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Influence of Origin and Salt Tolerance on Biofilm Formation by Halotolerant Isolates

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SAŽETAK

Biofilm production is one of the key adaptive strategies employed by soil microorganisms to withstand abiotic stressors, particularly increased salinity. Moreover, biofilm formation plays an essential role in the colonization of plant roots by rhizobacteria and in establishing beneficial plant-microbe interactions. In this study, we examined the significance of biofilm production as a mechanism enabling bacterial isolates from Solonchak and Molisol soils to tolerate salinity stress, as well as its potential dependence on isolate origin (bulk soil or rhizosphere). Biofilm production was examined using the microtiter plate method. Bacterial cultures were grown in wells containing 50% diluted medium and 50% diluted medium supplemented with 2% NaCl. After incubation, the biofilms formed in the wells were stained with crystal violet, and the optical density (OD) was measured at 595 nm. Since no correlation was observed between salt tolerance and biofilm production, it is likely that other mechanisms, such as osmoprotectant accumulation, contribute to salt tolerance. Conversely, rhizosphere bacteria exhibited higher biofilm production compared to bulk soil isolates, particularly when the medium was supplemented with 2% NaCl, suggesting that biofilm formation is an important strategy for root colonization and this interaction is enhanced under saline conditions. Further, biofilm formation declined by about 49–61% in moderately salt-tolerant isolates but increased by 220% in highly salt-tolerant ones, indicating that these changes occurred under increased salt concentrations. This suggests that biofilm development may become more effective as a protective strategy only at higher salinity levels, but likely in combination with other adaptive mechanisms. Rhizobacterial isolate HT3 showed the highest biofilm production in diluted media (OD595 = 1.820), eventhough this ability decreased in the medium supplemented with 2% NaCl. Notably, only two of 31 isolates (HT2 and HB13) showed increased biofilm production under saline conditions.

Key words
Biofilm, bulk soil, PGPR, rhizosphere, salinity stress.

Introduction

A biofilm is defined as “aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or to a surface” (Vert et al., 2012). Biofilms may consist of a single species, multiple species of the same taxonomic group, or even organisms from different taxonomic groups such as bacteria, fungi, archaea, algae, and yeasts (Sharma et al., 2023). They are ubiquitously distributed and can colonize almost all abiotic and biotic surfaces. They are also widely recognized in food industry facilities, water distribution systems, on the surface of animal tissue and on plant surfaces, particularly on the rhizoplane (Sharma et al., 2023). Like all living organisms, bacteria are continuously exposed to diverse abiotic and biotic stressors, and biofilm formation represents one of their key adaptive strategies to withstand these challenges. Among various stress factors, disbalans in osmotic potential is particularly significant, as it can lead to cytoplasmic lysis and cell death, thereby strongly influencing biofilm development (Cappellari et al., 2023). In saline environments, bacteria secrete EPS and a variety of metabolites that perform multiple protective functions, including reactive oxygen species (ROS) scavenging, osmoprotection, and facilitation of nutrient acquisition (Bhat and Roach, 2025). Biofilm formation is also essential for PGPR (Plant Growth Promoting Rhizobacteria), as it enables their stable colonization of the rhizosphere and rhizoplane, where fluctuating moisture, salinity, and nutrient conditions can otherwise limit microbial persistence. Within biofilms, rhizobacteria can communicate through quorum sensing, coordinate metabolic activities, and create protective microenvironments that enhance their survival and plant growth-promoting functions (Goszcz et al., 2025). Moreover, biofilms improve the efficiency of root colonization, facilitating nutrient exchange and biocontrol activities that benefit the host plant (Cappellari et al., 2023, Ambarita et al., 2024).

Biofilm-forming PGPR have strong potential to colonize plant roots and enhance plant growth, particularly under salinity stress (Ambarita et al., 2024). For example, bacterial strains from saline and acidic soils that produced IAA and solubilized phosphate significantly promoted maize growth, and

Azotobacter spp. strains with higher biofilm-forming capacity exerted a greater positive effect on maize compared to weaker strains (Cam et al., 2022). However, this trait is not uniformly distributed among rhizobacteria, as only four out of twelve isolates from the Arabian Sea coasts were capable of forming biofilms (Kapadia et al., 2022). Salinity can both stimulate and inhibit biofilm formation depending on the species: elevated NaCl promoted biofilm development in *Pseudomonas* spp. (Cappellari et al., 2023) and *Halomonas aquamarina*/*meridiana*, whereas *Kushneria indalinina* formed substantial biofilms only under non-saline conditions (Quarashi and Sabri, 2012). Conversely, high salinity suppressed biofilm formation in *Salmonella* Newport 193 (Gu et al., 2020) and reduced it in *Pseudomonas aeruginosa* (Bazire et al., 2007), highlighting species-specific responses to salinity.

This study aims to elucidate the relationship between salt tolerance and biofilm-forming capacity of halotolerant bacterial isolates isolated from bulk soil and rhizosphere samples, as well as to evaluate the effect of isolate origin on biofilm formation.

Material and methods

Isolation of halotolerant isolates

All microorganisms were isolated from either Solonchak or mollisol soils (both from South Bačka region, Vojvodina, Serbia), originating from both bulk soil and rhizosphere samples (Table 1). Serially diluted soil suspensions were inoculated onto Meat Peptone Agar (MPA) (g/L: 3 meat extract, 10 peptone, 20 NaCl, 2.5 K_2HPO_4, 16 Agar) and King B agar (Biolab, Hungary), both supplemented with 2% NaCl. Plates were incubated at 28 °C for 3–5 days, after which morphologically distinct colonies were selected and purified for further analysis. Morphological differentiation of the isolates was based on colony shape, color, margin characteristics, and fluorescence capability.

Table 1.
List of bacterial isolates with their origin and isolation medium.

Isolates	Sample origin	Medium used for isolation
HB1–HB14	Bulk Solonchak soil	MPA + 2% NaCl
HB15–HB17	Bulk Solonchak soil	KingB agar + 2% NaCl
HT1 and HT2	*Trifolium* sp. rhizophere soil on Solonchak	MPA + 2% NaCl
HT3	*Trifolium* sp. rhizophere soil on Solonchak	KingB agar + 2% NaCl
HE1 and HE2	*Eragrostis* sp. rhizosphere soil on Solonchak	MPA + 2% NaCl
HE3 and HE4	*Eragrostis* sp. rhizosphere soil on Solonchak	KingB agar + 2% NaCl
HC1 and HC2	*Cynodon* sp. rhizosphere soil on Solonchak	MPA + 2% NaCl
HC3 and HC4	*Cynodon* sp. rhizosphere soil on Solonchak	KingB agar + 2% NaCl
HP1 and HP2	*Capsicum annuum* rhizosphere soil on Molisol	MPA + 2% NaCl
HP3	*Capsicum annuum* rhizosphere soil on Molisol	KingB agar + 2% NaCl

Salt tolerance assay

All isolates were inoculated onto MPA or King B agar plates (depending on the isolate; the isolates demonstrating fluorescence capacity were inoculated onto King B agar medium) supplemented with varying NaCl concentrations (2%, 3%, 4%, 5%, 6%, and 7%) and incubated for 24 h at 28 °C. The highest NaCl concentration at which visible growth was observed (one level below the concentration that completely inhibited growth) was considered the upper salt tolerance limit for each isolate.

Biofilm formation microtiter plate assay

All isolates were evaluated for biofilm formation using a microplate assay following the method of Djordjevic et al. (2002), with modifications adapted for this study. Each isolate was first cultured in 50 mL of Meat Peptone broth (MPB) or King B broth (depending on the isolate) and incubated for 24 h at 28 °C on an orbital shaker. Subsequently, 150 µL of a 50% diluted medium (MPB or King B broth) was inoculated into the wells of a microtiter plate. The 50% diluted medium was experimentally determined as the optimal nutrient concentration for inducing biofilm formation in the selected isolates (data not shown). To assess the effect of salinity on biofilm formation, NaCl was added to achieve a final concentration of 2% in the test wells. Thus, two experimental treatments were established:
diluted medium alone and diluted medium supplemented with 2% NaCl, each in duplicate. The microtiter plates were then incubated for 24 h at 28 °C.

Medium was removed from wells and microtiter plate wells were washed five times with sterile distilled water to remove loosely associated bacteria. Plates were air dried for 45 min and each well was stained with 150 µL of 1% crystal violet solution in water for 45 min. After staining, plates were washed with sterile distilled water five times. The quantitative analysis of biofilm production was

performed by adding 200 µL of 95% ethanol to destain the wells. One hundred microliters from each well was transferred to a new microtiter plate and the level (OD) of the crystal violet present in the destaining solution was measured at 595 nm.

Statistical analysis

Spearman's rank correlation was used to assess the relationship between salt tolerance and biofilm production in diluted medium and diluted medium supplemented with 2% NaCl. Analysis of variance (ANOVA) was applied to evaluate the effect of isolate origin (bulk soil or rhizosphere) on biofilm formation under both conditions. All statistical analyses were performed using STATISTICA 14.1. software (Tibco software inc.).

Results and discussion

Biofilm formation represents an important adaptive strategy of soil microorganisms to withstand abiotic stressors such as salinity. This protection is largely mediated by the extracellular polysaccharide matrix, which provides structural stability, resilience, and defense against environmental stress. The presence of these polysaccharides was detected using the crystal violet assay, where crystal violet binds to the biofilm matrix, forming a complex that produces a characteristic purple coloration in the wells. Salt tolerance and biofilm production in diluted media and diluted media supplemented with 2% NaCl for all bacterial isolates are presented in Table 2.

Table 2.
Biofilm production (OD_{595}) and salt tolerance of isolates

Isolate	Biofilm (OD_{595}*) in diluted media	Biofilm (OD_{595}) in diluted media + 2% NaCl	Upper salt tolerance (%)
HB1	-0.001	-0.026	2
HB2	-0.002	-0.031	7
HB3	0.003	-0.018	4
HB4	0.303	0.421	3
HB5	1.016	0.056	3
HB6	0.000	-0.039	4
HB7	0.005	-0.035	7
HB8	0.009	-0.025	5
HB9	0.298	-0.027	5
HB10	-0.009	0.034	7
HB11	0.044	-0.022	3
HB12	-0.005	-0.008	2
HB13	0.105	0.311	7
HB14	0.018	0.013	7
HB15	0.005	-0.019	5
HB16	0.001	0.003	3
HB17	0.290	0.052	3
HT1	0.891	0.915	7
HT2	0.217	0.892	7
HT3	1.193	0.317	2
HE1	0.063	0.010	7
HE2	0.010	0.005	7
HE3	-0.020	0.005	7
HE4	1.820	0.761	3
HC1	0.015	0.025	7
HC2	0.042	0.027	7
HC3	0.012	0.024	2
HC4	0.098	0.215	3
HP1	0.986	0.715	2
HP2	0.866	0.174	2
HP3	0.037	0.001	2

* The final OD_{595} value is calculated by subtracting the OD_{595} of the control (non-inoculated medium) from the measured OD_{595} of the sample.

Reduced nutrient availability was one of the factors used to induce biofilm formation in the study of

Djordjevic et al. (2002). In addition to this condition, 2% salinity was introduced to further stimulate biofilm production in halotolerant isolates. However, the correlation between salt tolerance and biofilm formation in diluted media, as well as between salt tolerance and biofilm formation in diluted media supplemented with 2% NaCl, was not significant ($r = -0.179$, $p = 0.325$; $r = -0.030$, $p = 0.829$, respectively). This lack of correlation suggests that these isolates may employ alternative mechanisms to cope with salinity stress, such as the production of osmoprotectants, metabolic adjustments, modifications of cell membranes and walls (e.g., alterations in mechanosensitive channels), or increased potassium uptake (Goszcz et al., 2025). In line with this, Kapadia et al. (2022) reported that only a few isolated strains exhibited biofilm-forming ability, attributing this to the genetic determination of biofilm production or its dependence on specific culture conditions.

Although the correlation between salt tolerance and biofilm production was not statistically significant, grouping the isolates according to their salt tolerance ability revealed a notable trend (Figure 1, Figure 2). This trend was particularly evident in rhizosphere isolates, where the average OD_{595} values of isolates tolerant to 2% and 3% NaCl were higher in the diluted medium (0.51 and 0.96, respectively) compared to the diluted medium supplemented with 2% NaCl (0.20 and 0.49, respectively) (Figure 2). In contrast, isolates capable of tolerating higher salt concentrations exhibited increased biofilm production in the salt-supplemented medium (from 0.05 to 0.16). This suggests that isolates with lower salt tolerance may rely more heavily on biofilm formation as a protective mechanism, which appears insufficient under higher salinity levels. Conversely, isolates tolerant to higher salt concentrations may employ biofilm formation as an additional mechanism alongside other adaptive strategies, as evidenced by their biofilm production exclusively in the salt-supplemented medium.

ANOVA showed that isolate origin had a significant effect on biofilm formation, particularly in diluted media supplemented with 2% NaCl, where a statistically significant difference was observed ($p = 0.017$). On the other hand, a isolate origin had no statistically significant effect on biofilm formation in diluted media ($\alpha = 0.05$, $p = 0.051$), although a trend was observed. This finding suggests that biofilm production plays an important role in the adhesion of rhizoplane microorganisms, especially in saline soils such as Solonchak. Ambarita et al. (2024) demonstrated that biofilm-forming halotolerant strains capable of solubilizing phosphate and producing auxins enhanced maize seedling height under saline conditions. Although biofilm formation is an important trait for effective root colonization, plant growth promotion ultimately depends on additional plant growth-promoting characteristics of PGPR. Similarly, the biofilm-forming halotolerant rhizobacterium *Pantoea agglomerans* FAP10 exhibited increased biofilm production at 125 and 250 mM NaCl, which enabled efficient colonization of the wheat rhizosphere and promoted plant growth and yield under saline conditions (Ansari et al., 2024). In our study, the rhizobacterial isolate HE4 exhibited the highest biofilm production ($OD_{595} = 1.820$). However, this ability decreased in the medium supplemented with 2% NaCl. In contrast, isolates HT2 and HB13 showed increased biofilm production under saline conditions, supporting the findings of Kapadia et al. (2022), who reported that growth conditions can significantly influence biofilm formation.

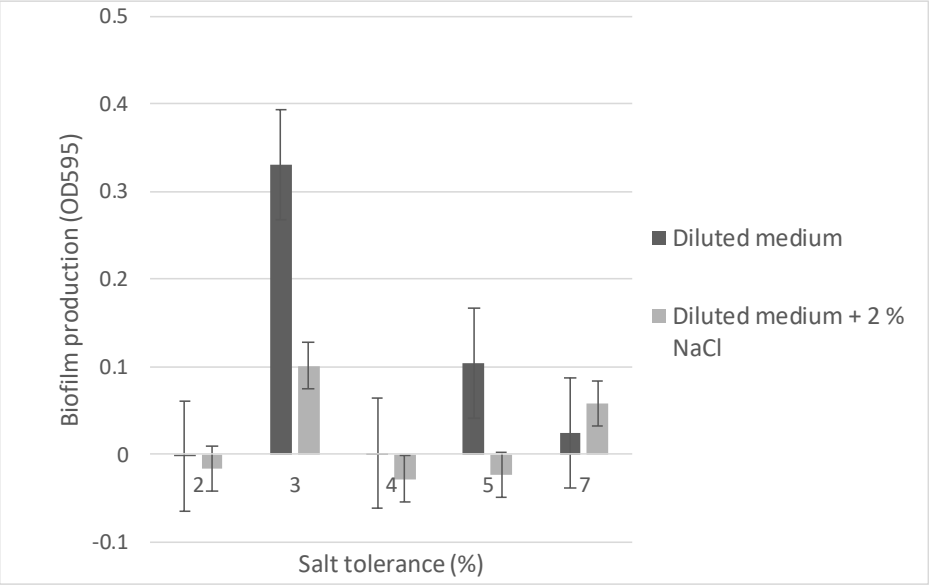

Figure 1. Average biofilm production of bulk soil isolates grouped according to their salt tolerance

ability

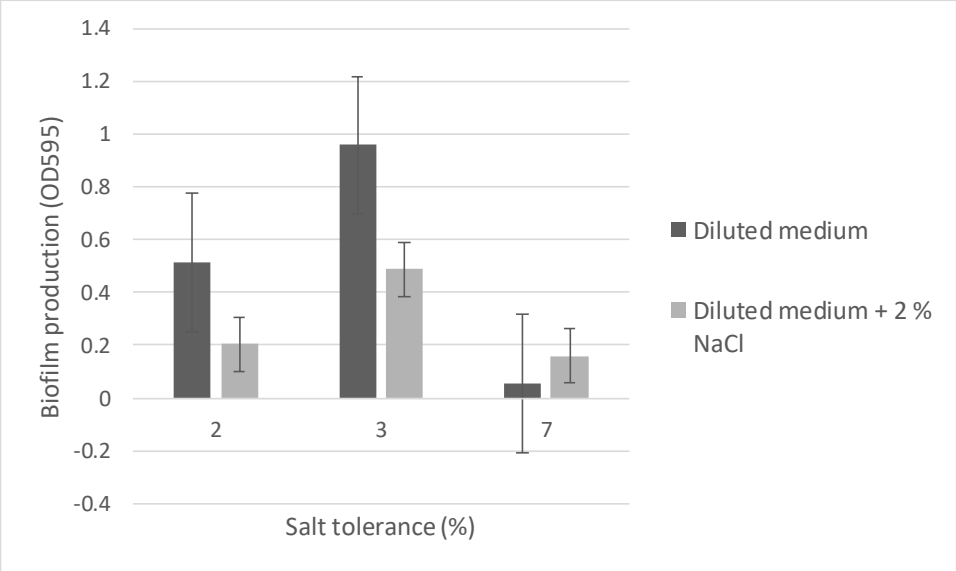

Figure 2. Average biofilm production of rhizosphere isolates grouped according to their salt tolerance ability

Conclusions

The results of our study demonstrated that salinity tolerance of the halotolerant isolates was not correlated with biofilm production, suggesting that these bacteria may rely on other mechanisms to cope with salt stress, such as osmoprotectant synthesis. Conversely, isolates from the rhizosphere exhibited enhanced biofilm formation when exposed to 2% NaCl, indicating that biofilm development may play an important role in root colonization and provide protection to both bacteria and plant roots in saline soils, such as Solonchak. The rhizobacterial isolate HE4 exhibited the highest biofilm production (OD_{595} = 1.820). However, this ability decreased in the medium supplemented with 2% NaCl. In contrast, isolates HT2 and HB13 exhibited increased biofilm production under saline conditions; however, only two out of 31 isolates displayed this response, which was a lower number than expected. In summary, biofilm formation declined by about 49–61% in moderately salt-tolerant isolates but increased by 220% in highly salt-tolerant ones, indicating that these changes occurred under increased salt concentrations. This suggests that biofilm development may become more effective as a protective strategy only at higher salinity levels, but likely in combination with other adaptive mechanisms. The effect of salinity and other abiotic stressors on biofilm production by the isolates should be further investigated in future studies aiming to elucidate the role of halotolerant bacteria in mitigating the impacts of abiotic stress on crops, as biofilm formation is crucial for successful colonization, particularly in harsh soil environments.

References

Ambarita, D. D. M., Mora, E., Fitriatin, B. N., Ruswandi, D., Simarmata, T. (2024). Biochemical traits and biological assessment of indigenous biofilm-forming rhizophosphate bacteria isolated from salinity and acidity stressed soils to enhance maize growth. J. Ecol. Eng. 25(9), 328-339. DOI: 10.12911/22998993/191150

Ansari, F. A., Ahmad, I., Pichtel, J., Husain, F. M. (2024). Pantoea agglomerans FAP10: A novel biofilm-producing PGPR strain improves wheat growth and soil resilience under salinity stress. Environ. Exp. Bot. 222, 105759. https://doi.org/10.1016/j.envexpbot.2024.105759

Bazire, A., Diab, F., Jebbar, M., Haras, D. (2007). Influence of high salinity on biofilm formation and benzoate assimilation by Pseudomonas aeruginosa. J. ind. microbiol. biotech. 34(1), 5-8. https://doi.org/10.1007/s10295-006-0087-2

Bhat, S. P., Roach, D. J. (2025). Bioactivity of microbial biofilms in extreme environments. Front. Microbiol. 16, 1602583. doi: 10.3389/fmicb.2025.1602583

Çam, S., Küçük, Ç., Cevheri, C. (2022). The effect of salinity-resistant biofilm-forming Azotobacter spp. on salt tolerance in maize growth. Zemdirbyste-Agriculture, 109(4). DOI 10.13080/z-a.2022.109.045

Cappellari, L. D. R., Bogino, P. C., Nievas, F., Giordano, W., Banchio, E. (2023). Exploring the differential impact of salt stress on root colonization adaptation mechanisms in plant growth-promoting rhizobacteria. Plants, 12(23), 4059. https://doi.org/10.3390/plants12234059

Djordjevic, D., Wiedmann, M., McLandsborough, L. A. (2002). Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation. Appl. Environ. Microbiol. 68(6), 2950-2958. https://doi.org/10.1128/AEM.68.6.2950-2958.2002

Goszcz, A., Furtak, K., Stasiuk, R., Wójtowicz, J., Musiałowski, M., Schiavon, M., Dębiec-Andrzejewska, K. (2025). Bacterial osmoprotectants-a way to survive in saline conditions and potential crop allies. FEMS Microbiol. Rev. fuaf020. https://doi.org/10.1093/femsre/fuaf020

Gu, L., Chen, Q., Guo, A., Liu, W., Ruan, Y., Zhang, X., Nou, X. (2020). Differential effects of growth medium salinity on biofilm formation of two Salmonella enterica strains. J. Food Prot. 83(2), 196-203. https://doi.org/10.4315/0362-028X.JFP-19-418

Kapadia, C., Patel, N., Rana, A., Vaidya, H., Alfarraj, S., Ansari, M. J., et al. (2022). Evaluation of plant growth-promoting and salinity ameliorating potential of halophilic bacteria isolated from saline soil. Front. Plant Sci. 13, 946217. https://doi.org/10.3389/fpls.2022.946217

Qurashi, A. W., Sabri, A. N. (2012). Biofilm formation in moderately halophilic bacteria is influenced by varying salinity levels. J. Basic Microbiol. 52(5), 566-572. https://doi.org/10.1002/jobm.201100253

Sharma, S., Mohler, J., Mahajan, S. D., Schwartz, S. A., Bruggemann, L., Aalinkeel, R. (2023). Microbial biofilm: a review on formation, infection, antibiotic resistance, control measures, and innovative treatment. Microorganisms. 11(6), 1614. https://doi.org/10.3390/microorganisms11061614

Vert, M., Doi, Y., Hellwich, K. H., Hess, M., Hodge, P., Kubisa, P., et al. (2012). Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). Pure Appl. Chem. 84(2), 377-410. http://dx.doi.org/10.1351/PAC-REC-10-12-04

Uticaj porekla i stepena tolerancije saliniteta na produkciju biofilma halotolerantnih izolata

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Sažetak
Produkcija biofilma predstavlja jednu od ključnih adaptivnih strategija koje mikroorganizmi u zemljištu koriste za prevazilaženje abiotskih stresova, posebno povećane saliniteta. Pored toga, formiranje biofilma ima ključnu ulogu u kolonizaciji korena biljaka od strane rizobakterija i uspostavljanju korisnih interakcija između biljaka i mikroorganizama. U ovom istraživanju ispitan je značaj formiranja biofilma kao mehanizma koji omogućava bakterijskim izolatima iz zemljišta tipa Solončak i Molisol da tolerišu stres izazvan salinitetom, kao i moguća zavisnost ovog procesa od porekla izolata (rizosferno ili nerizosferno zemljište). Produkcija biofilma ispitana je metodom u mikrotitar ploči. Bakterijske kulture su gajene u bunarčićima koji su sadržali 50% razblažen medijum i 50% razblažen medijum sa dodatkom 2% NaCl. Nakon inkubacije, formirani biofilmovi su obojeni kristal-violetom, a optička gustina (OD) je merena na 595 nm. Kako nije utvrđena korelacija između tolerantnosti na so i formiranja biofilma, verovatno je da drugi mehanizmi, poput akumulacije osmoprotektanata, doprinose tolerantnosti na so. S druge strane, rizosferne bakterije su pokazale veću produkciju biofilma u poređenju sa izolatima iz nerizosfernog zemljišta, naročito u medijumu sa dodatkom 2% NaCl, što ukazuje na to da je formiranje biofilma važna strategija za kolonizaciju korena i da je ova interakcija pojačana u uslovima povišenog saliniteta. Dalje, formiranje biofilma je opalo za oko 49–61% kod umereno halotolerantnih izolata, dok je povećano za 220% kod visoko halotolerantnih. Ovi rezultati sugerišu da biofilm postaje efikasniji kao zaštitni mehanizam tek pri većim nivoima saliniteta, verovatno u kombinaciji sa drugim adaptivnim mehanizmima.Rizosferni izolat HT3 pokazao je najveću produkciju biofilma u razblaženom medijumu (OD595 = 1.820), iako je ta sposobnost opala u medijumu sa 2% NaCl. Važno je istaći to da su samo dva od ukupno 31 izolata (HT2 i HB13) pokazala povećanu produkciju biofilma u uslovima saliniteta

Ključne reči
Biofilm, zemljište, PGPR, rizosfera, osmotski stres.

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