EVALUATING EFFICACY OF PLANT GROWTH PROMOTING RHIZOBACTERIA AND POTASSIUM FERTILIZER ON SPINACH GROWTH UNDER SALT STRESS

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Abstract

Soil salinity is a major constraint for crop production as it negatively impacts the supply of nutrients and produces ethylene that hampers crops productivity. Several studies have been conducted so far to devise robust strategies for alleviation of soil salinity stress on crops. Inoculation of plant growth promoting bacteria (PGPR) is one of the potential strategy to decrease salinity stress on the plants. The PGPR can produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which decreases stress of ethylene generated by salinity. On the other hand, improving potassium (K) uptake is also an effective strategy to minimize the negative effects of salt stress on crops. Therefore, a pot study was conducted with co-application of two pre-isolated ACC deaminase producing PGPR (i.e. Bacillus amyloliquefaciens and Alcaligenes faecalis) with half (HRK) and full recommended dose of K (RK) fertilizer in spinach (Spinacia oleracea) under salt stress (control = 3.0 and 5.0 dS m⁻¹). Co-application of B. amyloliquefaciens + RK significantly enhanced leaf and root fresh and dry weights of spinach. Maximum significant improvement in chlorophyll a (93%), chlorophyll b (50%), total chlorophyll (76%), K concentrations in leaf (70%) and root (60%), and reduction (26%) in leaf electrolyte leakage validated the effectiveness of B. amyloliquefaciens and A. faecalis with RK to mitigate salt stress. It is concluded that both B. amyloliquefaciens and A. faecalis with RK can alleviate salt stress in spinach.

Key words: ACC deaminase; Chlorophyll pigments; Electrolyte leakage; Morphological attributes.

Introduction

Plant response to salt stress has been widely investigated by researchers (Lu et al., 2003; Qu et al., 2012; Shahzad et al., 2012; Yasseen et al., 2013; Yasseen et al., 2019). It is well documented that 20% of cultivable and half of irrigated area of world is saline (Abogadallah, 2010). Cultivation of crops in saline soils results in development of toxicities, production of reactive oxygen species and osmotic stresses that significantly disturb plants growth (Locy et al., 1996; Flowers & Flowers, 2005; Munns et al., 2006; Zöbl et al., 2019). In salt affected soils, Na⁺ and Cl⁻ restrict the development of root and optimum uptake of water by decreasing the water potential in rhizosphere (Rout & Shaw, 2001). Stress induced by salinity accelerates the synthesis of ethylene (Stearns & Glick, 2003; Roychoudhury et al., 2019) that imposes deleterious effects on plants (Van Loon & Fontaine, 1984; Stearns & Glick, 2003). The elevated level of ethylene in plants minimizes the supply of energy and restricts the water uptake due to poor development of root (Taiz & Zeiger, 2010; Ricardo, 2012). Higher rate of transpiration, minimum biological nitrogen fixation, stomatal closure and evoking of physiological responses are also major indicators of elevated ethylene biosynthesis and accumulation (Tamimi & Timko, 2003; Wang et al., 2003; Tanaka et al., 2005; Roychoudhury et al., 2019).

Many scientists have observed that salinity stress stimulates ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (Shah et al., 1998; Glick et al., 1999; Glick, 2004; Albacete et al., 2008; Roychoudhury et al., 2019). For mitigation of salinity stress, inoculation with ACC deaminase (ACCD) producing plant growth promoting rhizobacteria (PGPR) is frequently recommended (Penrose & Glick, 1997; Glick, 2004; Zahir et al., 2009; Zafar-ul-Hye et al., 2014; Ijaz et al., 2019). The enzyme deaminase breakdown ethylene into α-ketobutyrate and NH₃ (Shah et al., 1998; Glick, 2004; Taiwo et al., 2018), and later one is mostly utilized by PGPR that inhibits the regeneration of ethylene (Shah et al., 1998; Roychoudhury et al., 2019; Ahmed et al., 2020).

Besides the use of PGPR, most of the researchers also suggested to improve the uptake of potassium (K) to reverse the negative effects of salinity on plants (Yahya, 1998; Tzortzakis, 2010). Presence of K ions in large amount increases the selectivity of K over Na that significantly reduces the intake of Na in plants (Volkov et al., 2004). Better uptake of K ultimately increased the K/Na ratio in mesophyll cells which play an imperative role in regulation of proper enzymatic functioning in crops under salt stress (Volkov et al., 2004). However, despite all modern reclamation and mitigation techniques for salinity stress, the natural causes and agricultural practices are continuously contributing towards the development of saline soils (Munns & Tester, 2008). The need of the time is to explore the approaches that are associated with the solution of salinity stress for better production of crops (Chinnusamy et al., 2005; Ashraf & Akram, 2009).

Spinach (Spinacia oleracea) is considered the richest source of beta carotene, calcium (Ca), vitamin C, iron (Fe), phosphorus (P) and potassium (K) (Dicoteau, 2000; Massa et al., 2018). It also plays an imperative role for the provision of supplement p-comaric acid derivatives that show a significant antioxidant activity and glucuronic acid derivatives of flavonoids (Bergman et al., 2001; Edenharder et al., 2001; Pandjaitan et al., 2005; Caprarotta et al., 2019). A lot of work has been so far done on inoculation of ACCD producing PGPR and K
individually for mitigation of salt stress in different crops. However, limited literature is available about co-application of ACCD producing PGPR and K for alleviation of salt stress. As spinach is moderately salt-sensitive crop (Zeng & Shannon, 1998; Maftoun et al., 2004) therefore, the current study was conducted to examine the effect of co-application of half and full recommended dose of K fertilizer with ACCD producing PGPR on growth attributes and pigments synthesis under salt stress. It is hypothesized that co-application of ACCD producing PGPR with full recommended K fertilizer could be an effective and better approach for mitigation of salt stress than half dose of potassium fertilizer.

**Materials and Methods**

**Inoculation:** Two pre-isolated PGPR strains *Bacillus amyloliquefaciens* and *Alcaligenes faecalis* were used in this study. For preparation of inoculum, Dworkin and Foster (DF) media without agar was used having (in mg/L) 4000 KH₂PO₄, 6000 Na₂HPO₄, 250 MgSO₄.7H₂O, 0.1 FeSO₄.7H₂O, 0.01 H₃BO₃, 0.01 MnSO₄.0.07 ZnSO₄, 0.05 CuSO₄, 0.01 MoO₃, 10000 glucose, 2000 gluconic acid, 2000 citric acid and 658 ACC (Dworkin & Foster, 1958). On rotatory shaker, the inoculum containing *B. amyloliquefaciens* and *A. faecalis* were incubated for 48 hours at 25 ± 2°C.

Healthy spinach seeds of variety “Desi Palak” were manually screened out to eliminate the factor of reduction in germination by damaged seeds. For sterilization, seeds were dipped in 0.1% HgCl₂ for 2 min. Traces of 0.1% HgCl₂ were washed 3 times with sterilized water (autoclaved at 120°C) for 20 min as described by Sadiq & Ali (2013). Ten sterilized seeds were placed in an autoclaved beaker having 1ml PGPR inoculum. Finally, seeds were top dressed with sterilized (autoclaved at 120°C for 20 min) peat and clay.

**Pot study:** Bulk sample of soil was collected from plough layer (15cm) at the research area of Department of Soil Science, Bahauddin Zakariya University and analyzed for texture by Hydrometer method (Gee & Bauder, 1986), soil organic matter by Walkley (1935), soil total nitrogen by Richards (1954), available P by Olsen & Sommers (1982), extractable K by Nadeem et al., (2013). The pre-experiment characteristics of the soil are provided in Table 1.

<p>| Table 1. Pre-experiment soil characteristics (prior to artificial development of salinity). |
|---------------------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Soil characteristics</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand</td>
<td>%</td>
</tr>
<tr>
<td>Silt</td>
<td>%</td>
</tr>
<tr>
<td>Clay</td>
<td>%</td>
</tr>
<tr>
<td>Texture</td>
<td>Sandy clay loam</td>
</tr>
<tr>
<td>pH</td>
<td>8.19</td>
</tr>
<tr>
<td>EC</td>
<td>dS m⁻¹</td>
</tr>
<tr>
<td>Organic matter</td>
<td>%</td>
</tr>
<tr>
<td>Organic nitrogen</td>
<td>%</td>
</tr>
<tr>
<td>Extractable phosphorus</td>
<td>mg kg⁻¹</td>
</tr>
<tr>
<td>Extractable potassium</td>
<td>mg kg⁻¹</td>
</tr>
</tbody>
</table>

Each pot was filled with 8 kg soil. Nitrogen 22 kg and P₂O₅ 15.6 kg ha⁻¹ were applied as urea and diammonium phosphate. Potassium was applied as per treatment plan. To maintain the half dose of recommended potassium fertilizer (HRK), 13.75 kg ha⁻¹ and for recommended potassium fertilizer (RK), 27.5 kg ha⁻¹ K₂O was applied as mure of potash (ZTBL, 2018).

**Treatments plan and experimental design:** Initially, soil electrical conductivity (EC) was 3.0 dS m⁻¹ (control) which was increased to 5.0 dS m⁻¹ by using NaCl to induce salt stress. At these two EC levels, following treatments were applied: (1) Half rate of recommended potassium fertilizer (HRK), (2) *B. amyloliquefaciens* + HRK, (3) *A. faecalis* + HRK, (4) Recommended potassium fertilizer (RK), (5) *B. amyloliquefaciens* + RK and (6) *A. faecalis* + RK. Treatments were arranged in two-factorial completely randomized design in four replicates.

Initially, 10 seeds pot⁻¹ were sown manually. After seven days of germination, 5 healthy seedlings were maintained. To fulfill the water requirement, the plants were irrigated frequently according to their demand. Plants were harvested after 65 days of sowing for examining the morphological and biochemical growth attributes. Leaves and roots fresh weight was assessed soon after harvesting. For determination of dry weight of leaves and root, samples were oven dried at 65°C for 24 hours.

**Leaf and root analysis:** Chlorophyll a, b and total in fresh leaves were determined by methodology of Arnon (1949) and Zafar-ul-Hye et al., (2019). Fresh leaves were grinded in 80% acetone and then filtered through a filter (Whatman filter paper no. 42) for measuring chlorophyll at an absorbance of 663 and 645 nm wavelength on a spectrophotometer. Final values were calculated by equations:

\[
\text{Chlorophyll a (mg g}^{-1}\text{f.wt.)} = \frac{12.7 (\text{OD 663}) - 2.69 (\text{OD 645})}{1000 (W)}
\]

\[
\text{Chlorophyll b (mg g}^{-1}\text{f.wt.)} = \frac{22.9 (\text{OD 645}) - 4.68 (\text{OD 663})}{1000 (W)}
\]

\[
\text{Total Chlorophyll (mg g}^{-1}\text{f.wt.)} = \frac{22.9 (\text{OD 645}) - 4.68 (\text{OD 663})}{1000 (W)}
\]

where V is for final volume made and W is g of fresh leaves sample.

Electrolyte leakage (EL) was assessed according to Lutts et al., (1996). Dust on fresh leaves was removed with deionized water and then equal discs of diameter 1 cm were cut by using the steel cylinder. In test tubes of 20ml, one gram of uniform size leaves discs were immersed and incubated at 25°C for 24h. After that, EC of incubated leaves (EC1) was measured on EC meter. The test tubes were heated for 20 min at 100°C in a water bath and again EC was measured (EC2). The EL was assessed by using the equation:

\[
\text{Electrolyte Leakage (EL)} = \frac{\text{EC1/EC2} \times 100}{
\]
Harvested plant samples were digested with the diacid mixture of HNO₃ and HClO₄ in 2:1 ratio as described by Chapman and Pratt (1961). After digestion, Na and K were analyzed on the flame photometer as described by Nadeem et al., (2013).

**Statistical analysis:** Data was analyzed using standard statistical procedures of Steel et al., (1997). Treatments were compared by ANOVA followed by using Tukey’s test at p≤0.05. Computer based program Statistix 8.1 was used for statistical computations.

**Results**

**Plant growth:** There were significant effects of treatments (T) and salinity (S) remained significant but their interaction (T x S) effect was non-significant on root and leaf fresh and dry weight. In case of root fresh weight, B. amyloliquefaciens + RK, A. faecalis + RK performed significantly better comparative to the control (HRK) (Table 2). For root dry weight, B. amyloliquefaciens + RK remained significantly better comparative to the control. The treatments RK and A. faecalis + RK did not differ to each other but performed significantly better as compared to the control for root dry weight. However, application of B. amyloliquefaciens + HRK and A. faecalis + HRK did not differ comparative to the control for both root fresh and dry weight. Maximum increase of 130% and 110% in fresh and dry weight of roots was noted as compared to the control in B. amyloliquefaciens + RK. For leaf fresh weight, B. amyloliquefaciens + RK differed significantly comparative to the control (Table 2). Application of A. faecalis + RK and RK alone also remained significantly better comparative to B. amyloliquefaciens + HRK, A. faecalis + HRK and control for fresh weight of leaf. Maximum increase of 120% in leaf fresh weight was noted as compared to the control in B. amyloliquefaciens + RK. In case of leaf dry weight, B. amyloliquefaciens + RK performed significantly best and all other treatments remained statistically alike to each other.

**Chlorophyll and electrolyte leakage:** Application of K fertilizer, PGPR and salinity stress had significant effects while their interaction did not significantly affect chlorophyll a, chlorophyll b, total chlorophyll and electrolyte leakage of spinach (Table 3). Statistical analysis showed that B. amyloliquefaciens + HRK and B. amyloliquefaciens + RK performed significantly best as compared to the control for chlorophyll a. Application of A. faecalis + RK and RK alone remained statistically alike to each other but performed significantly better as compared to the control for chlorophyll a. However, A. faecalis + HRK and the control did not differ to each other for chlorophyll a. Maximum increase of 93% in chlorophyll a content was noted comparative to control where B. amyloliquefaciens + RK was applied. In case of chlorophyll b, HRK and RK did not differ significantly (Table 3). Application of B. amyloliquefaciens + HRK, A. faecalis + HRK, B. amyloliquefaciens + RK and A. faecalis + RK did not differ to each other but significantly different as compared to the control. Maximum increase of 50% in chlorophyll b content was noted comparative to control where A. faecalis + RK was applied. For total chlorophyll, B. amyloliquefaciens + RK and A. faecalis + RK performed significantly best comparative to control (Table 3). The performance of B. amyloliquefaciens + HRK and RK was also significantly better comparative to control for total chlorophyll. Maximum increase of 76% was noted comparative to control where B. amyloliquefaciens + RK was applied. For electrolyte leakage, B. amyloliquefaciens + HRK, RK, B. amyloliquefaciens + RK and A. faecalis + RK remained statistically alike to each other but significantly different comparative to A. faecalis + HRK and control. A significant reduction by 26% in electrolyte leakage was noted when compared to the control in the B. amyloliquefaciens + RK treatment.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Root fresh weight (g plant⁻¹)</th>
<th>Soil ECE (dS m⁻¹)</th>
<th>Root dry weight (g plant⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IE (T x S)</td>
<td>ME (T)</td>
<td>IE (T x S)</td>
</tr>
<tr>
<td>HRK</td>
<td>0.59 ± 0.06</td>
<td>0.33 ± 0.05</td>
<td>0.46BC</td>
</tr>
<tr>
<td>B. amyloliquefaciens + HRK</td>
<td>0.87 ± 0.06</td>
<td>0.46 ± 0.03</td>
<td>0.67C</td>
</tr>
<tr>
<td>A. faecalis + HRK</td>
<td>0.96 ± 0.07</td>
<td>0.42 ± 0.05</td>
<td>0.69C</td>
</tr>
<tr>
<td>RK</td>
<td>0.93 ± 0.09</td>
<td>0.74 ± 0.03</td>
<td>0.83BC</td>
</tr>
<tr>
<td>B. amyloliquefaciens + RK</td>
<td>1.20 ± 0.09</td>
<td>0.93 ± 0.06</td>
<td>1.06A</td>
</tr>
<tr>
<td>A. faecalis + RK</td>
<td>1.11 ± 0.09</td>
<td>0.72 ± 0.04</td>
<td>0.91AB</td>
</tr>
<tr>
<td>ME (S)</td>
<td>0.94A</td>
<td>0.60B</td>
<td></td>
</tr>
</tbody>
</table>

Means ± standard error; Mean sharing different letters are significantly different (p≤0.05); No letter means non-significant effect.

**Table 2. Effect of treatments on root and leaf fresh and dry weight of spinach plant grown under normal (3.0 dS m⁻¹) and salinity stressed conditions (5.0 dS m⁻¹).**
Concentration of Na and K: The Na and K concentration in leaves and root of spinach were significantly influenced by K fertilizer, PGPR and salinity stress whereas non-significant of their interaction was observed for Na and K concentration (Table 4). For Na in leaves and root, B. amyloliquefaciens + RK and A. faecalis + RK remained significantly best comparative to control. Application of RK also differs significantly comparative to control for Na in leaves and root. However, B. amyloliquefaciens + HRK remained significant for Na concentration in leaves but non-significant for Na concentration in the root comparative to control. A significant reduction of 45% and 82% in leaves and root Na concentration was noted respectively comparative to control where A. faecalis + RK was applied. In case of K concentration in leaves and root, B. amyloliquefaciens + RK and A. faecalis + RK differ significantly comparative to control. Application of RK performed significantly better for K concentration in leaves and root comparative to control. However, B. amyloliquefaciens + HRK and A. faecalis + HRK differ significantly for leaf K concentration but remained non-significant for root K concentration comparative to control. Maximum increase in leaf and root K concentration was noted 70% and 60% comparative to control in A. faecalis + RK and B. amyloliquefaciens + RK respectively.

Discussion

Soil salinity stress markedly decreased the growth of spinach in the present study. The reduction in fresh and dry weight of leaves and roots of spinach might be due to higher biosynthesis of ethylene and disturbance in the minerals uptake that induced ion toxicity. The findings of Xu & Mou (2016) are in agreement with our results. They argued that ionic imbalance under salinity stress induced toxicity of ions that significantly decreased the fresh and dry weight of spinach. It has also documented that under abiotic stress, salinity or drought, a significant increase in ethylene biosynthesis decreases the growth attributes of the crops (Glick et al., 1995; Zafar-ul-Hye et al., 2007; McDonnell et al., 2009; Zahir et al., 2011; Ahmad et al., 2014; Bano, 2018; Danish et al., 2020). Ali et al., (2014) and Glick et al., (1998) suggested that ACC deaminase plays a key role in mitigation of salinity stress by decreasing the ethylene accumulation in the crops. According to Glick et al., (1998), the ACC deaminase enzyme breaks the molecules of rhizospheric ethylene into an intermediate compound called HN₃ and α-ketobutyrate. As a result of ethylene reduction in rhizosphere, the stress generating ethylene in plants root moved outside along the concentration gradient in the rhizosphere due to which ethylene accumulation in plants is decreased. A significant improvement in root and leaves fresh and dry weight in spinach plants inoculated with B. amyloliquefaciens and supplied with HKR validated the efficacious functioning of ACC deaminase in the presence of K under salinity stress. It was also noted that salinity stress significantly increased electrolyte leakage where HK treatment was applied (Table 3). However, B. amyloliquefaciens and A. faecalis along with RK treatment significantly decreased the electrolyte leakage.
leakage in spinach plants which might be due to reduction in ethylene accumulation. Senaratna & McKersie (1983) suggested that more permeability of cell membrane due to damage caused by abiotic stress usually resulted in higher electrolyte leakage. The findings of Nadeem et al., (2017) justified our argument as they reported a significant reduction in the electrolyte leakage where they applied Pseudomonas fluorescens in combination with biochar under abiotic stress. A significant reduction in the chlorophyll a, b and total in plants also indicated the generation of ethylene stress in HRK where no PGPR was applied (Table 3). Matile et al., (1997) observed in their study that when ethylene become in contact with the chloroplast then it activates the chlorophyllase (chls) gene. As a result of chlorophyllase activation the membrane loses its integrity and destruction of chlorophyll started. Higher synthesis of holorophyll a, chlorophyll b and total chlorophyll in current study indicates the imperative role of B. amyloliquefaciens and A. faecalis along with RK for mitigation of salinity stress by lowering the ethylene. Greater K concentration in leaves and root might also be an allied factor for the mitigation of salinity stress in spinach plants (Table 4). It was also noted that the concentration of K was significantly improved but Na was decreased where RK was applied along with inoculation of B. amyloliquefaciens and A. faecalis compare to control. According to Zhao et al., (2001), a significant reduction in the chlorophyll contents of cotton was due to the less intake of K in the plants. This indicates that better uptake of K might also be a pronounced allied factor that can also play an important role in mitigation of salinity stress in plants.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Na concentration (%) in leaves</th>
<th>Soil ECE (dS m⁻¹)</th>
<th>Na concentration (%) in root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na concentration (%) in leaves</td>
<td>IE (T x S) ME (T)</td>
<td>IE (T x S) ME (T)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>HRK</td>
<td>0.69 ± 0.03</td>
<td>0.79 ± 0.03</td>
<td>0.74 A</td>
</tr>
<tr>
<td>B. amyloliquefaciens + HRK</td>
<td>0.57 ± 0.02</td>
<td>0.71 ± 0.03</td>
<td>0.64 B</td>
</tr>
<tr>
<td>A. faecalis + HRK</td>
<td>0.56 ± 0.04</td>
<td>0.75 ± 0.03</td>
<td>0.65 AB</td>
</tr>
<tr>
<td>RK</td>
<td>0.48 ± 0.02</td>
<td>0.58 ± 0.03</td>
<td>0.53 C</td>
</tr>
<tr>
<td>B. amyloliquefaciens + RK</td>
<td>0.31 ± 0.04</td>
<td>0.42 ± 0.05</td>
<td>0.37 D</td>
</tr>
<tr>
<td>A. faecalis + RK</td>
<td>0.37 ± 0.02</td>
<td>0.45 ± 0.03</td>
<td>0.41 D</td>
</tr>
<tr>
<td>ME (S)</td>
<td>0.60 B</td>
<td>0.62 A</td>
<td>0.64 D</td>
</tr>
<tr>
<td>K concentration (%) in leaves</td>
<td>2.01 ± 0.15</td>
<td>1.46 ± 0.09</td>
<td>1.74 D</td>
</tr>
<tr>
<td>K concentration (%) in root</td>
<td>2.72 ± 0.16</td>
<td>2.16 ± 0.08</td>
<td>2.44 BC</td>
</tr>
<tr>
<td>HRK</td>
<td>2.50 ± 0.11</td>
<td>2.09 ± 0.11</td>
<td>2.29 C</td>
</tr>
<tr>
<td>A. faecalis + HRK</td>
<td>2.81 ± 0.11</td>
<td>2.06 ± 0.07</td>
<td>2.44 BC</td>
</tr>
<tr>
<td>RK</td>
<td>2.91 ± 0.20</td>
<td>2.56 ± 0.11</td>
<td>2.74 AB</td>
</tr>
<tr>
<td>B. amyloliquefaciens + RK</td>
<td>3.38 ± 0.13</td>
<td>2.52 ± 0.16</td>
<td>2.95 A</td>
</tr>
<tr>
<td>A. faecalis + RK</td>
<td>2.72 A</td>
<td>2.14 B</td>
<td>2.74 A</td>
</tr>
<tr>
<td>ME (S)</td>
<td>2.72 A</td>
<td>2.14 B</td>
<td>7.24 A</td>
</tr>
</tbody>
</table>

Means ± standard error; Means sharing different letters are significantly different (p<0.05); No letter means non-significant effect; ME (T) = Main effect of treatments; ME (S) = Main effect of salinity; IE = Interactive effect; RK = Full recommended dose of potassium; HRK = Half recommended dose of potassium

Conclusion

It is concluded that ACC deaminase containing B. amyloliquefaciens and A. faecalis have the potential to reduce the salt stress in spinach plants when applied with recommended K fertilizer. Both B. amyloliquefaciens and A. faecalis were effective but B. amyloliquefaciens was more efficacious comparative to A. faecalis along with recommended K fertilizer to mitigate salinity stress in spinach.

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