# Summary of Ph.D. dissertation

## Comprehensive examination of glyphosate-tolerant bacteria with plant growth-promoting and biocontrol potential

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## INTRODUCTION

The extensive utilisation of synthetic agrochemicals, such as pesticides and fertilisers, including the globally-used herbicide glyphosate has resulted in soil and water contamination, representing serious risk to beneficial soil microorganisms, bees, fish, and mammals. A variety of microbial species can enhance plant growth and protect crops using different direct and indirect mechanisms, including the synthesis of compounds that are beneficial for plants, such as ammonia, siderophores, a phytohormone indole-3-acetic acid (IAA), the solubilisation of phosphorus and potassium, and the secretion of bioactive metabolites. Therefore, beneficial strains are considered as effective and promising alternatives to hazardous agrochemicals to enhance crop productivity and to maintain soil fertility in an environment-friendly way. However, the successful utilisation of potential biofertilisers and biopesticides requires the comprehensive eco-physiological characterisation of the beneficial strains, particularly the examination of the level of their stress tolerance, as certain abiotic factors can remarkably limit their activity *in vivo*.

In the study, a total of 30 glyphosate-tolerant bacterial strains were isolated. Among 10 selected strains, 3 isolates possessing various characteristics that are beneficial for plant growth and protection, and tolerance to adverse abiotic stress factors might be considered as microbial agents for potential agricultural applications.

## OBJECTIVES

1. Isolation and identification of glyphosate-tolerant bacteria from soil samples previously treated with a glyphosate-containing herbicide

2. Examination of the potential beneficial properties (plant growth-promotion and biocontrol activity) of the isolated strains

3. Detailed characterisation of the isolates possessing beneficial features

## METHODS

*Metagenome analysis:* Shotgun sequencing of a control and a glyphosate-treated soil sample, bioinformatical data analysis

*Isolation and identification of bacterial strains:*

- Isolation: Enrichment-culture techniques using glyphosate as the sole carbon and nitrogen source from a soil sample previously exposed to glyphosate
- Identification: PCR amplification and sequence analysis of fragments of the 16S rRNA and RNA polymerase (*rpoB*) genes
- Phylogenetic reconstruction: E-INS-i iterative refinement method

*Examination of the plant growth-promoting potential:*

- Tomato growth promotion: Soaking tomato seeds in bacterial cultures, examination of germination and seedling growth
- IAA biosynthesis: Colorimetric assays using Salkowski reagent, High Performance Liquid Chromatography (HPLC)-Mass Spectrometry (MS) analysis
- Siderophore production: Chromeazurol S (CAS) dye technique
- Ammonia production: Colorimetric assays using Nessler's reagent
- Phosphate solubilisation: Incubation on solid Pikovskaya Medium (PM) amended with $Ca_3(PO_4)_2$

*Investigation into the biocontrol potential:*

- Quorum-sensing (QS) inhibition: Agar diffusion method using *Chromobacterium violaceum* and *Serratia marcescens* as model organisms
- *In vitro* antagonistic activity: Dual-culture method using 9 fungal and 5 bacterial plant pathogens
- Carbon source utilisation tests: Broth microdilution technique using 80 different compounds
- The influence of carbon sources on the antagonistic potential: Dual-culture method using 13 carbon sources utilised by both the glyphosate-tolerant strains and *Agrobacterium tumefaciens*
- Testing the inhibitory effect of the cell-free culture filtrates (CCF): Broth microdilution of CCFs obtained from the culture supernatant the strains grown in 7 different media including minimal medium (MM)

amended with carbon sources providing the highest inhibitory effect previously (50, 25, and 12.5%)

- Siderophore- and ROS-mediated suppression: Broth microdilution of CCFs possessing significant inhibitory effect (50 and 25%), with the addition of $FeCl_3$ and ascorbic acid was applied to eliminate possible iron sequestration and the generation of reactive oxygen species (ROS), respectively
- Extracellular enzyme activity assays: Spectrophotometric assays of esterases, trypsin- and chymotrypsin-like proteases using chromogenic substrates
- MS-MS analysis and the identification of bioactive metabolites: HPLC-High-resolution mass spectrometry (HRMS) of CCF from all 7 tested media

*Eco-physiological characterisation:*

- pH spectrum: Britton-Robinson buffer (pH 6.09-8.95)
- Heavy metal tolerance: 10 heavy metal salts, broth microdilution (1.0, 0.5, and 0.1 mM)
- Pesticide sensitivity: 33 pesticides, broth microdilution (25.0, 12.5, and 6.25 µg/ml)
- Salinity tolerance: NaCl, broth microdilution (100-1.6 g/l)
- Drought tolerance: Polyethylene glycol/PEG 6000, broth microdilution (250-3.9 g/l)

*Whole genome sequence analysis*: Next-generation sequencing, followed by bioinformatical data analysis

*Statistical analysis and data visualisation:* Parametric analyses: Analysis of Variance (ANOVA), t.test, and correlation. Non-parametric alternatives (in cases the data do not meet assumptions): Kruskal-Wallis, Wilcoxon, Kendall coefficient (*tau*), respectively. All data analyses and visualisation were performed using the R and the RStudio Desktop software.

## RESULTS

*Metagenome analysis:* Glyphosate was found to have a remarkable influence on the diversity of microbial communities in the examined soil samples.

*Isolation and identification of bacterial strains:*

- In total, 30 glyphosate-tolerant bacteria were isolated. Ten strains with the highest tolerance to glyphosate and different morphology were selected and identified as:

- *Ensifer adhaerens* (2), *Ochrobactrum anthropi* (1), *Pseudomonas resinovorans*, genotype I (3) and II (4)

*Examination of the plant growth-promoting potential*

- Tomato growth promotion: *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875 significantly promoted tomato root length and consequently total length ($p<0.05$). These 2 strains were selected for the detailed characterisation as promising plant growth-promoting species together with *P. resinovorans* (genotype I) SZMC 25872 which was

selected as the most active strain during the biocontrol studies.

- IAA biosynthesis: *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype I) SZMC 25872 produced 0.28-0.23 and 0.004-0.02 µg/ml indole-3-acetic acid (IAA) with and without tryptophan, respectively.

- Siderophore production: *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872, and *P. resinovorans* (genotype II) SZMC 25875 could synthesise 21.66, 24.59, and 13.73% of siderophores, respectively.

- Ammonia production: *E. adhaerens* SZMC 25856, *P. resinovorans* (genotype I) SZMC 25872, and *P. resinovorans* (genotype II) SZMC 25875 were found to produce 0.11, 0.13, and 0.14 mg/ml ammonia, respectively.

- Phosphorus solubilisation: None of the studied strains showed phosphorus solubilising ability.

*Investigation into the biocontrol potential:*

- Anti-quorum sensing activity: 200 and 300 µl of the supernatant of all 3 studied glyphosate-tolerant strains resulted in QS inhibition of *S. marcescens* SZMC 0567.

- *In vitro* antagonistic activity: Living cultures of *P. resinovorans* strains could inhibit the growth of *A. tumefaciens* SZMC 21407. The subsequent studies revealed the inhibitory effect of *P. resinovorans* strains on 6 *A. tumefaciens* and 6 *A. vitis* strains. *P. resinovorans* (genotype I) SZMC 25872 was found to possess the highest antagonistic activity, thus it was selected for all further studies together with the plant

growth-promoting isolates (*E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875).

- Carbon source utilisation tests: 13 compounds (2-keto-D-gluconic acid, D-fructose, D-glucuronic acid, D-glucose, glycerol-l-monoacetate, L-alanine, L-asparagine, L-glutamic acid, L-glutamine, L-malic acid, sodium pyruvate, starch, and succinic acid) utilised by both *P. resinovorans* isolates (SZMC 25872 and SZMC 25875) as the most promising biocontrol agents, and *A. tumefaciens* SZMC 14557 as the most susceptible pathogenic strain) were selected to test the effect of carbon sources on the suppression of *A. tumefaciens* by *P. resinovorans*.

- The influence of carbon sources on the antagonistic potential: Among the 13 tested substances, the growth of *A. tumefaciens* SZMC 14557 was remarkably inhibited by *P. resinovorans* (genotype I) SZMC 25872, and the inhibitory effect varied depending on the carbon source. The highest inhibition zones of 17.67, 20.00, 25.33, and 19.00 mm were found on plates containing D-glucose, L-alanine, sodium pyruvate, and succinic acid, respectively.

- Testing the inhibitory effect of CCF: CCF samples obtained from MM amended with L-alanine of both *P. resinovorans* strains (SZMC 25872 and SZMC 25875) at 25% and 50% concentrations resulted in a significant growth inhibition of *A. tumefaciens* SZMC 14557 (58.69-73.01 and 83.03-85.63%, respectively) compared to the positive control ($p<0.05$). CCF of MM amended with succinic acid of *P. resinovorans* SZMC 25872 at 50% concentration also significantly inhibited (56.80%) the growth of *A. tumefaciens* ($p<0.05$). Based

on the highest significant inhibition ($p<0.05$), L-alanine and succinic acid were found as the most promising carbon sources for the identification of metabolites of *P. resinovorans* with antimicrobial activity against *A. tumefaciens*.

- Siderophore- and ROS-mediated suppression mode: Significant positive correlation was found between the siderophore amount detected in the CCF samples and the degree of growth inhibition. The inhibitory effect caused by CCF of *P. resinovorans* grown in MM amended with succinic acid was completely eliminated in the presence of an extra Fe source. The inhibitory effect of CCF of both *P. resinovorans* strains grown in MM amended with L-alanine remained unaffected in the presence an additional Fe supplement. The inhibitory effect of CCF of both *P. resinovorans* obtained from L-alanine or succinic acid-containing MM remained unchanged with the supplement of ascorbic acid.

- Extracellular enzyme activity assays: The highest activities of *P. resinovorans* (genotype I) SZMC 25872 for both trypsin- and chymotrypsin-like proteases were found in MM amended with sodium pyruvate or L-alanine as the carbon source. Therefore, it can be assumed that extracellular protease activities might play an additional role in the inhibition of *A. tumefaciens*, particularly in the case of MM amended with L-alanine, as 25 and 50% CCF obtained from these media significantly inhibited the growth of *A. tumefaciens* SZMC 14557. Esterase activities were also detected only in the case of *P. resinovorans* (genotype I) SZMC 25872. As the highest esterase activity was found in the

CCF from LB, and CCF obtained from this medium did not suppress *A. tumefaciens*, it might be concluded that esterases do not play role in the inhibitory mechanism.

- MS-MS analysis and identification: A total of 24 previously unknown metabolites with tentative inhibitory potential against *A. tumefaciens* SZMC 14557 were detected in CCFs obtained from *P. resinovorans* cultures grown in L-alanine and succinic acid-containing MM (12-12 compounds in each media). The inhibition caused by the CCFs of SZMC 25872 and 25875 strains was most likely due to the presence of these novel metabolites as they were detected at substantial quantities only in these 2 media compared to the remaining 5 tested media (without significant inhibitory effect), containing no or just negligible traces of these metabolites. Based on the identical molecular weight and fragmentation pattern, 9 compounds detected in CCF obtained from MM amended with succinic acid can be grouped in three chemical classes, with 4, 3, and 2 compounds in each group. Moreover, all the 9 compounds were found to have similar composition to homoserine lactones, but their main fragment differs from that of lactones reported in the literature. Metabolites detected in CCF derived from MM supplied with L-alanine possess unique molecular weight and fragmentation pattern, therefore, they are supposed to represent various metabolites.

*Eco-physiological characterisation:*

- pH spectrum: The optimal pH for all isolates fell in the range 6.59-8.95.

- Heavy metal tolerance: All the examined strains could grow sufficiently in the presence of most of the tested heavy metals, such as Fe, Mn, Pb, and Cu at 0.1 mM. *P. resinovorans* (genotype I) SZMC 25872 was tolerant to Fe and Pb up to 1.0 mM, while *E. adhaerens* SZMC 25856 could grow in the presence of 1.0 mM Fe, Mn, and Pb. Besides the tolerance to Fe, Mn, and Pb, *P. resinovorans* (genotype II) SZMC 25875 was also able to grow in the presence of 1.0 mM Zn and Al.

- Pesticide sensitivity: The majority of the tested 33 pesticides, including herbicides, such as glyphosate, bensulfuron-methyl, chlorotoluron, chlorpropham, cinosulfuron, diuron, fenuron, isoproturon, primisulfuron-methyl, propham, and 2,4-dichlorophenoxyacetic acid (2,4-D); fungicides, such as carbendazim, carboxin, fenarimol, flutriafol, imazalil, thiabendazole, and thiophanate-methyl as well as the insecticide diflubenzuron had no detrimental effect on the three examined strains.

- Salinity tolerance: Salinity up to 6.3 g/l NaCl did not limit the growth of the tested glyphosate-tolerant bacteria, which can be considered as moderate halotolerance.

- Drought tolerance: All strains could grow without significant inhibition up to 125 g/l PEG concentration, suggesting them as tolerant to moderate drought conditions.

*Whole genome sequence analysis*: The presence of various genes playing role in the degradation of xenobiotics, the tolerance to heavy metals and antibiotics, quorum sensing, as well as the synthesis and

tolerance to biogenic amines has been revealed. Numerous genes including benzoate 1,2-dioxygenase, protocatechuate 3,4-dioxygenase, quinone oxidoreductase, catechol 1,2-dioxygenase, Pb-Cd-Zn-Hg transporting ATPase) were detected in all the 3 tested strains.

## SUMMARY

The glyphosate-tolerant *E. adhaerens* SZMC 25856 and *P. resinovorans* (genotype II) SZMC 25875 strains significantly promoted tomato growth, and possessed various characteristics that are beneficial for plant growth, such as the synthesis of IAA, as well as the production of siderophores and ammonia. *P. resinovorans* SZMC 25872 (genotype I) was found to have the mentioned plant growth-promoting traits, in addition, both the living culture, as well as its siderophores, extracellular proteases, and yet undiscovered bioactive metabolites could significantly inhibit the growth of *A. tumefaciens*. All the three strains showed remarkable tolerance to abiotic stress factors due to the presence of various genes involved in stress tolerance, suggesting their potential for successful utilisation in agricultural applications.

## LIST OF PUBLICATIONS

MTMT Author ID: 10079221
**Cumulative impact factor: 6.729**

### 1. Publications required for the doctoral process

**Zhumakayev, A. R.,** Vörös, M., Szekeres, A., Rakk, D., Vágvölgyi, C., Szűcs, A., Kredics, L., Škrbić, B. D., Hatvani, L. (2021). Comprehensive characterization of stress tolerant bacteria with plant growth-promoting potential isolated from glyphosate-treated environment. *World Journal of Microbiology & Biotechnology* 37(6):94.
https://doi.org/10.1007/s11274-021-03065-8
($IF_{2020}$: 3.312)

Allaga, H., **Zhumakayev, A.,** Büchner, R., Kocsubé, S., Szűcs, A., Vágvölgyi, C., Kredics, L., Hatvani, L. (2021) Members of the *Trichoderma harzianum* species complex with mushroom pathogenic potential. *Agronomy* 11(12):2434.
https://doi.org/10.3390/agronomy11122434
($IF_{2020}$: 3.417)

### 2. In extenso publications in referred journals

**Zhumakayev, A. R.,** Vörös, M., Szekeres, A., Rakk, D., Vágvölgyi, C., Szűcs, A., Kredics, L., Škrbić, B. D., Hatvani, L. (2021). Comprehensive characterization of stress tolerant bacteria with plant growth-promoting potential isolated from glyphosate-treated environment.

*World Journal of Microbiology & Biotechnology* 37(6):94.
https://doi.org/10.1007/s11274-021-03065-8
($IF_{2020}$: 3.312)

## 3. Conference abstracts

**Zhumakayev, A. R.,** Vágvölgyi, C., Hatvani, L. (2021). Studies on stress-tolerant *Pseudomonas resinovorans* strains with biocontrol potential against *Agrobacterium* species. In 18th Wellmann International Scientific Conference, 84

**Zhumakayev, A. R.,** Vörös, M., Kredics, L., Manczinger, L., Škrbić, B., Vágvölgyi, C., Hatvani, L. (2019). Characterization of *Ensifer adhaerens* strains isolated from glyphosate-treated soil. In 21st Danube-Kris-Mures—Tisa (DKMT) Euroregion Conference on Environment and Health, 34.

**Zhumakayev, A. R.,** Vörös, M., Kredics, L., Manczinger, L., Škrbić, B., Vágvölgyi, C., Hatvani, L. (2019). Examination of pesticide-degrading bacteria isolated from agricultural soils. In 17th Wellmann International Scientific Conference, 105-106

**Zhumakayev, A. R.,** Vágvölgyi, C., Hatvani, L. (2018). Potential application of bacteria for xenobiotic removal from agricultural soils. In PhD Hallgatók 3. Környezettudományi Konferenciája, 6

**Zhumakayev, A. R.,** Kredics, L., Manczinger, L., Škrbić, B., Vágvölgyi, C., Hatvani, L. (2018). Potential application of microbes for xenobiotic-removal in agricultural systems. In 16th Wellmann International Scientific Conference, 118

## 4. Other publications

**Zhumakayev, A. R.,** Syzdykova, G. T. (2015). Economic effectiveness of buckwheat cultivation on moisture saving technology in the steppe zone of Akmola region. *Young Scientist* 5(85), 221-223

**Zhumakayev, A. R.,** Syzdykova, G. T. (2015). Initial element in technological line of production buckwheat products. *Kazakhstan giliminin zhanaliktary* 2(124), 187-198 [in Kazakh]

**Zhumakayev, A. R.,** Syzdykova, G. T. (2012). Graded technology of the buckwheat's cultivar "Shortandinsky krupnozernaya" in Akmola region. In International scientific-practical conference, 99-101 [in Russian]

## Declaration

I declare that the contribution of Anuar R. Zhumakayev was significant in the listed publications and the doctoral process is based on the publications listed. The results reported in the Ph.D. dissertation and the publications have not been used to acquire any PhD degree previously and will not be used in the future either.

Szeged, April 27, 2022

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Dr. Lóránt Hatvani

. ……
Prof. Dr. Csaba Vágvölgyi