The protective effect of Klebsiella on grassland plants under saline soil conditions

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Abstract: The inoculation of grassland plants, cultivated under saline conditions, with bacteria of the Klebsiella genus (K. planticola and K. pneumoniae) showed a protective effect of these bacteria. The previously unknown property of Klebsiella pneumoniae to ensure normal plant growth at high sodium chloride levels was identified in this study. Plant inoculation with these bacteria induced optimization of the morphological and physiological properties of grassland plants and increased the number of mitoses in the apical portion of the root, resulting in a less severe decline in mitotic index under saline conditions.

This study also determined the capacity of Klebsiella planticola to penetrate the plant and, hence, facilitate activation of protective mechanisms that enhance the immune status in the plant and improve its resistance to salinity.

Keywords: bacteria, grassland plants, protection

Introduction

The development of urban landscapes has generally been hampered by inorganic soil contamination induced by the use of anti-icing agents on roads during winter (Gladkov 2003, Kalasnikova 2003). This leads to problems such as environmental disturbances, green cover deterioration and a reduction in grassland density and longevity.

Over the past several years, microbial products derived from a variety of microorganisms of the genera Azospirillum, Bacillus, Klebsiella, Pseudomonas and others (Emtev 1994, Stankevič 2002, Jegorov 2003, Belimov 2004, Raghvendra et al. 2010).
Harbans 2008) have been actively used to protect plants against the effects of environmental stress factors. Major benefits of the bioremediation method include duration of effect and harmonization within the biosphere. This method has been recognized as an inexpensive and safe soil purification technology that does not cause later damage to the environment. As the contribution of microorganisms to reducing the effects of salinity stress on plants has still not been sufficiently investigated, this study was aimed at providing further insight into this matter.

**Material and Methods**

The bacteria used in this study included *K. planticola* (TSHA-91 and Rif²⁰⁰ strains) obtained from the Department of Microbiology, Russian State Agrarian University, Moscow Timiryazev Agricultural Academy and *K. pneumoniae* (strain 204 obtained from the Tarasevich State Institute of Standardization and Control of Biomedical Preparations).

Luria Bertani (LB) nutrient agar (Miller 1976) and LB with an addition of rifampicin were employed for the cultivation of *K. planticola* and *K. pneumoniae* were grown on Worfel-Ferguson (WF) agar (Mirošničenko 1980).

Meadow fescue (cv. Vik and Duet) and red oatgrass (cvs. Tatjana and Juliška) plants were used in evaluating the protective effect of *K. planticola* on grassland plants under saline soil conditions. Chinese cabbage cv. Lastavica was used as a test crop in assessing the protective effect of the test bacteria against cadmium penetration into the plants.

The quantitative determination of the protective effect of *K. planticola* TSAH-91 and *K. pneumoniae* 204 strains on the plants under saline soil conditions included grassland plant assessment for the following parameters: seed germination (GOST-12038-84), length of the largest leaf and average root length. The stressants used included NaCl, KCl or CaCl₂. The level of NaCl was 1.0-2.0-3.0 and CaCl₂ and KCl were applied at identical levels of 0.2-0.3-0.5 M (in the trial with *K. planticola* TSAH-91). The level of NaCl used in the trial with *K. pneumoniae* 204 was 0.1-0.2-0.3 M. The grassland plant seeds were inoculated for 30 minutes with a 24-hour culture suspension of live *K. planticola* TSAH-91 or *K. pneumoniae* 204 diluted with distilled water to a concentration of 10⁹ microbial cells. The results on meadow fescue and red oatgrass were read within 7 and 14 days, respectively.

The cytophotometric analysis of the test grassland plants was conducted in order to determine the duration of cell cycle phases using an Opton SMP-20 cytospectrophotometer. The seeds of meadow fescue cv. Duet were germinated and inoculated with *K. planticola* TSAH-91 following the established methods at a level of 0.1-0.2-0.3 M NaCl. Seven days later, root meristem preparations were made and dyed after Feljgen (Siffa, Megsk reagent).

The mitotic activity of the root meristem was estimated in meadow fescue cv. Duet. Seeds were germinated and fixation was performed on day 7, following which the squash preparation of root meristems was made and mitotic indices assessed by light microscopy using generally accepted methods (Pauševa, 1970).
The vegetative trial was set up during 2003-2004 using soil culture (Žurbickij, 1968) to estimate the adaptability of \( K. \) \( \text{planticola} \) \( \text{TSHA-91} \) and Rif\(^{200} \) within the phytosnake according to the method described by Tepper (1987) and determine certain quality parameters in plants under saline soil conditions. NaCl was incorporated into the soil at a rate of 0.05-0.1-0.2% per soil mass. The total level of mineral nutrition was established by incorporation of \( \text{NH}_4\text{PO}_4 \) and \( \text{KH}_2\text{PO}_4 \) salts.

Before sowing, the seeds of meadow fescue cv. Duet and red oatgrass cv. Tatjana were inoculated for 10 minutes using a 24-hour culture of \( K. \) \( \text{planticola} \) Rif\(^{200} \) (initial culture titre was \( 10^9 \) microbial cells per cm\(^3 \)). During winter, the dishes containing the grassland plants were buried into trenches at soil level. The capacity of \( K. \) \( \text{planticola} \) TSHA-91 Rif\(^{200} \) to colonize the rhizosphere, rhizoplane and phylosphere was determined by assessing the number of colonies following inoculation of the test material with 200 mg/cm\(^3 \) rifampicin on solid LB medium. The number of live bacteria in both rhizosphere and rhizoplane was determined following the method described by Tepper et al. 1987.

**Plant quality indicators.** During the shoot growth stage, the grassland plants were estimated for the contents of dry matter (Ganzar et al. 2006), nitrate nitrogen (Morion-OK2 nitrate-tester ), chlorophyll (Jagodin et al. 1987), ß-carotene (Jagodin et al. 1987), proline (Bates et al. 1973), selenium and ascorbic acid. The tissue contents of potassium, sodium, barium, chlorine and cadmium ions were determined by ion-selective electrodes in an aqueous extract (Expert-001).

A vegetation trial was established in order to evaluate the effect of \( K. \) \( \text{planticola} \) on the root and leaf content of cadmium in Chinese cabbage cv. Lastavica under saline soil conditions as well as on an increase in soil cadmium levels. The substrate used was common chernozem incorporated with 10 mg \( \text{Cd(CH}_3\text{COO})_2\cdot2\text{H}_2\text{O} \) and 500 mg NaCl per kg of soil. Plant seeds were inoculated with \( K. \) \( \text{planticola} \) TSHA91 before sowing. The plants were collected during the growing season. The level of cadmium was determined in both root and the leaf. The cadmium ion level was determined by using ion-selective electrodes in an aqueous extract (Expert-001).

The obtained data were subjected to statistical analysis following Fisher-Student’s criteria using Statistica 6.0 programme (Borovikov 2001).

**Results and discussion**

**Evaluation of the effect of \( K. \) \( \text{planticola} \) and \( K. \) \( \text{pneumoniae} \) on seed germination, growth and development of grassland plants under saline soil conditions.**

The effect of \( K. \) \( \text{planticola} \) on seed germination and development of different varieties of grassland plants was evaluated \textit{in vitro} at different sodium, potassium and calcium salt levels (Table 1).

In all grassland plant varieties, the critical salt level that did not induce seed germination was 0.3-0.4 M. Inoculation with \( K. \) \( \text{planticola} \) caused a reduction in phytotoxicity of the salts used in this study. The germination rate in the test grassland plants at the salt level of 0.3 M under all treatments was 60-90%. 

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The increase in the germination capacity of grassland plant seeds suggests the protective effect of *K. planticola* on plants under salinity-induced abiotic stress. Importantly, the survival potential and expression of the protective effect under saline conditions is evidently associated with both the coefficient of fatty acid saturation and the indicator of bacterial membrane stability (hydrophylicity) (Lothagen *et al.* 1995) which enable the grassland plants to adapt to extreme environmental factors. This study showed that the strain *K. planticola* Rif200 exhibited an increased value of the coefficient of fatty acid saturation and a decrease in the cell membrane hydrophylicity indicator.

Tab. 1. The effect of *K. planticola* TSHA-91 and *K. pneumoniae* 204 on root length and largest leaf length of grassland plants under different NaCl levels.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Meadow fescue cv. Duet</th>
<th>Red oatgrass cv. Tatjana</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germination, %</td>
<td>Average root length, mm</td>
</tr>
<tr>
<td>Control (H&lt;sub&gt;2&lt;/sub&gt;O)</td>
<td>99.9±0.9</td>
<td>42.7±3.5</td>
</tr>
<tr>
<td>0.1 M NaCl</td>
<td>93.0±2.6</td>
<td>30.2±2.6</td>
</tr>
<tr>
<td>0.2 M NaCl</td>
<td>83.0±3.7</td>
<td>21.4±2.2</td>
</tr>
<tr>
<td>0.3 M NaCl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>K. planticola</em></td>
<td>93.0±2.6</td>
<td>34.1±2.5</td>
</tr>
<tr>
<td><em>K. planticola</em> +</td>
<td>93.0±2.6</td>
<td>29.3±2.3</td>
</tr>
<tr>
<td>0.1 M NaCl</td>
<td>90.0±3.0</td>
<td>28.0±2.2</td>
</tr>
<tr>
<td><em>K. planticola</em> +</td>
<td>90.0±3.0</td>
<td>28.0±2.2</td>
</tr>
<tr>
<td>0.3 M NaCl</td>
<td>57.0±4.9*</td>
<td>9.6±1.7*</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>87.0±3.4</td>
<td>34.6±3.7</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> +</td>
<td>90.0±3.0</td>
<td>29.4±2.9</td>
</tr>
<tr>
<td>0.1 M NaCl</td>
<td>93.0±2.6</td>
<td>26.6±2.7</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> +</td>
<td>77.0±4.2*</td>
<td>16.6±2.0*</td>
</tr>
<tr>
<td>0.2 M NaCl</td>
<td>93.0±2.6</td>
<td>26.6±2.7</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> +</td>
<td>77.0±4.2*</td>
<td>16.6±2.0*</td>
</tr>
</tbody>
</table>

N.B.: significance of difference between the treatments 4 and 8; 4 and 12

* - p≤0.01; 0.001

Similarities were found between *K. planticola* TSHA-91 and *K. pneumoniae* 204 with respect to the protective effect of the grassland plants under 0.1-0.2-0.3 M NaCl salinity. At a lethal level of 0.3 M NaCl for the grassland plants, seed inoculation with *K. planticola* TSHA-91 and *K. pneumoniae* 204 led to a rather high seed germination capacity of up to 57-93% (Table 2). Under all bacterial treatments, the length of the largest leaf and root length increased as compared to the control. The largest length and the root were longer under treatment with *Klebsiella* strains than in the control even at maximum NaCl levels (0.3 M). Analogous data were obtained for meadow fescue cv. Vik and red oat grass cv. Juliska.
The above results suggest that *K. planticola* and *K. pneumoniae* exhibited a protective effect on grassland plants under salinity stress. Furthermore, grassland inoculation with *Klebsiella* under the said conditions led to seed germination as compared to the saline solution. In view of the estimated importance of checking the survival potential of the bacteria incorporated into the rhizosphere of the test plants under saline soil conditions, a two-year vegetation trial (soil culture, after Žurbicki 1968) was established. During 2003, the ability of *K. planticola* to survive in the rhizosphere of grassland plants under saline soil conditions was assessed during the winter period. Following the artificial salinization conducted in May, the grassland plant seeds previously inoculated with *K. planticola* TSHA-91 Rif20 were sown in the soil. The results were obtained across plant growth stages after sowing of the test material in solid LB medium. The count of *K. planticola* TSHA Rif20 in the rhizoplane of red oatgrass (cv. Tatjana) and meadow fescue (cv. Duet) ranged during the growing season from $10^6$–$10^7$ col/g of root (first-leaf stage) to $10^2$–$10^3$ col/g root (end of season, September) under all treatments. Following the winter period, *K. planticola* counts were determined in the rhizoplane, phylo- and rhizospheres. *K. planticola* was found to retain its vitality in the rhizoplane under salinity treatments after the termination of the winter period during which the dishes were kept under field conditions. Its vitality was $10^2$–$10^3$ col/gr root. *K. planticola* was not detected in the control (without salinization).

The test bacteria, as shown by this study, were found in the rhizoplane of the grassland plants during the growing season and retained their viability during the entire autumn-winter period. Under saline soil conditions, *K. planticola* most likely 'moved' from the soil and rhizosphere into the root and stem of the plants in search of a new environmental niche, where they survived during autumn and winter.

**Evaluation of the effect of *K. planticola* on the morphological and physiological indicators in grassland plants under saline soil conditions.**

Since *K. planticola* was found to stimulate the growth of grassland plants under saline soil conditions, trials were set up to determine the mitotic index and distribute DNA across cell cycle phases that could be altered under stress, salinization in particular. Studies were conducted by cytophotometric analysis of the preparation of the root apical meristems of grassland meadow fescue cv. Duet.

*K. planticola* was found to exhibit a positive effect on the mitotic activity of the root germ meristems of meadow fescue under saline soil conditions (Table 2).

At a maximum level of NaCl (0.3 M), *K. planticola* stimulated cell division in cv. Duet, inducing an increase in MI of the root meristem to 53.7% as compared to the treatment without bacteria.

An important informative parameter used in evaluating the functional activity of root apical meristem cells of meadow fescue cv. Duet is DNA distribution across cell cycle phases including variations in the distribution. The cell analysis results revealed that NaCl changed the distribution of root cells in cv. Duet across the interphase periods. The 0.2 M NaCl level induced cell numbers to increase up to 72% and G2-phase to prolong, whereas the levels of 0.1 and 0.3 M NaCl resulted in the prolongation of phase G1. This may testify to
the adaptation of the root meristem of meadow fescue cv. Duet to the effect of salinity stress by cells entering a state of dormancy required for survival under stress conditions (Georgieva et al. 1994, Lucenko et al. 2005). The use of K. planticola under saline soil conditions caused a redivision of cells during the interphase period and cell domination in the postsynthetic phase. The changes were rate-dependent.

Tab. 2. The mitotic index of root apical meristem cells in Meadow fescue cv. Duet under treatment with K. planticola under saline soil conditions.

<table>
<thead>
<tr>
<th>Number</th>
<th>Treatments</th>
<th>Mitotic index, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control (H$_2$O)</td>
<td>8.9±0.6</td>
</tr>
<tr>
<td>2</td>
<td>K. planticola</td>
<td>9.1±0.3</td>
</tr>
<tr>
<td>3</td>
<td>0.1 M NaCl</td>
<td>8.1±0.2</td>
</tr>
<tr>
<td>4</td>
<td>0.2 M NaCl</td>
<td>4.8±0.2</td>
</tr>
<tr>
<td>5</td>
<td>0.3 M NaCl</td>
<td>2.4±0.1</td>
</tr>
<tr>
<td>6</td>
<td>K. planticola + 0.1 M NaCl</td>
<td>8.5±0.2</td>
</tr>
<tr>
<td>7</td>
<td>K. planticola + 0.2 M NaCl</td>
<td>7.6±0.2$^*$</td>
</tr>
<tr>
<td>8</td>
<td>K. planticola + 0.3 M NaCl</td>
<td>3.8±0.1$^*$</td>
</tr>
</tbody>
</table>

Observations: 1) mitotic index (MI) – the percent contribution of cells undergoing mitosis to the total number of cells in the test tissue; 2) significance of difference between the treatments 8 and 5; 7 and 4$^*$ - p<0.01.

Therefore, NaCl reduces the mitotic index and K. planticola increases the number of mytoses in the root apical meristem of meadow fescue. The use of bacteria under chloride soil salinization induces a less intensive decrease in the mitotic index. During the cell cycle interphase, cell division is observed and cells dominate the postsynthetic phase (G$_2$), which induces normalization of the growth process in plants.

**Evaluation of the effect of K. planticola on some properties of grassland plants grown under saline soil conditions.**

The obtained data showed that K. planticola substantially improved grassland plant condition under chloride salinity. Therefore, it was necessary to explain whether the indicators of the chemical composition of the plants cultivated under saline soil conditions underwent any changes.

The leaves of the test grassland plants were used in this study and analyzed for dry matter, nitrate, selenium, ascorbic acid, proline, chlorophyll and ß-carotene contents and potassium, sodium, barium, chlorine and cadmium ions. The potassium to sodium ratio was calculated.

In response to stress conditions, the leaf content of proline in the grassland plants increased 2.4- to 14-fold as compared to the control. The increased proline content is known to be able to protect proteins against denaturation by salt ions due to increased solubility of the latter (Olmos et al. 1996, Pronina 2000, Mutlu and Bozkuk 2005). At a level of 0.2 % NaCl per mass of soil under treatment with K. planticola, a
3- to 19-fold increase in proline content was observed as compared to the control, which attests to increased plant resistance to the salts.

Therefore, the protective effect of plant inoculation with *K. planticola* under saline soil conditions can be associated with the increased proline content in grassland plants.

The indicators for grassland plants tested under salinity and bacterial inoculation conditions did not exhibit any other substantial changes.

*K. planticola* was found to reduce the cadmium content in grassland plants under saline soil conditions, the reduction being 1.5- to 2.3-fold.

In order to confirm the detected ability of *K. planticola* to reduce the content of cadmium in other plants, a vegetation trial was established involving Chinese cabbage cv. Lastavica. The obtained results are given in Table 3.

Tab. 3. The effect of *K. planticola* TSHA-91 on the root and leaf contents of cadmium in Chinese cabbage under soil salinity and cadmium pollution

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cadmium, mg/kg root fresh weight</th>
<th>Cadmium, mg/kg leaf fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.91</td>
<td>0.03</td>
</tr>
<tr>
<td>Cd</td>
<td>5.64</td>
<td>0.25</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.36</td>
<td>0.19</td>
</tr>
<tr>
<td>NaCl+Cd</td>
<td>2.62</td>
<td>0.53</td>
</tr>
<tr>
<td><em>K. planticola</em></td>
<td>1.93</td>
<td>0.06</td>
</tr>
<tr>
<td><em>K. planticola</em>+Cd</td>
<td>1.05*</td>
<td>0.05*</td>
</tr>
<tr>
<td><em>K. planticola</em>+NaCl</td>
<td>1.20*</td>
<td>0.04*</td>
</tr>
<tr>
<td><em>K. planticola</em>+NaCl+Cd</td>
<td>2.29*</td>
<td>0.13*</td>
</tr>
</tbody>
</table>

Observation: 1) Cd- 10mg/kg Cd (CH₃COO)₂·2H₂O; 2) NaCl- 500 mg/kg; 3) significance of difference in indicators within related groups *-p<0.01.

The results showed that the inoculation of Chinese cabbage seeds with *K. planticola* at the established NaCl level resulted in a 1.14-fold and 4-fold decrease in cadmium level in the root and the leaf, respectively, as compared to the non-inoculated treatment. The effect is attributed to the apparent capacity of *Klebsiella* bacteria to inhibit the effect of cadmium resulting from the deposition of cadmium using H₂S, by converting it into CdS unavailable to plants (Sharma *et al.* 2000).

Primarily, therefore, *K. planticola* Rif²⁰⁰ and TSHA-91 strains were determined to have the capacity to induce a reduction in cadmium content in the test plants under salinity and cadmium pollution conditions.

The results obtained in this study evidenced that *Klebsiella*, due to its adaptability to extreme environments and its physiological and biochemical properties, can be suitably used under grassland plants to protect the plants under salinity stress.
Conclusion

A protective effect of bacterial inoculation of grassland plants grown under saline soil conditions with Klebsiella was determined. The bacteria reduced the phytotoxic effect of different salts (NaCl, KCl, CaCl₂), thereby facilitating seed germination and stem length in grassland plants.

A comparison of the ability of two species of Klebsiella (K. planticola and K. pneumoniae) to ensure normal growth of the plants under salinity conditions showed that both K. planticola and K. pneumoniae facilitate seed germination in grassland plants, the germination capacity reaching 57-93%, even at rather high levels of NaCl (0.3 M). K. pneumoniae exhibited the previously unknown property to normalize plant growth at high NaCl levels.

Seed inoculation with the test bacteria under sodium chloride salinity induced bacterial colonization of the entire phytoplane (root, stem, leaf). However, following the autumn/winter period during which the dishes containing grassland plants were kept under field conditions, viability was preserved only in the bacteria dwelling in the rhizoplane (10⁻²⁻¹⁰⁻³ col/g) of the plants grown under saline conditions. Bacteria were not detected in the non-saline soil.

A level of 0.3 M NaCl induced a substantial (4-fold) decline in the mitotic index of the plants. Bacterial inoculation caused an increase in mytosis number in the root apical meristem, leading to a less intensive decline in mitotic index irrespective of salinity. Cell distribution occurred during the interphase and cells dominated in the postsynthetic phase.

Under NaCl salinity, seed inoculation with K. planticola caused an increase in proline content in grassland plants, contributing to increasing plant resistance to salt.

This is the first record of the seed inoculation with K. planticola of plants (grassland plants, Chinese cabbage) grown under NaCl and Cd conditions leading to a decrease in Cd level in the root and leaf of the plants.

References


ZAŠTITNI EFEKAT KLEBSIELLA NA GAZONSKE TRAVNJAKE GAJENE U USLOVIMA ZASLANJENIH ZEMLJIŠTA

- originalni naučni rad -

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Rezime

Inokulacijom gazonskih trava, gajenih u uslovima zaslanjenosti, bakterijama roda Klebsiella (K. planticola i K. pneumonia) utvrđen je zaštitni efekat. Isholjeno je ranije nepoznato svojstvo K. pneumonia da obezbedi normalan rast biljaka u uslovima visoke koncentracije NaCl. Inokulacija biljaka sa ovim bakterijama uslovljava optimizaciju morfofizioloških svojstava biljaka gazonskih trava, povećava broj mitoza u apikalnim delu korena, što dovodi do manje intenzivnog pada mitotičkog indeksa u uslovima zaslanjenja.

Utvrđena je sposobnost K. planticola da prodire u biljku, što, očigledno, doprinosi aktivaciji zaštitnih mehanizama, koji povećavaju imuni status biljaka, zahva-ljujući čemu ispoljavaju otpornost na zaslanjenje.