control plants; likewise, these parameters were increased in MF3- and MF4-fertilized plants. The weight of plant tissues increased in response to inoculated bacteria possibly because of increased root growth, thereby occupying greater soil volume for efficient nutrient uptake; indeed, biomass production increased. Enhanced nutrient concentrations in plant tissues were reported by bacterial inoculation under stress conditions (Nadeem *et al*., 2006).

Soluble protein content: Under salt stress condition, the soluble protein contents of MF3- and MF4-treated plants were higher than those of MF1-, MF2-, and NMF-treated plants. Approximately 500% more soluble proteins in

MF3 and MF4 were recorded in the leaves of inoculated plants compared with those of the respective control plants under salt stress (Fig. 5). Thus, MF3 and MF4 treatment may help alleviate salt stress and promote cotton growth. Based on the results, our conclusion is that MF3 and MF4 could effectively protect Rs-198; this strain possibly prevented soluble protein degradation, thereby maintaining soluble protein content at high levels. High soluble protein contents elicited regulatory effects on intracellular osmotic potential when plants were exposed to salt stress. Changes in osmotic potential may also be attributed to the upregulation of protein biosynthesis because proline, as an osmolyte, can help maintain cell water status and protect membranes and proteins from degradation (Yoshiba *et al*., 1997). Islam (2014) also showed that *P. aeruginosa* inoculations in non-contaminated soil can increase total soluble protein up to 8.7 percent.

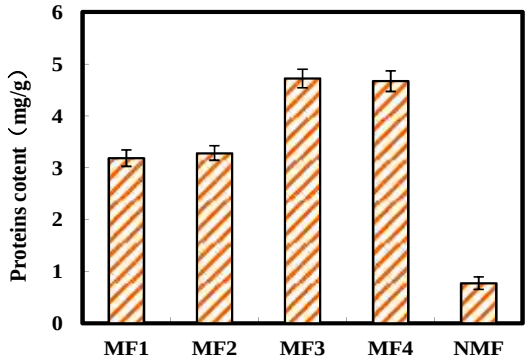

Fig. 5 Effect of different treatments on the soluble proteins concentration of cotton seedling. Error bars are standard error of means (± SE).

Proline content: Proline, which is usually considered as an osmoprotectant, may also be involved in reducing oxidative damage by scavenging free radicals (Vendruscolo *et al*., 2007). Changes in proline content were determined to investigate the salt stress-alleviating effect of PGPR. A significant decrease in proline content was recorded in plants grown in soil inoculated with Rs-198 compared with that in plants grown in uninoculated soil under stress conditions (Fig. 6). Notably, the proline contents of plants in MF3 and MF4 treatments were reduced at a greater extent than those of the respective uninoculated plants under salt stress.

Compared with the inoculated plants, the uninoculated plants under soil salinity conditions showed increased antioxidant activity and proline concentration (Han and Lee, 2005). However, (Sandhya *et al*., 2011) also reported that inoculation with *Bacillus* species increases proline content of maize seedlings during drought.

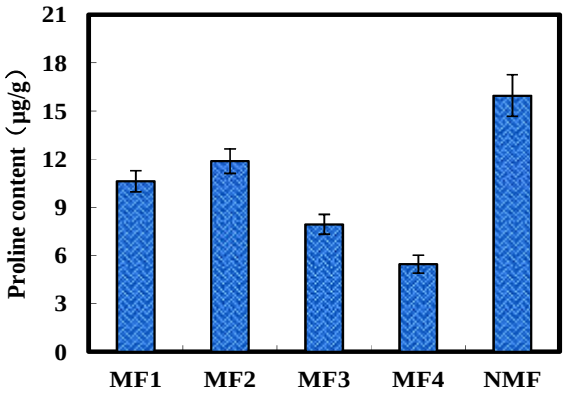

Fig. 6 Effect of different treatments on the proline concentration of cotton seedling. Error bars are standard error of means (± SE).

MDA content: It is quite clear that MDA accumulation during stress is one of the mechanisms of tolerance. It was observed that although MDA content, a measure of lipid peroxidation, decreased in all inoculation in contrast to respective non-inoculation plants during salt stresses (Fig. 7). MF3- and MF4-treated plants showed that MDA contents were decreased by a maximum of 47.73% and 54.55%, respectively, under salt stress compared with their respective uninoculated controls.

Han and Lee (2005) also reported that MDA content in inoculated plants, under soil salinity conditions had a decrease compared to the un-inoculated plant. The results suggested that inoculation of salt-stressed plant with PGPR strains could alleviate salinity stress. The findings are similar to Islam (2014), who noted a reduced concentration of MDA in bacterial inoculation under Zn stress.

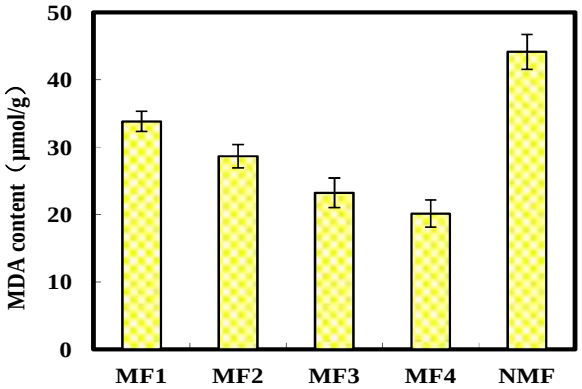

Fig. 7 Effect of different treatments on the MDA concentration of cotton seedling. Error bars are standard error of means (± SE).

Conclusions: Experimental results showed that nutrient carriers, additives, and major chemical fertilizer components were compatible with Rs-198 and the

number of viable bacteria in the formulations with these substances was significantly higher than those in the control up to > 10^8 CFU/ml after 180 day storage. These results indicated that these substances elicited a protective effect on bacteria by reducing bacterial mortality and prolonging the shelf life of bacterial formulations. In addition, pot experiment results showed that *P. putida* Rs-198 formulations MF3 and MF4 treatments significantly increased the biomass, soluble protein content and decreased proline, MDA accumulation in cotton seedlings under salt stress conditions. These functions might have a key role in tolerance to salinity of cotton. That is to say, MF3 and MF4 can protect Rs-198 effectively and can be used to partially or eliminate the effects of salt stress on the growth of cotton. Furthermore, this study provided a strategy to the promising application of PGPR liquid formulations that can be used to enhance plant tolerance to various stress.

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