



Improvement of salinity stress in cumin (*Cuminum cyminum*) seedling by inoculation with Rhizobacteria

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ABSTRACT

Cumin (*Cuminum cyminum* L.) is the second most popular spice in the world, after black pepper, which is sensitive to salinity. In order to investigate the effect of seed bio-inoculation with plant growth promoting Rhizobacteria on germination and seedling indices of cumin under salinity stress, an experiment was laid out based on a completely randomized design with two factors and four replications in 2017 at Seed Science and Technology Laboratory, Yasouj University, Iran. Experimental factors included bio-inoculation with 3 strains, viz. PF2, PF25, and CHA0 of *Pseudomonas fluorescens*, and *Bacillus subtilis* and non-primed (control) and three levels of salinity stress (0, -4 and -8 bar). Results indicated that salt stress reduced germination (up to 40%) and seedling indices of cumin under -4 and -8 bar, the highest germination percentage, germination rate, seedling length, and seedling vigor index were achieved in the seeds inoculated with *P. fluorescens*, CHA0. At all levels of stress, seed bio-inoculation were increasing the seed soluble protein content compared to non-primed. The effect of this treatment was more obvious under salinity potential of -8 bar. The results indicated that salinity can affect cumin seed germination and the beneficial effect of PGPR could be used for improving its salt tolerance.

Key words: *Bacillus subtilis*, Medicinal plant, Osmotic stress, *Pseudomonas fluorescens*, Seed germination

Cumin is an annual plant with medicinal properties including numerous stimulus appetites, strengthen the stomach, anti-flatulence, which has low germination, poorly vigor, low storage substances and weak establishment in the soil (Dhanalakshmi *et al.* 2003). The origin of this plant is known as Iran, Turkey, Egypt and western Mediterranean (Dehaghi M and Mollafilabi A 2011). A major constraint to seed germination is soil salinity, a common problem in irrigated areas of Iran, with low rainfall (Kaya *et al.* 2003). Although, the cumin plant is relatively salt resistant during adult growth and reproductive stages (Hassanzadeh *et al.* 2013), but as a rule, seed germination and seedling growth are the stages most sensitive to salinity (Ibrahim HIM 2016, Shoor *et al.* 2014) that finally resulted in reduction of germination percentage, germination rate, root and shoot length, root and shoot weight, seedling length and weight vigor index (Neamatollahi *et al.* 2009, Roodbari *et al.* 2013). One method of increase germination and seedling indices is inoculation of seeds with beneficial organisms (seed bio-priming).

Many studies have shown that the use of seed pretreatments including beneficial microbes can resist plant against adverse environmental stress, such as drought (Piri *et al.* 2016) and salinity (Habib *et al.* 2016). Potential of seed treatment with Plant Growth Promoting Rhizobacteria (PGPR) in improving the resistance against salt stress in cumin seeds has never been explored. In this regard, a laboratory experiment was conducted to study the effect of seed inoculation with *Pseudomonas fluorescens* and *Bacillus subtilis* on germination of cumin (*Cuminum cyminum* L.) under salinity stress.

MATERIALS AND METHODS

Seed preparation: Cumin seeds were collected from Sabzevar region of Khorasan province, Iran. A factorial based experiment was performed on the completely randomized design with four replications in 2017 at Seed Science and Technology Laboratory of Yasouj University, Iran. Experimental factors were included seed inoculation in five levels (inoculation with PF2, PF5 and CHA0 strains of *P. fluorescens* and one strain of *B. subtilis* and dry seeds (as inoculation control)) and salinity stress in three levels (0, -4 and -8 bar imposed by NaCl). Van't Hoff (1887) equation was used for calculating salinity osmotic potential.

$$\Psi_s = -MRT$$

where M, molality (moles/1000 g); I, ionization constant;

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R, gas constant ($0.0083 \text{ l} \times \text{MPa/mol}$), T, temperature (K).

Preparation of culture medium and suspension: In order to prepare growth medium and suspension of bacteria NA (nutrient agar), the culture medium was prepared by adding 5 g NA and 25.1 g agar to 250 ml distilled water. Bacterial isolates were cultured on NA with a loop test tube and in the zigzag shape and incubated for 24-48 h for growing. The bacterial population was adjusted to 10^8 colonies per ml of distilled water using spectrophotometer at a wavelength of 600 nm (Weller and Cook 1983). Before inoculation, the seeds were disinfected for 30 sec with 2% sodium hypochlorite and were washed three times with distilled water; the seeds were then immersed for 1 h in 20 ml bacterial suspension (for the inoculated treatments) at room temperature (20–25°C).

Seed germination: After applying the treatments, the seeds were sown immediately on top of a two-layer filter paper in 90 mm Petri dishes and moistened with 3 ml of distilled water. The seeds were then incubated at 20–30°C for 14 days (ISTA 2010). During the experiment, the numbers of germinated seeds were counted daily. The maximum monitoring period was 14 days. After 14 days, 10 seedlings were randomly selected from each petri-dish, and the seedling length was measured. Germination percentage (GP) and germination rate (GR) and seedling vigor index (SVI) were calculated using the following equations as per Abdul-baki and Anderson (1970), Ellis and Roberts (1981).

$$\text{GP} = \frac{\text{Total number of germinated}}{\text{Seeds total number of seeds}} \times 100$$

$$\text{GR} = \sum \frac{N_i}{T_i}$$

where N_i , number of germinated seeds per day; T_i , day from the start of testing.

Seedling Vigor Index = Seedling length $\times$ Germination percentage

Seed soluble protein content: For measuring seed soluble protein content, seeds were sampled after phase II of imbibition (germination *sensu stricto*) before root protrusion occurs. For this purpose, the seed was imbibed for 48 hours in specified osmotic stress, then 0.6 g of imbibed seeds were weighted and biochemical traits were measured. Protein was extracted in 2 ml of extraction buffer containing 100 mM KH_2PO_4 and 100mM NaOH (pH 6.8).

The homogenate was then centrifuged at $12000 \times g$ for 30 min at 4°C. The supernatant was used for total protein and enzymatic assays. Soluble protein content was measured by (Bradford 1976) method.

Statistical analysis: The data were analyzed by two way analysis of variance (ANOVA) by using SAS statistical software version 9.1. Mean comparison of data was done by using the least significant difference (LSD) test at 5% error probability. If main treatments and interactions were significant, mean comparison of interactions was merely described in results after slicing.

RESULTS AND DISCUSSION

The results obtained from variance analysis show that the salinity and biopriming effects, and their interactions were significant for cumin's germination, seedling length, seedling vigor index, and soluble protein content (Table 1).

The soluble protein content of cumin seeds decreased with increasing salinity stress. Among different stress levels, the highest seed soluble protein was achieved from non-stressed and *Bacillus* treated seeds (1.18 mg/g FW) that had no significant differences with CHA0 treated one. Also, lowest seed soluble protein (0.46 mg/g FW) related to inoculation control and -8 bar salinity (Fig 1A). The results showed that the germination of cumin seeds was significantly ($P \leq 0.05$) affected by the PGPR isolates under different salt concentrations. Seed germination declined by 22 and 44% with -4 bar and -8 bar salinity, respectively. Bacterial inoculation significantly enhanced germination percentage in the 0, -4 and -8 bar of NaCl. PGPR enhanced seed germination in by 16–32% with the higher effect at -8 bar salinity. Among the studied isolates CHA0 inoculated seeds had the highest germination which had no significant difference with *Bacillus subtilis* treated seeds (Fig 1B).

Like germination percentage, salt stress affected significantly ($P \leq 0.05$) germination rate. Germination declined significantly with increasing salinity stress. The highest (2.91 seed/d) and the lowest rate of germination (1.29 seed/d) were observed at non-salinized and -8 bar salinity stressed seeds, respectively. The bio-inoculation has allowed improvement of germination of all stress levels; this difference was not significant at 0 bar salinity.

At -4 and -8 bar, the highest germination rate was related to CHA0 that in -4 bar had no significant difference

Table 1 Analysis of variance (mean square) for the effect of seed bio-priming on some traits characteristics of cumin under salinity stress

Seedling vigor index	Seedling length	Germination rate	Germination percentage	Soluble protein content	df	S O V
15.59**	12**	1.15**	943.06**	0.0740**	4	Bio-priming
71.55**	9.77**	13.63**	0.12035**	0.1166**	2	Salinity
0.856**	0.198**	0.162*	73.46*	0.0035**	8	Bio-priming $\times$ Salinity
0.091	0.024	0.078	21.77	0.0001	14	Error
7.0	2.60	12.82	6.81	11.88	-	C V (%)

* and ** significant at 5 and 1% probability level

with *Bacillus subtilis* (Fig 1C). Seedling length of cumin seeds was significantly ($P \leq 0.05$) affected by the PGPR isolates under salt concentrations. At all levels of salinity, CHA0 strain had the highest seedling length and the lowest of seedling length was allocated to the control (Table 2).

The results demonstrated that salinity adversely affected seedling vigor. This decrease was 35 and 72% at -4 and -8 bar NaCl, respectively. Like other studied characteristics application of PGPR isolates significantly ($P \leq 0.05$) improved seedling vigor under both stress conditions. This enhancing effect was more obvious under -8 bar salinity which CHA0 inoculated seeds showed seedling vigor about three fold higher than that of non-inoculated one (Table 2).

The study revealed that increasing salt stress leads to a decrease in germination and seedling indices of cumin seeds, but seed inoculation with PGPR reduced the adverse effects. Decreasing cumin seed germination with increasing salinity

Table 2 Mean comparison of seedling length and seedling vigor index of cumin seed affected by bio-inoculation at different levels of salinity

Salinity levels	Seedling vigor index	Seedling length (cm)	Treatment
Non- stress	6.25 b	6.65 c	<i>Bacillus subtilis</i>
	5.47 c	6.6 c	P.F2
	7.19 a	7.82 a	CHA0
	6.38 b	7.1 b	P.F25
	3.8 d	5.07 d	Control
-4 bar	5.1 b	6.15 c	<i>Bacillus subtilis</i>
	4.87 b	6.25 c	P.F2
	6.57 a	7.22 a	CHA0
	5.47 b	6.67 b	P.F25
	2.5 c	4.47 d	Control
-8 bar	2.35 b	5.47 c	<i>Bacillus subtilis</i>
	2.33 b	5.82 b	P.F2
	2.95 a	6.15 a	CHA0
	2.19 b	5.35 c	P.F25
	1.1 c	3.55 d	Control
LSD (P=0.05)	0.63	0.19	-

*Data are means of four replications of 25 seeds each. In each salinity level, means with the same letter are not significantly different according to LSD test at $P=0.05$.

levels is also observed by another researcher (Roodbari *et al.* 2013, Mohammadizad *et al.* 2014, Shoor *et al.* 2014, Piri *et al.* 2016). Reduced the rate and extent of water absorption due to osmotic effects of salinity (De and Kar 2004, Keshavarzi 2011) on the one hand and reduction of metabolic activity due to ionic effects of this stress on the other hand (Datta *et al.* 2009), inhibits the root and shoot

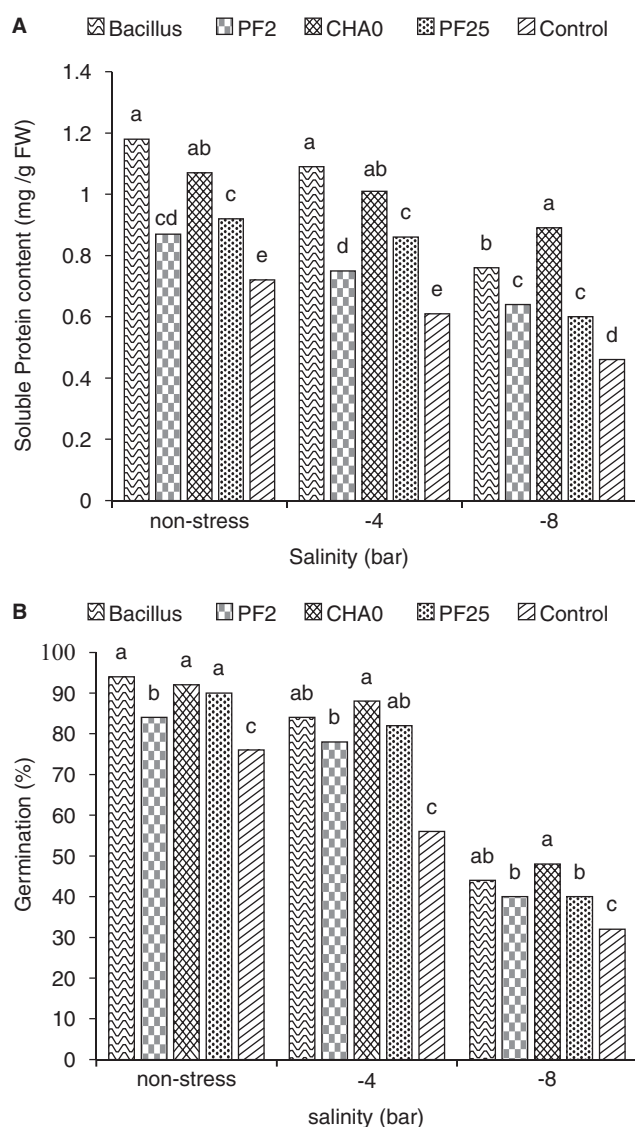


Fig 1 Mean comparison of soluble protein content (A) germination percentage (B) and germination rate (C) of cumin seed affected by bio-inoculation at different levels of salinity. Seed germination values are means from four replications of 25 seeds each. At each salinity level, means with the same letter are not significantly different according to LSD test ($P=0.05$). PF2, CHA0, and PF25 are *Pseudomonas fluorescens* isolates.

elongation and increases the time required to radicle protrude out of seed coat and thereby decreases germination rate (Mohammadizad *et al.* 2014). In contrast, pretreatment with bacteria isolation of PGPR enhanced germination percentage under salt stress conditions that *Bacillus subtilis* and CHA0 had a significant effect on seed germination of cumin under optimal and salt stress conditions.

The study showed that salinity stress affected cumin seed protein content. The reduction of soluble protein under salinity stress was previously recorded by Metwalil *et al.* (2015). Soluble proteins have been shown involved in osmotic regulation in the plant, playing an important role in tolerance of a plant to salinity stress and may use as a protective strategy to alleviate Na⁺ toxicity (Metwalil *et al.* 2015). Seed inoculation with PGPR sets in motion many germination-related activities such as early reserve mobilization such as a protein that facilitate the transition of quiescent dry seeds into a germinating state and lead to improvement of germination potential (Ibrahim 2016). In addition, the treated seeds increased the compatible solutes such as proline, maintaining ions balance (Shoor *et al.* 2014, Habib *et al.* 2016) total sugars (Ghezal *et al.* 2016) and α -amylase activity, soluble carbohydrate and free amino acids (Metwalil *et al.* 2015) and exhibited earlier initiation of protein, RNA, and DNA synthesis. Consequently, when the seed is set out for germination, cellular events are much advanced.

The ameliorative effects of seed enhancing treatment on cumin seed germination rate under salinity stress have been also reported in previous studies (Neamatollahi *et al.* 2009). This enhancing effect appeared to be linked to the better genetic repair, i.e. earlier and faster synthesis of DNA, RNA, and proteins (Srivastava 2003). Also, it seems that an increase in germination percentage is due to the impact of PGPR on increasing is the production of some hormones particularly GA and cytokinin (Demidchik Grosskinsky *et al.* 2016). GA by activating some of the enzymes such as α -amylase that are involved in starch metabolism affected the germination (Kaymak *et al.* 2009). In research, Ghezal *et al.* (2016) found that seed pretreatment alleviated the inhibitory effect of salt stress of pea germinated seeds. It has reported that the lower reduction in germination parameters of PGPR inoculated seeds in the salinity stress may be due to the ability of PGPR to limit Na⁺ and Cl⁻ transport into the seed (Metwalil *et al.* 2015). Concerning the effect of the seed inoculation with PGPR on germination rate demonstrated that this treatment limited the negative impact of salinity because plants developed from pretreated seeds recorded better germination and growth than plants developed from non-treated seeds and they showed significant amelioration in the absence and in the presence of NaCl.

The results showed that salinity causes a decrease in germination and seedling of cumin. Among biological treatments, CHA0 strain of *Pseudomonas fluorescens* emerged as the best treatment agent for enhancing seed germination and growth of cumin seedlings under salinity stress.

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