

Effect of *Brevibacterium iodinum* RS16 and *Methylobacterium oryzae* CBMB20 Inoculation on Seed Germination and Early Growth of Maize and Sorghum-sudangrass hybrid Seedling under Different Salinity Levels

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Salinity is one of the most relevant abiotic factor limiting crop yield and its net primary productivity. In addition, salinity induces an increased stress ethylene synthesis in plants which, in turn, exacerbate the responses to the stressor. Bacterial single or co-inoculation effect was tested using previously characterized plant growth promoting (PGP) bacteria *Brevibacterium iodinum* RS16 and *Methylobacterium oryzae* CBMB20 on maize and sorghum-sudan grass hybrid under different concentrations of NaCl. Non-inoculated maize and sorghum-sudangrass hybrid showed 33.4% and 20.0% reduction in seed germination under highest NaCl (150 mM) level tested. However, under the same NaCl concentration, co-inoculation with *B. iodinum* RS16 and *M. oryzae* CBMB20 PGP strains increased the seed germination in maize (16.7%) and sorghum-sudangrass hybrid (4.4%). In Gnotobiotic growth pouch experiments conducted for maize and sorghum-sudangrass hybrid, co-inoculation of PGP *B. iodinum* RS16 and *M. oryzae* CBMB20 mitigated the salinity stress and promoted root length by 22.9% and 29.7%, respectively. Thus the results of this study could help in development of potential bioinoculants that may be suitable for crop production under saline conditions.

Key words: Salinity stress, NaCl, *Brevibacterium iodinum* RS16, *Methylobacterium oryzae* CBMB20, maize, Sorghum-sudangrass hybrid.

Introduction

Salinity is one of major abiotic stress in arid and semi-arid regions, more than half of the irrigated arable land area of the world are affected with salts, thus it substantially reduce crop yield by more than 50% (Bray et al. 2000). It is one of the most important factor limiting crop production, especially in sensitive crops (Zadeh and Naeni, 2007). Saline soils contain sufficient soluble salts to suppress plant growth through a series of interacting factors such as osmotic potential effect, ion toxicity and antagonism; they in turn induce nutrient imbalances (Neumann, 1995). Salinity stress affects all stages of crop growth, especially, seed germination and seedling growth stages are more sensitive for most plant species (Cuartero et al. 2006). Seedling growth of

plant is the most critical stage in plant development and is highly responsive to the environmental conditions (Saritha et al. 2007). The effects of salinity on germination could be related to the imbalance in endogenous levels of growth hormones (Zholkevich and Pustovoytova, 1993; Jackson, 1997). Therefore, seeds with rapid germinating ability under salinity stress may be expected to achieve seedling establishment and more salinity tolerance and hence higher yields (Munns, 2002; Bybordi and Tabatabaei, 2009).

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize the rhizosphere soil or in phyllosphere region and it helps in enhancement of plant growth and yield (Vessey, 2003). The mechanisms of plant growth promotion includes phytohormone production (Egamberdiyeva, 2007; Shaharouna et al. 2006), asymbiotic N₂ fixation (Salantur et al. 2006), production of siderophores, synthesis of antibiotics, enzymes and/or fungicidal compounds (Bharathi et al. 2004 and Jeun et al. 2004; Ahmad et al. 2007) and

solubilization of mineral nutrients (Cattelan et al. 1999). Under salinity stress, PGPR have positive effects on plants specifically on germination rate and seedling growth (Kokelis-Burelle et al. 2006). Importantly the PGPR containing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase were considered to effectively facilitate the early growth of plants, especially under stressful conditions such as flooding, heavy metals, phytopathogens, drought and high salinity (Grichko et al. 2001; Belimov et al. 2005; Mayak et al. 2004). Ethylene is an important phytohormone connected with fruit ripening, however, overproduction of ethylene under stressful conditions can result in the inhibition of plant growth or death, especially for seedlings (Glick et al. 1997 and Ghosh et al. 2003). PGPR containing ACC deaminase can hydrolyze ACC, the immediate precursor of ethylene, to α -ketobutyrate and ammonia, leading to reduction in ethylene levels, thus promoting plant growth (Glick et al. 1998). In general, ethylene production, ACC concentration, ACC synthase (ACS), and ACC oxidase (ACO) enzyme activities increase with increasing levels of salinity stress. Inoculation with ACC deaminase producing PGPR reduced ethylene production, ACC concentration, ACS and ACO activities in salinity stress (Siddikee et al. 2011a). ACC deaminase-producing PGPR may assist plant growth by alleviating deleterious effects of stress ethylene. Moreover, PGPR are found to tolerate high salinity levels, which are often detrimental to growth of important crop plants (Bacilio et al. 2004).

The objective of this work was to examine in the effect of ACC deaminase producing PGP strains inoculation on seed germination and early growth of maize (*Zea mays* L.) and sorghum-sudangrass (*Sorghum bicolor* L.) hybrid under different salinity levels.

Materials and Methods

Bacterial strains and culture inoculum preparation

The halotolerant *Brevibacterium iodinum* RS16 was isolated from coastal saline soil of the Yellow sea, Incheon, South Korea. It possesses plant growth promoting (PGP) traits like nitrogen fixation and ACC deaminase activity (Siddikee et al. 2011b). The culture was maintained in Tryptic soy agar (TSA) medium supplemented with 5% (~0.85 M) NaCl (pH 7.2). For experimental studies 1.0 mL of the initially mass multiplied culture (1×10^8 cfu mL⁻¹) was transferred to 100 mL fresh TSB

supplemented with 5% (0.85 M) NaCl and allowed to grow for 24 h. The other bacteria used in this study was pink-pigmented facultative methylotroph (PPFM) *Methylobacterium oryzae* strain CBMB20, which was isolated from stem tissues of rice and well characterized in related to PGP activities (Madhaiyan et al. 2007). The culture was maintained in ammonium mineral salt (AMS) media with 0.5% sodium succinate supplemented at pH 7.2. For experimental studies, 1.0 mL initially mass multiplied culture (1×10^8 cfu mL⁻¹) was transferred to 100 mL fresh AMS broth and allowed to grow for 72 h.

Effect of NaCl concentration on maize and sorghum-sudangrass hybrid seed germination

Maize seeds were surface sterilized by immersion in 70% ethanol for 1 min and 6% NaOCl for 5 min, followed by thorough rinsing with sterile distilled water (7-10 times). Sorghum-sudangrass hybrid seeds were surface sterilized by immersion in 70% ethanol for 2 min and 1% NaOCl for 3 min, followed by thorough rinsing in sterile distilled water (7-10 times). For conducting germination test, ten seeds were maintained per treatment on sterilized filter paper (Whatman No. 2) soaked in a 5 mL solution of 0, 50, 100 and 150 mM NaCl in Petri dishes. Treatment without NaCl addition was considered as control and each NaCl concentration was assigned as separate treatment, the experiment was conducted in triplicate. Germination potential was observed till 3 days at $28 \pm 1^\circ\text{C}$ under dark condition in a plant growth chamber (DS 54 GLP, DASOL Scientific Co., Ltd., Republic Korea). The number of germinated seeds was recorded every 24 h.

Bacterial inoculation effects on maize and sorghum-sudangrass hybrid seed germination under salinity stress

Germination potential of the seeds under different NaCl (50, 100 and 150 mM) concentrations was tested in presence of single inoculant or under co-inoculated condition. Surface sterilized maize and sorghum-sudangrass hybrid seeds were soaked in bacterial suspension (1×10^8 cfu mL⁻¹) of each bacteria and imbibed for 4 h. Single inoculation treatments received 20 mL of culture as inoculum, and in case of co-inoculation, 10 mL of each culture were used. In case of control no seed bacterization was carried out. The rate of germination was estimated using a modified

Timson index of germination velocity: germination velocity = $\sum G/t$, where G is the percentage of seed germination at 24 h intervals and t is the total germination period (Khan and Ungar, 1985).

Assessment of bacterial inoculation effects on maize and sorghum-sudangrass hybrid seedling using gnotobiotic growth pouch assay under salinity stress

Surface sterilized seeds of maize and sorghum-sudangrass hybrid were imbibed in *B. iodinum* RS16 and *M. oryzae* CBMB20 cell suspensions for 4 h. Salinity levels were maintained at 50, 100 and 150 mM of NaCl and bacterial treatments included either single or dual inoculation of cultures. Three sprouted seeds of maize and five seeds of sorghum-sudangrass hybrid were aseptically transferred to growth pouches (CYG seed germination pouch, Mega International Manufacturer, USA) which were filled with 20 mL of 50, 100 and 150 mM NaCl solution. The pouches were incubated in the growth chamber at $28 \pm 1^\circ\text{C}$, a relative humidity of 70% and a dark/light cycle beginning with 12 h of dark followed by 12 h of light. After 3 days of incubation, 2 mL water containing 50, 100 and 150 mM NaCl (for salinity stress imposed treatments) were added to the pouches. The length of primary roots formed after 7 days was measured.

Statistical analysis All data were subjected to analysis of variance (ANOVA). Significance at 5% level was tested by Least Significant Difference (LSD) using SAS package, Version 9.1 (SAS, 2009).

Results and Discussion

Effect of salinity stress on maize and sorghum-sudangrass hybrid seed germination

Salinity stress slowed down water uptake by seeds, thereby inhibiting their germination and root elongation (Uhvits, 1946; Simon, 1984; Werner and Finkelstein, 1995). Salinity affects seed germination due to osmotic inhibition of water availability, toxic effects and nutritional imbalance caused due to salt ions. In the life cycle of plant germination, seedling and flowering stages are more critical for salinity stress. Maize and sorghum-sudangrass hybrid seeds in the control dishes (0 mM NaCl) had the highest germination percentage (96.7%) after 72 h of incubation. However, germination percentage decreased as NaCl concentration increased (Table 1 and Table 2). Significant reduction on seed germination was observed with all the three levels of salinity (50, 100 and 150 mM) stress imposed on the maize and sorghum-sudangrass hybrid. At 50 mM NaCl concentration, germination of maize seeds were reduced by 6.7%,

Table 1. Germination percentage of maize seeds under different levels of salinity stress.

Treatment	Germination percentage (%)		
	24 h	48 h	72 h
Control	6.7 \pm 3.3a	86.7 \pm 3.3a	96.7 \pm 3.3a
50 mM NaCl	3.3 \pm 3.3a	50.0 \pm 5.7b	90.0 \pm 5.8a
100 mM NaCl	3.3 \pm 3.3a	36.7 \pm 3.3c	83.3 \pm 3.3ab
150 mM NaCl	3.3 \pm 3.3a	26.7 \pm 6.6c	63.3 \pm 3.3b

Each value represents the average of three replicates per treatment \pm SE (standard Error). In the same column, significant differences according to LSD at $P \leq 0.05$ levels are indicated by different letters.

Table 2. Germination percentage of sorghum-sudangrass hybrid seeds under different levels of salinity stress.

Treatment	Germination percentage (%)		
	24 h	48 h	72 h
Control	93.3 \pm 6.7a	93.3 \pm 6.7a	96.7 \pm 3.3a
50 mM NaCl	50.0 \pm 5.8b	80.0 \pm 5.8ab	86.7 \pm 3.3ab
100 mM NaCl	40.0 \pm 5.8bc	70.0 \pm 5.8b	86.7 \pm 6.7ab
150 mM NaCl	23.3 \pm 6.7c	70.0 \pm 5.8b	76.7 \pm 6.7b

Each value represents the average of three replicates per treatment \pm SE (standard Error). In the same column, significant differences according to LSD at $P \leq 0.05$ levels are indicated by different letters.

However, severe reduction (33.4%) was observed at 150 mM NaCl (Table 1). For sorghum-sudangrass hybrid, germination was reduced by 10.0% at 50 mM NaCl and 20.0% reduction at 150 mM NaCl concentration tested (Table 2). The germination percentages of maize and sorghum-sudangrass hybrid germination was severely reduced (> 50%) at 150 mM NaCl. Several earlier studies also documented the relationship between salt stress and reduction in seed germination (Gill et al. 2002; Almodares et al. 2007; Blanco et al. 2007).

Effect of inoculation of PGP strains on germination of maize and sorghum-sudangrass hybrid seed under salinity stress

Inoculation of *B. iodinum* RS16 and *M. oryzae* CBMB20 and their co-inoculation increased germination of maize seeds compared to non-inoculated control by 16.7% at 150 mM NaCl (Table 3). *M. oryzae* CBMB20 and co-inoculation increased germination of

sorghum-sudangrass hybrid seeds compared to non-inoculated control by 4.4% at 150 mM NaCl (Table 4). In general, rate of maize and sorghum-sudangrass hybrid seeds germination decreased with increasing levels of salinity. However, *B. iodinum* RS16 or *M. oryzae* CBMB20 alone and their co-inoculation treatments showed increased rate of maize and sorghum-sudangrass hybrid seeds germination compared to non-inoculated control at different levels of salinity stress (Figure 1). In an earlier study under *in vitro* condition, seed treatment with PGPR strains improved seed germination, seedling vigor, seedling emergence and seedling stand over the control (Egamberdieva, 2009). In particular, lettuce seed germination was enhanced under salt stress with the help of *Azospirillum* inoculation, it further improved the fresh and dry weights accumulation and biomass partition to the aerial portions of the plant (Barassi et al. 2006).

Table 3. Effect of *B. iodinum* RS16, *M. oryzae* CBMB20 inoculation on germination of maize seeds under different levels of salinity stress.

Treatment	Germination percentage (%)		
	50 mM NaCl	100 mM NaCl	150 mM NaCl
Non-inoculation	90.0 ± 5.8a	83.3 ± 3.3a	63.3 ± 3.6b
<i>B. iodinum</i> RS16	93.3 ± 3.3a	86.7 ± 3.3a	80.0 ± 5.8a
<i>M. oryzae</i> CBMB20	90.0 ± 5.8a	93.3 ± 3.3a	80.0 ± 5.8a
Co-inoculation	96.7 ± 3.3a	93.3 ± 3.3a	80.0 ± 5.8a

Each value represents the average of three replicates per treatment ± SE (standard Error). In the same column, significant differences according to LSD at $P \leq 0.05$ levels are indicated by different letters. The germination percentage were measured on 96 hour after sowing.

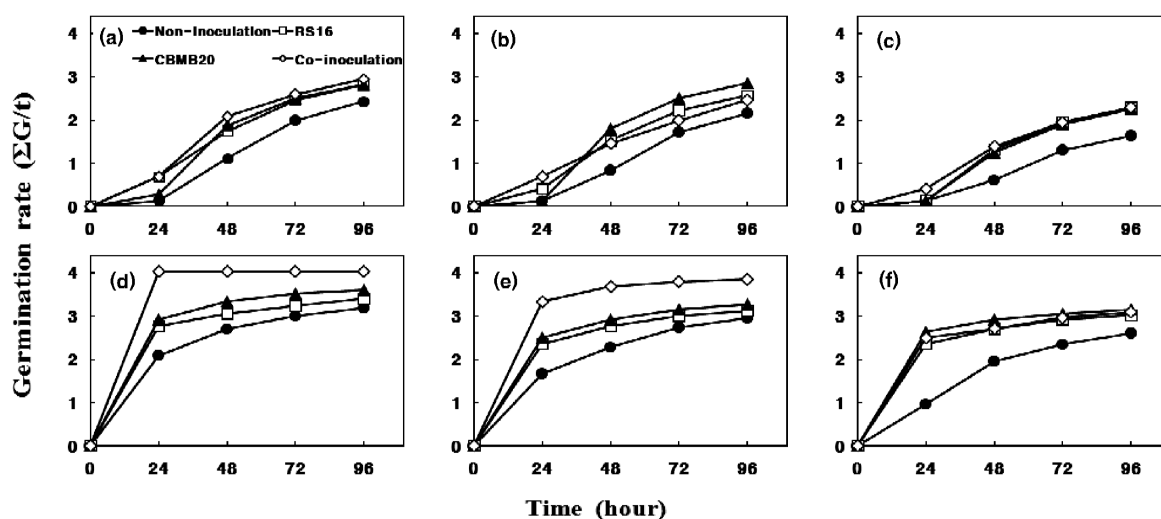


Fig. 1. Effect of *B. iodinum* RS16 and *M. oryzae* CBMB20 inoculation on germination rate ($\Sigma G/t$) of maize and sorghum-sudangrass hybrid under different levels of salinity stress (a; maize + 50 mM NaCl, b; maize + 100 mM NaCl, c; maize + 150 mM NaCl, d; sorghum + 50 mM, e; sorghum + 100 mM, f; sorghum + 150 mM). In absence of NaCl and bacterial inoculation on maize and sorghum-sudangrass hybrid germination rate ($\Sigma G/t$) was 2.98 and 3.95, respectively at 96 h.

Effect of salinity stress on maize and sorghum-sudangrass hybrid seedling growth in gnotobiotic growth pouch assay

Growth of maize and sorghum-sudangrass hybrid seedlings exposed to salinity stress for 7 days were significantly affected compared to seedlings grown in the absence of NaCl and bacterial inoculation (statistical results not included). Similar results were found in the study by Mohammad et al. (1998) where both root length and root surface area per plant decreased significantly under higher salinity

conditions. NaCl level at 150 mM decreased root length in both maize and sorghum-sudangrass hybrid compared to seedlings grown in the absence of NaCl and bacterial inoculation (Table 5 and Table 6). Root length of maize was 13.4% and 62.7% lower at 50 mM and 150 mM NaCl treatments respectively, when compared to seedlings grown in the absence of NaCl and bacterial inoculation (21.7 cm). Root length of sorghum-sudangrass hybrid seedling was 16% and 55.4% lower at 50 mM and 150 mM NaCl treatments compared to seedlings without

Table 4. Effect of *B. iodinum* RS16, *M. oryzae* CBMB20 inoculation on germination of sorghum-sudangrass hybrid seeds under different levels of salinity stress.

Treatment	Germination percentage (%)		
	50 mM NaCl	100 mM NaCl	150 mM NaCl
Non-inoculation	90.0 ± 5.7a	86.7 ± 6.7a	80.0 ± 5.8a
<i>B. iodinum</i> RS16	93.3 ± 6.8a	83.3 ± 3.3a	80.0 ± 5.8a
<i>M. oryzae</i> CBMB20	93.3 ± 3.3a	86.7 ± 3.3a	83.3 ± 6.7a
Co-inoculation	96.7 ± 3.3a	96.7 ± 3.3a	83.3 ± 6.7a

Each value represents the average of three replicates per treatment ± SE (standard Error). In the same column, significant differences according to LSD at $P \leq 0.05$ levels are indicated by different letters. The germination percentage were measured on 96 hour after sowing.

Table 5. Effect of *B. iodinum* RS16, *M. oryzae* CBMB20 inoculation on early growth of maize under different levels of salinity stress.

Treatment	Root length (cm plant ⁻¹)		
	50 mM NaCl	100 mM NaCl	150 mM NaCl
Non-inoculation	18.8 ± 0.6b	13.7 ± 1.5b	8.1 ± 1.1b
<i>B. iodinum</i> RS16	19.8 ± 0.3b	16.2 ± 0.4a	9.0 ± 0.9b
<i>M. oryzae</i> CBMB20	19.5 ± 0.3b	15.2 ± 0.3ab	8.5 ± 0.3b
Co-inoculation	21.7 ± 0.5a	16.4 ± 0.5a	10.5 ± 0.5a

Each value represents the average of three replicates per treatment ± SE (standard Error). In the same column, significant differences according to LSD at $P \leq 0.05$ levels are indicated by different letters. In the absence of NaCl and bacterial inoculation root length was 21.7 ± 1.2 cm.

The root length were measured 7 days after sowing (DAS).

Table 6. Effect of *B. iodinum* RS16, *M. oryzae* CBMB20 inoculation on early growth of sorghum-sudangrass hybrid under different levels of salinity stress.

Treatment	Root length (cm plant ⁻¹)		
	50 mM NaCl	100 mM NaCl	150 mM NaCl
Non-inoculation	14.7 ± 0.9b	12.3 ± 1.2a	7.8 ± 0.9b
<i>B. iodinum</i> RS16	17.6 ± 1.1a	13.4 ± 1.5a	8.8 ± 0.8b
<i>M. oryzae</i> CBMB20	14.6 ± 1.5b	13.9 ± 0.4a	11.1 ± 1.1a
Co-inoculation	18.7 ± 1.4a	13.9 ± 1.5a	9.9 ± 0.2b

Each value represents the average of three replicates per treatment ± SE (standard Error). In the same column, significant differences according to LSD at $P \leq 0.05$ levels are indicated by different letters. In the absence of NaCl and bacterial inoculation root length was 17.5 ± 0.6 cm.

The root length were measured 7 days after sowing (DAS).

NaCl and bacterial inoculation treatments (17.5 cm). Previously, roots have been reported to be less sensitive to salinity than leaves (Rendig and Taylor, 1989). However, in the present study, root length was severely affected with the highest salinity level tested in this study.

Effect of salinity stress and bacterial inoculation on the maize and sorghum-sudangrass hybrid seedlings root growth under Gnotobiotic pouch assay Inoculation with PGP strains *B. iodinum* RS16 and *M. oryzae* CBMB20 significantly promoted growth of maize seedling under different NaCl concentrations. In general, inoculation of PGPR resulted in early seedling growth and development (Egamberdiyeva, 2009) but normal plant growth will be reduced by increasing salinity. However, inoculation of PGPR was effective in the presence of 120 mM of NaCl (Nadeem et al. 2006). In the present work single inoculation of *B. iodinum* RS16 or *M. oryzae* CBMB20 and their co-inoculation increased growth of maize and sorghum-sudangrass hybrid seedlings compared to non-inoculated plants at different levels of salinity stress (Table 5 and Table 6). In particular, co-inoculation significantly increased root length of maize seedling by 22.9% as compared to non-inoculated plants at 150 mM NaCl concentration (Table 5) and inoculation of *M. oryzae* CBMB20 significantly increased root length of sorghum-sudangrass hybrid seedling by 29.7% as compared to non-inoculated control at 150 mM concentration (Table 6). It was observed that inoculation with these PGP strains significantly improved root length at all salinity levels tested. Inoculating ACC-deaminase containing rhizobacterial strains reduced the stress ethylene level and increased shoot and root growth significantly under high NaCl (200 mM) conditions (Mayak et al. 2004). This kind of alleviation of salt stress by an ACC-deaminase possessing bacterial strain was demonstrated under green house and field conditions and in another study, mechanism was conclusively proved using ACC-deaminase minus mutants in canola plants (Saravananakumar and Samiyappan, 2006; Cheng et al. 2007).

Conclusion

The results of this study proved that inoculation of seeds with PGPR strains containing ACC-deaminase

(*B. iodinum* RS16 and *M. oryzae* CBMB20) promotes germination and root growth of maize and sorghum-sudangrass hybrid grown under high NaCl levels. ACC-deaminase containing bacteria are already known to promote plants growth under salinity stress condition. The strains used in this study have enhanced tolerance of maize and sorghum-sudangrass hybrid under high salinity level, which eventually lead to better germination, root and shoot growth compared to non-inoculated plants. Further work is needed to explore other synergistic PGP mechanisms associated with crop establishment under salinity stress condition. Thus this study could pave way for evaluation of PGP activities of *B. iodinum* RS16 and *M. oryzae* CBMB20 under reclaimed soil conditions.

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