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PLANT PROTECTION AND STORAGE PRODUCTS

EFFECT OF HALOTOLERANT METHYLOTROPHS ISOLATED FROM SALINE SOIL RHIZOSPHERE ON DIMINUTION OF SALT STRESS IN WHEAT (*TRITICUM AESTIVUM* L.)

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Conflict of Interest

None declared.

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Abstract

Two aerobic, halotolerant, facultatively methylotrophic strains M1K and M7 were isolated from the Suaeda prostrata Pall. and Plantago maritima L. rhizospheres from saline soils of the Solikamsk technogenic ecosystem. The isolates showed growth at 5–10% NaCl concentration. Based on 16S rRNA and mxaF gene sequence analysis, the obtained strains were classified as Methylophaga nitratireducenticrescens M1K and Paracoccus sp. M7. Within 5 days of the inoculation experiment, the wheat seedlings colonized with the isolated strains under 170 mM of NaCl showed an increase in growth parameters and synthesis of plant pigments over the control samples. The ability of analyzed cultures to influence indole-3-acetic acid (IAA) synthesis from L-tryptophan in the culture medium was found. The obtained data suggest the possibility of using isolated symbiotic bacterial strains for the creation of biofertilizer – plant growth promoter in the salt affected soils.

Keywords: *rhizosphere,·halotolerant methylotrophic bacteria,·pigments, indole-3-acetic acid,·wheat, salt stress*

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1 Introduction

Soil salinization is one of the major issues in agricultural production. It affects an estimated 45 million hectares of irrigated land, and is expected to increase due to global climate changes and as a consequence of many irrigation practices (Roy et al., 2014). Since most of the crop plants are sensitive to salinity, enhancement of natural drought and salt resistance of crops due to microbial fertilizer presowing treatment is one of the most promising methods of crop protection from drought and salinization.

Plant growth-promoting bacteria (PGPB) are naturally occurring microorganisms that colonize plant surfaces or tissues and may benefit plants by providing growth promotion. Aside from well described earlier species of PGPB a number of PGP methylotrophic bacteria like *Methylobacterium*, *Methylophilus, Methylovorus, Paracoccus, Xanthobacter* have been also reported (Fedorov et al., 2011; Saharan and Nehra, 2011; Patil, 2013). Analysis of phytosymbiotic interactions of aerobic methylotrophic bacteria and plants showed that the products of plant metabolism (methane, methanol, methylamines) are used for the growth of these bacteria (Kutchera, 2007). Due to a major role of plants' emission in the world budget of methanol, methylotrophic bacteria are ubiquitous in nature and colonize probably all land plants (Abanda-Nkpwatt et al., 2006). Besides, since some methylotrophic bacteria are able to fix nitrogen, produce and secrete phytohormones, amino acids and osmoprotectors, the methanol-consuming bacteria are viewed as coevolved partners of plants (Kutchera, 2007). In this connection, it should be noted that methylobacteria, especially obligate methylotrophs, appear to be a preferable biotechnology object, particularly in IAA synthesis because consuming one-carbon substances, they are less prone to cause pathogenesis.

The main objective of this study was to assess the effects of methylotrophic bacteria isolated from the rhizosphere of saline soil plants on wheat seedlings growth parameters under salt stress.

2 Materials and methods

Plant specimens were collected from a *Suaeda prostrata* Pall. and *Plantago maritima* L. rhizospheres near the waste banks and salt lakes of Solikamsk salt mines (Perm Krai, Russia) in September 2012 (59°34'0.00"C, 56°45'60.00"B; 59°37'55.00"C, 56°45'54.00"B). Microbial cultures were isolated using modified selective liquid Galchenko medium with 0.5 – 1.0% (*v/v*) methanol. Isolates were purified by repeated culturing on liquid and solid media with methanol as the sole carbon source using the dilution plate count method. All the subsequent experiments were conducted after raising a fresh bacterial culture. Series of routine

methods based on cultural, physiological and biochemical characteristics were carried out.

The fragments of 16S rRNA genes were amplified by the PCR with universal eubacterial primers and under standard PCR conditions (Lane, 1991). The methanol dehydrogenase gene (*mxaF*) was amplified with known primer sets: f1003, r1561, 1555r (McDonald and Murrell, 1997; Neufeld et al., 2007) and newly designed *Methylophaga*-specific primers MFGmxa2-f (GGA ACG AAA CCA TGC GTC CTG G) and MFGmxa2-r (CCC TGG TTG TGG AAA CCC AT). The reaction conditions used for MFGmxa2-set were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 20 sec, 54°C for 20 sec, and 72°C for 30 sec, and a final extension step consisting of 72°C for 5 min. The 16S rRNA and *mxaF* gene sequence data reported in this paper appear with the following accession numbers (GenBank numbers of 16S rRNA: KM192265, KM192266; GenBank number of *mxaF* KT728194).

The *Triticum aestivum* L. seeds of Gornoural'skaya cultivar were taken to estimate the influence of isolates on the cereal growth and its aboriginal microflora. Seeds were washed thoroughly three times with distilled water and germinated in sterile distilled water in the Petri plate for 48 h at room temperature. 20–30 seedlings were transferred into sterile (1.0 l) glass bottles containing 8 ml of saltless water or 170 mM of NaCl solution and bacterial suspension (10^8 cells per ml). Examples without bacterial suspension were used as negative controls. Plants were grown at 25±3°C and essential light intensity. At harvest (1, 3 and 5 days post-inoculation), the root and shoot fresh weight and length of random three to five seedlings were determined.

For pigment determination the 5–110 mg of biomass were detached for each series from the plants after incubation. The leaf tissues were homogenized in 80% acetone at 4°C. The supernatant was taken to determine the photosynthetic pigments using the Cary 100 (Agilent Technologies, USA). Pigment content was further converted into μg g^{-1} of fresh weight (Sohrabi et al., 2012).

IAA production from 1 mM of L-tryptophan was determined with Salkowski's reagent (0,05M $FeCl_3$ in 35 % $HClO_4$). Pink color was estimated by measurement of absorbance at a wavelength of 530 nm using spectrophotometer. The IAA level was assessed in accordance with a standard curve prepared from serial dilutions of the standard IAA stock solution (Gordon and Weber, 1951).

For morphological parameters (shoot and root length and fresh weight) and pigment content means and standard deviations were calculated by Excel 2007 software to evaluate the significance of differences between treatments.

3 Results and discussion

Two strains of methylotrophic bacteria were isolated on selective media from the *Suaeda prostrata* Pall. (synonym *Suaeda maritima* L.) and *Plantago maritima* L. rhizospheres from saline soil around Solikamsk salt mine spoil banks. Notably these plants are well known as typical inhabitants of sea and salt lake shores, salt marshes and arid areas.

Strain M1K showed the highest (1406 nt) 16S rRNA gene sequence similarity to *Methylophaga nitratireducenticrescens* JAM1^T (98.3% identity with GenBank entry CP003390). *Methylophaga nitratireducenticrescens* strain M1K is aerobic, moderately halophilic, facultatively methylotrophic. Grows at temperatures 15–37 ºC and 0.5–10.0% NaCl. Optimum pH is 8.0 but it grows between pH 7.0–9.0. The almost complete 16S rRNA gene (1315 nt) of strain M7 was amplified and sequenced. Database search by using BLAST on NCBI showed that strain M7 was related with group of strains of *Paracoccus* genus. The closely related species are *P. marcusii* (99.8%) and *P. carotinifaciens* (99.9% from 99.5% of coverage). *Paracoccus* sp. strain M7 is aerobic, halotolerant, facultatively methylotrophic. Grows at temperatures 15–30 ºC and 0.05–5.0% NaCl. Optimum pH is 8.0 but it grows between pH 7.0–10.0. PCR products of *mxaF* gene of M1K strain were obtained only with the *Methylophaga*-specific primers MFGmxa2-f × MFGmxa2-r constructed for this study. According to BLAST analysis of partial 368 nt methanol dehydrogenase gene sequencing, strain M1K showed 97.6% sequence similarity with *M. nitratireducenticrescens* JAM1^T followed by *M. frappieri* JAM7^T (90.4%), *M.* lonarensis MPL^T (85.3%) and *M. thiooxydans* DMS010^T (85.5% from 98.9% of coverage). Using described primer systems for amplification of methanol dehydrogenase gene we did not get any PCR product for strain M7.

To investigate the effect of isolated methylobacteria on wheat seedlings, pigment content, length and fresh weight of root and shoot were measured during the incubation with cultures M1K and M7 supported with 0 mM or 170 mM of NaCl. Our results show that salinity negatively affected the growth parameters and pigment content in wheat seedlings even. The length, fresh weight and pigment content data of plants in control with 170 mM of salt were significantly less than those with no salt. Analysis of the morphometric data in the inoculation experiment revealed that on the first and third days of investigation isolated strains did not have statistically significant effect on affected seedlings under 170 mM of NaCl. Meanwhile pigment content demonstrated significant increase of chlorophyll content in the test sample with M7+170 mM of salt. High mean concentration of green pigments for M1K+170 mM of NaCl over the control was also shown. However, carotenoid content did not reveal significant difference between the trials and control.

The final harvest measurements after five days showed that among all plant growth parameters analyzed, the maximum mean values were obtained in plants treated with M1K under 0 mM of NaCl. Test sample of M7 with no salt showed similar results. In contrast, the length and weight of the seedlings with 170 mM of NaCl in both, trials and control underwent strong inhibitory impact which reduced these morphometric characteristics. Nevertheless, we can observe a significant increase in the length of the shoot in the sample with isolate M1K at 170 mM (300% of control) (Figure 1). M7 mean values were slightly higher than in control, though the length of root system in all cases remains insufficient. The results of measuring fresh weight of seedlings also showed active development of wheat in the presence of culture M1K with 170 mM of NaCl. Weight indices of M1K with 170 mM of NaCl reached values by 1.4 and 9.3 times (compared to the control) for shoot and root, respectively. In sample with M7 and 170 mM of NaCl after five days the fresh weight of root system exceeded the control by 650% in despite of less length. Additionally, it was noted that the root weight of seedlings with M7 under 0 mM of NaCl was 188% and 155% for M1K with no salt compared with control (Figure 1). After five days we could observe a general decrease of green pigments' concentration in plant fresh weight in 170 mM of NaCl, as well as in 0 mM, which apparently indicates a stress impact not related solely with salt stress. The mean values of chlorophyll content in all test samples were higher than

control ones, but no statistically significant alterations at $p<0.05$ were found (Figure 2a), meanwhile for carotenoid content in trials under 170 mM statistically significant difference over the control samples was shown for both, M1K and M7 (Figure 2b). The diminution of chlorophyll content in controls was 43% and 30% for 0 mM and 170 mM of NaCl comparatively to previous measurements. At the same time, carotenoid showed to decrease in fresh weight of control by 2 and 4 times for 0 mM and 170 mM of NaCl, respectively. Similar parameters were found in test samples with saltless water (by 1.5 times for M1K and equal carotenoid content for M7), but with 170 mM in trials a significant increase was shown (up to 3.2 and 1.4 times for M1K and M7, respectively). The difference between test and control samples was as follows; under 0 mM of NaCl the chlorophyll content was 177 μg g^{-1} (123% of control) and 221 μg g^{-1} (154% of control) for M1K and M7, respectively, but for 170 mM of salt concentration the values were 145 μg g^{-1} (190% of control) and 186 μg g^{-1} (245% of control), respectively. The carotenoid concentration in the test fresh weight was slightly above the control value, meanwhile at 170 mM the carotenoid content was by 8 and 9 times higher for M1K and M7, respectively.

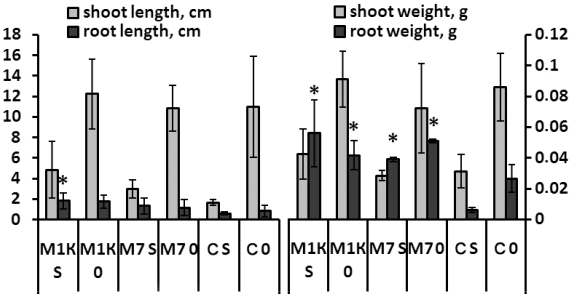

Fig. 1 - Shoot and root length (cm) and fresh weight (g) of wheat seedlings inoculated with isolates of microorganisms under 170 mM or 0 mM of NaCl after five days. Values are expressed as means±standard deviation (n = 3). S or 0 mean variants with or without NaCl, C means control. Ststisticslly significant differences with $p<0.05$ are marked with *.

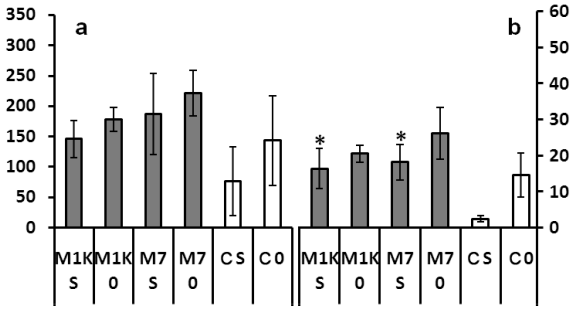

Fig. 2 - Total chlorophyll (**a**) and carotenoid (**b**) content (μg g^{-1}) in inoculated wheat seedlings after five days. Values are expressed as means±standard deviation (n = 3). S or 0 mean variants with or without NaCl, C means control. Ststisticslly significant differences with $p<0.05$ are marked with *.

After the experiments with seedlings the 1 mM of L-tryptophan was added to the rest of suspension. Within three days of incubation the IAA was detected in all test samples. It was also shown that the synthesis of IAA occurs in the control samples by means of native wheat microflora, but in much smaller quantities and under salt stress only, while in the experiment in all cases IAA is synthesized more in vessels with 170 mM of NaCl, reaching values 10 and 5 μg ml^{-1} for M1K and M7, that was by 1.4 and 2.5 times higher than those ones without salt. The IAA amount of M1K under 0 mM of NaCl was by 2.3 times more than in control with 0 mM. A largest amount of auxin synthesized by M1K with 170 mM of NaCl was 10 μg ml^{-1}. Also it has been previously shown that isolates M1K and M7 in mixed cultures were able to synthesize IAA up to 70 and 10 μg ml^{-1}, respectively, incapable of doing it in a pure culture (unpublished data).

4 Conclusion

The beneficial effects of the bacteria analyzed in this study and their ability to synthesize IAA in cooperation with local microflora encourage us to consider isolated phytosymbiotic halotolerant methylotrophic strains for the production of microbial biofertilizer which can be able to stimulate cereal crops growth in salt stress conditions.

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