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Growth promotion of maize by desiccation tolerant *Micrococcus luteus*-chp37 isolated from Cholistan desert, Pakistan

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Abstract

Present study deals with the isolation and characterization of desiccation tolerant bacteria *Micrococcus luteus*-chp37 from rhizosphere of plant growing in the Cholistan desert of Pakistan. To check the desiccation tolerance, strain was incubated at various levels of relative humidity (5, 27 and 100%) for a period of 6, 12 and 18 days. The surviving fractions of *M. luteus*-chp37 after 18 days of desiccation, under RH 5, 27 and 100% were 2, 4 and 0.6%, respectively. *M. luteus*-chp37 incubated under RH 5% and 100% lost its viability at much faster rates as compared to RH 27%. To check the plant growth promoting ability of this strain, pot experiments with *Zea mays* (under sandy and pure field soil) were conducted and various growth parameters (germination, root and shoot length, number of leaves, dry weight per gram fresh weight, chlorophyll contents) were measured after 90 days maize plants. *M. luteus*-chp37 inoculation resulted 8.5, 13, 23 and 54% increases in number of leaves, shoot length, root length and dry weight g^{-1} fresh weight, respectively, as compared to un-inoculated control maize plants. Beside this there were 159, 80, 84% and increase in chlorophyll a, chlorophyll b and total carotenoids respectively in pure field soil. This growth promotion was attributed as a result of production of plant growth promoting substances cytokine and hydrogen cyanide.

Keywords: Bacteria; cytokinin; exopolysaccharide; maize; phyto-stimulation.
Abbreviations: EPS - exopolysaccharide; RH - Relative humidity; HCN - hydrogen cyanide; IAA - indole acetic acid; PGPR - plant growth promoting rhizobacteria; PS - pure soil; MS - mixed soil; TSB - Tryptic soya broth; TGY - tryptone glucose yeast; CFU - colony forming units .

Introduction

Desiccation tolerance is the ability of vegetative cells to undergo nearly absolute dehydration through air drying, without being killed and eventually leading to deactivation of metabolic activities (Vriezen et al., 2007). Many bacteria survive in dry conditions by producing spores or cysts around them (Leggett et al., 2012), but others do not have such structures and they have evolved some other alternate mechanisms such as by producing compatible solutes (Welsh, 2000). Now a day's desiccation and salinity are the two biggest problems faced by agriculture sector which effect crop yield (Zahran, 1999). Desiccation tolerance in soil bacteria could be an important trait, as soil bacteria must be active and positively affect the plant growth when it needs, especially in water deficient conditions (Humann et al., 2009). The quantity and the type of bacteria present in different soils types are influenced by the soil physico-chemical conditions including temperature, moisture, salts, chemicals and the presence various types of plants found in those soils (Peixoto et al., 2010). The effect of soil bacteria on plant may be beneficial, harmful, or neutral (Nascimento et al., 2012; Berggren et al., 2001). Plant growth promoting bacteria showed various mechanisms through which they can promote plant growth such as enhanced nutrients uptake (Yang et al., 2013), secretion of enzymes (Choudhary, 2011), production of inhibitory compounds (Norman et al., 2011), secretion of plant growth regulatory (Karnwal and Kaushik, 2011) and solubilization of phosphate (Ren et al., 2013). Maize is one of the most important cash crops of the world. It is grown in various regions of Pakistan as food and feed. Numerous investigators studied the positive impact of bacteria on maize plants (Gholami et al., 2009; Cevheri, 2012; Jarak et al., 2012). Plant growth promoting bacterial strains may be plant-specific for maize root colonization (Babalola et al., 2003). Pakistan is facing serious problem of water shortage for crop production and it has been estimated that water for irrigation purposes may be reduced up to 50%. To cope with such situation the bacterial inocula applied should be tolerant to high temperature and desiccation. Under abiotic stress bacterial strains produce trehalose which is an important weapon against heat shock and drought (Reina-Bueno et al., 2012). *In vitro* studies have shown that trehalose preserves structural and metabolic activities in drought and heat stress (Ricker et al., 2003). Moreover, directed identification of desiccation tolerant plant growth promoting bacteria would allow the production and development of dry seed inocula (Vriezen et al., 2007). In present study we used EPS producing desiccation tolerant bacterial strain *Micrococcus luteus*-chp37 for the growth responses of maize plant.

Results

Isolation and identification of bacterial strain

Initially twenty bacterial strains were isolated from fifteen different soil and plant samples. Out of twenty strains, one

Table 1. Morphological and biochemical characteristics of strain *Micrococcus luteus*-chp37.

Characteristics	*Micrococcus luteus*-chp37	Characteristics	*Micrococcus luteus*-chp37
Colony shape	Circular	O.F test	A
Colony elevation	Convex	Oxidase	+ve
Colony size (mm)	06	Catalase	+ve
Colony margin	Entire	Nitrate reduction	-ve
Cell shape	Cocci	Denitrification	+ve
Gram staining	+ve	Gas production	-ve
Capsules staining	+ve	Starch hydrolysis	+ve
Spore staining	-ve	Citrate	-ve
Motility	-ve	H_2S production	-ve
Urease	+ve	MRVP	-ve
IAA	-ve	Phosphate solubilization	-ve
Cytokinin	+ve	HCN	+ve

Antibiotics resistance					
Ampicillin (10 µg ml^{-1})	Erythromycin (15 µg ml^{-1})	Kanamycin (30 µg ml^{-1})	Chloroamphenicol (30 µg ml^{-1})	Oxy-tetracycline (300 µg ml^{-1})	Streptomycin (300 µg ml^{-1})
-ve	-ve	-ve	+ve	-ve	-ve

OF, Oxidation fermentation; -, Negative; +, Positive; A, aerobe; MRVP, Methyl Red Voges Proskaur; IAA, indole acetic acid; HCN, hydrogen cyanide.

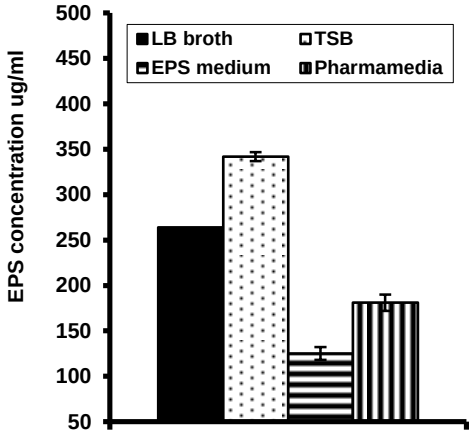

Fig 1. Impact of various growth media (LB broth, Tryptic soya broth medium, EPS media and Pharmamedia) on the exopolysaccharides production in *Micrococcus luteus*-chp37 cells incubated at 37 °C for 72 hrs in an incubating shaker at 150 rpm.

bacterium which showed extraordinary EPS production was selected for further study. Isolate was identified as *Micrococcus luteus*-chp37 by 16S rRNA gene and submitted in gene bank under accession number JF275856. *M. luteus*-chp37 is gram positive aerobic, non-motile cocci with catalase and oxidase positive. The major characteristics of *Micrococcus luteus*-chp37 are given in the Table 1. Strain also showed variable resistance to various antibiotics used.

Plant growth promoting traits

M. luteus-chp37 was screened for various plant growth promoting activities (cytokinin, siderophores, HCN, phosphate solubilization) before going to the field trial. Among phytohormones, the strain *M. luteus*-chp37 was able to produce cytokinin whereas it showed negative results for indole acetic acid. In contrast to cytokinin the strain was unable to produce siderophores and fails to solubilize inorganic phosphates. Moreover *M. luteus*-chp37 also gave positive results for HCN production (Table 1).

Quantitative estimation of EPS

The 72 hrs old culture of *M. luteus*-chp37 grown under different media were subjected to EPS extraction. Fig. 1 showed that maximum EPS production (341 µg ml^{-1}) was achieved when cell were grown in TSB medium. Strain was able to produce 267, 177 and 123 µg ml^{-1} of EPS, under LB broth, Pharmamedia and EPS medium, respectively (Fig. 1).

Desiccation tolerance of the strain

The survival of *M. luteus*-chp37 cells to desiccation at 5, 27 and 100% relative humidity (RH) under ambient pressure was assessed by plate count analysis following incubation periods of 0, 5, 11, and 18 days (Fig. 2). Cell viability of *M. luteus*-chp37 decreased significantly i.e., 40 and 1.4% at 5 and 100% RH, respectively, after 5 days incubation. But at 27% RH, the cell survival rate was better (i.e. 80%) after 5 days of incubation. After 18 days, cell viability decreased to 0.6, 4 and 2% at RH of 100, 27 and 5%, respectively (Fig. 2).

Plant growth experiments

Maize plants were grown till maturity in two different soil conditions (pure soil and in mixed soil) under bacterial inoculation and un-inoculated control. Plants grown in pure soil in inoculated and control condition responded differently in term of seed germination. Apparently bacterial inoculation had negligible effect on seed germination. Table 2 showed that seed germination was enhanced up to 5.6% in case of bacterial inoculated plants grown in PS when compared with

Table 2. Effect of *Micrococcus luteus*-chp37 inoculation on seed germination, number of leaves, shoot length, root length and dry weight per gram fresh weight of *Zea mays* grown in pure and mixed soil.

Soil type	Plant growth parameters	Control	Inoculated	p value[1]
Pure Soil	Germination (%)	94.4±0.33 [a]	100.0±0.0 [b]	<0.001
	Number of leaves	11.8±0.36 [a]	12.8±0.64 [a]	0.207
	Shoot length (cm)	59.6±1.2 [a]	67.8±2.2 [b]	0.01
	Root length (cm)	33.6±1.73 [a]	41.3±1.89 [b]	0.01
	Dry weight g^{-1} fresh weight (g)	0.13±0.01 [a]	0.20±0.07 [a]	0.43
Mixed Soil	Germination (%)	100.0±0.0 [a]	97.2±0.33 [b]	<0.001
	Number of leaves	9.4±0.53 [a]	12.2±0.29 [b]	0.001
	Shoot length (cm)	56.0±2.5 [a]	62.0±1.6 [a]	0.066
	Root length (cm)	35.3±3.29 [a]	47.0±2.05 [b]	0.013
	Dry weight g^{-1} fresh weight (g)	0.16±0.06 [a]	0.20±0.10 [a]	0.75

[1]p value was calculated by T-test. Different letters indicate significant difference between mean of treatments (p = 0.05).

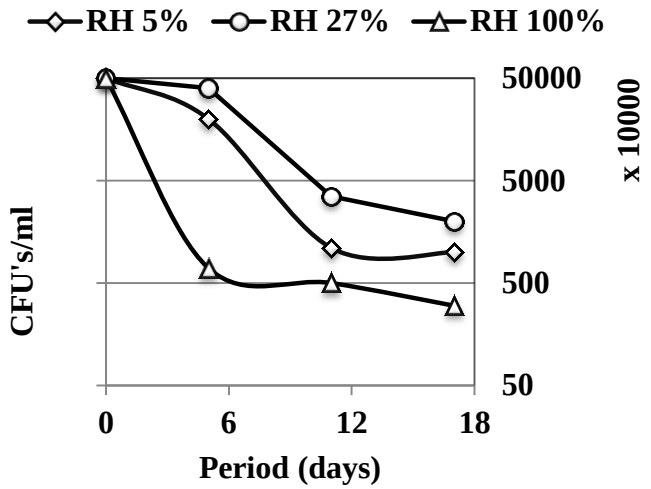

Fig 2. Survival fraction of *Micrococcus luteus*-chp37 cells stored at different relative humidity (5, 27 and 100%) for a period of 0, 6, 12 and 18 days.

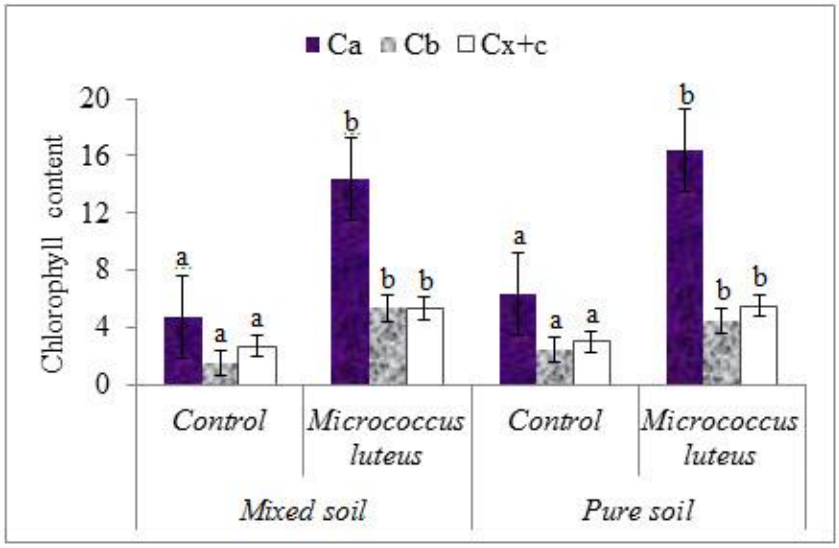

Fig 3. Effect of bacterial inoculation on chlorophyll a, b and carotenoid content of 30 days old *Zea mays* plants. Ca, chlorophyll a; Cb, chlorophyll b and Cx+c, carotenoids. Bars represents mean ± SD. Different letters indicate significant difference between mean of treatments using T-test (p = 0.05).

un-inoculated control. While in case of plants grown in mixed soil the situation is reversible i.e., bacterial inoculation had inhibitory effect on seed germination in comparison to control. In all other parameters inoculated plants showed over all better growth in term of number of leaves, root and shoot length, fresh weight and dry weight Bacterial inoculation caused an enhancement of 54 and 25% in case of dry weight per gram fresh weight in comparison to un-inoculated control maize plants grown under pure soil and mixed soil, respectively (Table 2). Plants were also analyzed for chlorophyll *a*, *b* content and total carotenoids after harvesting (Fig. 3). A marked increase 159 and 199% in chlorophyll *a* and *b* content was observed in case of inoculated plants grown both in pure soil and mixed soil, respectively. The

same trend was observed in case of total carotenoids content (Fig. 3).

Discussion

The morphological and biochemical characterization of the strain *M. luteus*-chp37 are given in Table 1. Results showed that the strain *M. luteus*-chp37 is a desiccation tolerant gram positive aerobic bacterium. *M. luteus*-chp37 was able to produce handsome amount of exopolysaccharide which helps the strain to survive in various abiotic stresses. Several bacterial strains especially *Pseudomonas* survives under abiotic stress by producing exopolysaccharides (Sandhya et al., 2009). Rhizosphere microorganisms, especially beneficial bacteria improve plant performance under stress conditions which results enhance crop yield (Dimkpa et al., 2009). Results of EPS showed that maximum production (341 µg ml^{-1}) was achieved when cell were grown in TSB medium (Fig. 1). *M. luteus*-chp37 was desiccated at three different relative humidity RH for 18 days at 37^{o}C. After 18 days of desiccation at 5, 27 and 100% RH, the survival fractions of *M. luteus*-chp37 was 2, 4 and 0.6%, respectively. Strain *M. luteus*-chp37 desiccated at (RH) 5% and RH 100%, lost the viability at much faster rates as compared to that treated under moderate humidity (i.e. RH 27%). There was a drastic loss (98.6%) after 5 days of desiccation in the surviving rate of strain under RH 100%. In contrast, 40% and 80% of the cells were still viable under RH 5% and RH 27% after 5 days of incubation (Fig 2). This indicates better survival of *M. luteus*-chp37 at RH 5% than at RH 100%. The
results are in compliance to previous studies that under higher RH is detrimental for the survival of bacteria under desiccation (McIntyre et al., 2007). In another study conducted by Bauermeister et al. (2011) which showed that low relative humidity is better for cell survival during long-term storage of desiccated *Deinococcus radiodurans* cells. Now a day's microbial inoculants that can promote plant growth and productivity is internationally recognized as a substitute for chemical fertilizers. In the present study inoculation of *M. luteus*-chp37 cause an increment in the seed germination of maize plant grown in pure soil. But some inhibition in seed germination was recorded in inoculated plant grown in mixed soil. Other investigators also observed the enhancement (Cezon et al., 2003) as well as inhibition in seed germination (Miche et al., 2000) of bacterial inoculated plants in comparison to un-inoculated ones. In the present case the EPS producing strain *M. luteus*-chp37 showed positive enhancement for root, shoot and number of leaves of maize plants in both soil treatment when compared with un-inoculated respective controls (Table 2). Inoculation with plant growth promoting bacteria had significant impact on various growth parameters including shoot, root and seedling length, dry weight of seedlings, seed germination of *Cynara scolymus* (Jahanian et al., 2012; Panachali and Chanadie, 2012). Jarak and co-workers also observed significant increase in height and weight of maize plants inoculated with plant growth promoting bacterial strains (Jarak et al., 2012). Beside this the wild strains of *Pseudomonas fluorescens*- PF-16W and *Pseudomonas polymyxa*- Pb-16W were found to augment the root elongation in maize plant (Natheer et al., 2012). Inoculation of desiccation tolerant *M. luteus*-chp37 in the pure and mixed soil demonstrates phyto-stimulatory effect on the growth of maize under green house conditions by increasing chlorophyll a, b and carotenoids content as compared to un-inoculated control plants. Previous studies also showed the plant growth promotion by EPS producing bacteria through the formation of stable soil aggregates (Ashraf et al, 2006). These soil aggregates determines the mechanical and physical properties of the soil (water movement and retention, aeration and temperature) which in turn effects germination and root growth (Dinel et al., 1992). Moreover, microbial EPS not only increase water holding capacity of the soil but also limit water loss during desiccation. The strain *M. luteus*-chp37 was able to produce HCN which might be a useful component in the growth promotion of maize plants (Table 1).
Ahmed et al. (2008) observed the phyto-stimulatory effect in plants through the inhibition of phytopathogens by HCN. Recently, the beneficial role of HCN in the control of plant root damaging subterranean termite has been studied (Devi et al., 2007). Several investigators have shown that numerous soil bacteria especially plant growth promoting bacteria can produce either cytokinins or gibberellins or both (Lorteau et al., 2001; Kang et al., 2009). Our results showed that strain *M. luteus*-chp37 produced plant growth phytohormone cytokinin. The phytohormone cytokinin control apical dominance and regulates root and shoot growth, leaf senescence and chloroplast development (Oldroyd, 2007). The above described several broad spectrum plant growth promoting attributes of the strain *M. luteus*-chp37 are likely to be the potential candidate for the enhancement of maize growth under drought and desiccated environment.

Materials and Methods

Soil sampling for bacterial isolation

Cholistan desert is located in south of Punjab province of Pakistan. Fifteen soil and plant samples were collected in sterile containers from Yazman Mandi, Cholistan desert, Pakistan. Various samples were transported to laboratory within 24 hrs and stored at 4 °C until further analysis. Modified TGY (mTGY) Agar was used for the isolation and purification of bacteria from desert samples. The isolated strain was characterized morphological, biochemically and physiologically following Gerhardt et al. (1994).

Screening for plant growth promoting traits

Whether the strain is capable of solubilization of inorganic phosphates, isolate was streaked on Pikovskaya's medium (Pikovskaya, 1948) and plates were incubated at 25 °C for 4 days. Clearing zones around the colonies of microorganisms indicate that phosphate has been solubilized in medium. Siderophore production was also determined by using Chrome Azurol-S (CAS) medium (Schwyn et al., 1987). Strain was grown on the plates and is incubated at 37 °C for 48 hrs. Appearance of yellow colored colonies indicates siderophore production. Cytokinin was detected following the method of Akiyoshi et al. (1987). Bacterial strain was grown on half side of the M9 medium, while cotyledons were placed on the other half. After incubation, chlorophyll content of the cotyledons was measured using spectrophotometer. Enhanced chlorophyll-*a* formation was taken as evidence for positive cytokinin activity. M9 plate supplemented with IBA served as positive control, while M9 plate containing *E. coli* served as negative control. Auxin estimation was carried out by the method of Tang and Borner (1979). Briefly, 5ml tryptone water was inoculated with bacterial isolate and incubated at 37 °C for 24 hrs. After incubation, Salkowski's reagent (Schwyn et al., 1987) was added to supernatant and allows it some time for color development. Appearance of pinkish color indicated auxin production. Bacterial isolate was screened for the production

of hydrogen cyanide (HCN) by the method described by Ahmed et al. (2008). Bacteria were streaked on L-agar plate and a filter paper soaked in 0.5% picric acid solution was placed on the top of the agar surface. Plates were incubated at 30 °C for 4 days. Development of orange to red color indicated HCN production.

Quantitative estimation of EPS

For the production of EPS, cells of *Micrococcus luteus*-chp37 were grown in four different media (LB broth, Tryptic soya broth, EPS media and Pharmamedia). Bacterial isolate was grown for 72 hrs at 37 °C with a constant stirring at 150 rpm. Cell free extracts were prepared by centrifugation at 16000 rpm (15 min at 25 °C) and filtered through 0.2 µm filter paper. Polysaccharides were precipitated out by 3 volumes of cold acetone and they were kept at -20 °C for 2 hrs. Precipitate was separated by centrifugation at 16000 rpm (15 min at 4 °C) and re-suspended in water. This suspension was lyophilized and stored at -20 °C. EPS concentration was determined by the phenol sulfuric acid method, described previously (Dubois et al., 1956). The concentration of EPS was determined with reference to standard curve prepared with glucose.

Screening for desiccation tolerance

Isolate was grown at 37 °C in 50 ml LB broth at pH 7 in 250 ml flasks with an aeration of 150 rpm. After 24 hrs incubation, cells were pelleted at 10,000 rpm for 5 min. Cell pellet was re-suspended in phosphate buffer saline and optical density (OD) of the culture was adjusted to 5 $x10^7$ CFU's/ml. 25 µl of the culture was transferred to prewashed, sterile glass slide (25 x 37.5mm) and evenly distributed on it by using the tip of the another glass slide. Glass slides were left to dry at room temperature (RH 33%) for 6 hrs. Slides were placed vertically on the perforated plates, in three different glass desiccators. The relative humidity of the desiccators was controlled by filling their base with silica gel (RH 5%) or sterile saturated solution of potassium acetate (RH 27%) or sterile distilled water (RH 100%). Desiccators were placed in sterile incubator at 37 °C. Survival rate of each isolate was determined up to 18 days on L-agar. Cells deposited over glass slides were re-suspended by adding 100µl sterile saline (0.9%) water and this suspension was transferred to 10 ml saline water and mixed well with micropipette. Suspension was further serially diluted up to 10^{-3} dilution and 100 µl from each of the 10^{-1} and 10^{-3} dilution was spread on the LB agar plate. Number of colony forming units (CFUs) was determined by incubating plates at 37 °C for 24 hours.

Plant growth experiments

The microbiological effect of strain *M. luteus*-chp37 on maize (*Zea mays* L.) plants was studied in the field trial. Certified seed of *Zea mays* were obtained from National Agriculture Research Center, Islamabad, Pakistan. Seed were surface sterilized with 5% (w/v) sodium hypochlorite. These sterilized seed were washed thrice with autoclaved distilled water and then inoculated with bacterial suspensions having cell density of approximately 10^7 CFU for 15 min, while control seeds were soaked in sterile distilled water for the same time period. After seed inoculation, 12 seeds were sown into each pot containing 3 kg of soil. Two types of soil were prepared: one that containing pure soil (PS) and the other one is mixed soil (1:1 sand and soil). Plants were harvested at maturity and various growths (seed germination, number of leaves, root and shoot length, dry weight per gram fresh weight) and biochemical (chlorophyll a, b and carotenoids content) parameters were recorded. Estimation of chlorophyll a, b and carotenoids were carried out by the method of Lichtenthaler and Wellburn (1983).

Statistical analysis

Data obtained was analyzed statistically following Steel and Torrie (1981). Least significant difference was also calculated.

Conclusion

Desiccation is one the most severe stress faced by bacteria in desert environment and these bacterial strains has evolved various mechanisms to survive in such water deficient conditions. Hence the desiccation tolerant plant growth promoting strain could be explored in agriculture sector in Pakistan especially in desert area, where there is low rain fall and water has become scarce.

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